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Isolation and Characterization of Abscisic Acid-Responsive, Embryo Specific Genes from *Zea mays*

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abscisic Acid-Responsive, Embryo-Specific Maize Genes

Abstract

Embryogenesis in plants, as in animals, requires the regulated expression of sets of genes involved in developmental processes. To gain insight into the processes regulating gene expression during embryogenesis differential screening was used to identify embryo-specific sequences in a cDNA library constructed from *Zea mays* embryo RNA. Four embryo-specific sequences and one constitutive sequence were characterized further by RNA blot hybridization and DNA sequence determination. The constitutive sequence and two of the embryo-specific sequences were found to encode parts of the previously-reported chloroplast 23S rRNA, Oleosin KD-18, and RAB-17 genes. Two sequences, named Emb5 and Emb564, were found to encode novel maize homologs of a gene expressed during late embryogenesis in a wide range of seed plants. These 5 genes exhibited differential temporal and spatial accumulation during development. Moreover, analysis of RNA from cultured embryos suggested that 4 of these genes were regulated by abscisic acid. The ABA-responsive genes could be divided into 3 classes, based on their developmental expression, tissue-specificity, and sensitivity to ABA. Antibodies raised against a ß-galactosidase:EMB564 fusion protein were used to analyze the accumulation of the EMB564 and/or EMB5 proteins. These polyclonal antibodies detected one or several polypeptides with a molecular weight less than 14 kD which exhibited patterns of developmental accumulation and regulation similar to Emb5 and Emb564 transcripts.

Résumé

L'embryogénèse, chez les plantes comme chez les animaux, nécessite l'expression contrôlée d'ensembles de gènes impliqués dans le processus développemental. De façon à élucider les étapes contrôlant l'expression de gènes durant l'embryogénèse, les séquences spécifiques à l'embryon ont été identifiées par sélection différentielle dans une librairie d'ADN complémentaire construite à partir d'ARN extrait d'embryons de *Zea mays*. Quatre séquences spécifiques à l'embryon et une séquence constitutive ont été caractérisées plus loin par hybridation sur blot d'ARN et par séquençage de l'ADN. La séquence constitutive et deux des séquences spécifiques à l'embryon encoderaient des sections de gènes rapportés précédemment d'ARN ribosomal 23S de chloroplaste, d'oléosine KD-18, et de RAB-17. Deux séquences nommées Emb5 et Emb564, encoderaient chez le maïs de nouveaux homologues d'un gène exprimé vers la fin de l'embryogénèse d'une grande variété d'angiospermes. Les cinq gènes démontrent des accumulations temporelles et spatiales distinctes lors du développement. De plus, l'analyse de l'ARN d'embryons cultivés *in vitro* suggère que quatre de ces gènes sont contrôlés par l'acide abscissique. Les gènes répondant à l'acide abscissique peuvent être divisés en trois classes, selon leur expression développementale, leur spécifité tissulaire et leur sensibilité à l'acide abscissique. Des anticorps contre une protéine-fusion de ß-galactosidase:EMB564 ont été utilisés pour analyser l'accumulation de protéines de EMB564 et/ou EMB5. Les anticorps polyclonaux détectent un ou des polypeptides d'un poids moléculaire inférieur à 14 kDa dont l'accumulation lors du développement et la régulation suivent un modèle similaire à celui des ARN messagers Emb5 et Emb564.

Contributions to Original Knowledge

1. I have demonstrated that two novel maize genes, Emb5 and Emb564, are members of a gene family sharing significant homology to late embryogenesis abundant proteins from a wide range of angiosperm species.

2. I have demonstrated that the transcripts of Emb5 and Emb564 accumulate during mid embryogenesis, disappear during germination, and accumulate precociously in isolated embryos cultured with abscisic acid. These genes were responsive to ABA only during early and mid embryogenesis. Transcripts of Emb5 and Emb564 did not accumulate in ABA- or water-stressed seedlings, conditions which did stimulate the accumulation of Rab-17 transcripts.

3. I have extended the characterization of Oleosin KD18 and Rab-17, demonstrating in detail the developmental modulation of their transcript levels and their regulation by ABA.

4. By comparing the accumulation of 4 ABA-responsive transcripts in developing wild-type and viviparous embryos, and in response to exogenous ABA in developing embryos and vegetative tissues, I have defined 3 classes of ABA-responsive maize genes.

5. I have generated antibodies to a ß-galactosidase:EMB564 fusion protein isolated from *E.coli*. I have used these antibodies to characterize the developmental accumulation and

regulation of the final polypeptide product of the Emb5 and Emb564 genes. I have also demonstrated the presence of similar polypeptides in the seeds of other plant species.

Preface and Acknowedgements

This thesis is assembled in accordance with the regulations of the faculty of Graduate Studies and Research. It consists of an Abstract, Résumé, Introduction (Chapter 1), Material and Methods (Chapter 2), Results (Chapter 3), Discussion (Chapter 4) and Literature Cited.

A preliminary description of some of the data presented here can be found in the following publications:

Williams B., Tsang A. (1991) A maize gene expressed during embryogenesis is abscisic acid-inducible and highly conserved. Plant Mol. Biol. 16:919-923

Williams B.A., Tsang A. (1992) Nucleotide sequence of an abscisic acid-responsive, embryo-specific maize gene. Plant Physiol. 100:1067-1068

An additional manuscript has been prepared and submitted for publication:

Williams B.A., Tsang A. Expression of multiple classes of abscisic acid-responsive genes during embryogenesis in *Zea mays*. Submitted to Developmental Genetics

All of the data presented in this thesis are the work of the author.

I am indebted to my supervisor, Dr. Adrian Tsang, for his advice and encouragement during the course of this work. I would also like to thank the people who served on my supervisory committee, Drs. G. Brown, R. Dhindsa, R. Poole, and D. P. Verma. In addition I gratefully acknowledge the generosity of Dr. R. Hamilton and Dr. B. Burr who supplied Co255 and viviparous seed, respectively. I am also indebted to Dr. B. Coulman and the staff at the Macdonald College Seed Farm for a place to grow my crops. Special thanks go to the members of our lab, past and present, for making it an enjoyable place to work: Caroline Grant, Gerard Bain, Michael Greenwood, Claire Bonfils (for helping with pollination), Anastasia Protopapas, Rob Wennington, Amalia Martinez, Gillian Kent, and Carolyn Kay. I would also like to express my gratitude to Francis Ouellette for introducing me to MS-DOS. Last, but not least, I thank Nathalie Chiasson for providing the French translation of my Abstract, for assisting in field work, and for her unflagging encouragement. This research was supported by grants from Natural Sciences and Engineering Research Council of Canada and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (Québec).

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Abbreviations

ABA	abscisic acid
ABRE	abscisic acid response element
bp	base pairs
cDNA	complementary DNA
Ci	Curie
cpm	counts per minute
DAG	diacylglycerol
Denhardt's	1X= 0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone
DNA	deoxyribonucleic acid
DTT	dethiothreitol
EDTA	ethylene diamine tetraacetic acid
g	gram
GA	gibberellic acid
GARE	gibberellic acid response element
h	hour
IP_3	inositol trisphosphate
kb	kilobase
kD	kilodalton
M	molar
min	minute
ml	milliliter

mm	millimeter
mM	millimolar
mRNA	messenger RNA
NaCl	sodium chloride
NaN_3	sodium azide
ORF	open reading frame
PBS	phosphate buffered saline (50 mM Na_2HPO_4/KH_2PO_4 pH 7.5, 150 mM NaCl)
PIP_2	phosphatidylinositol 4,5-bisphosphate
poly(A)$^+$	polyadenylated
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSC	saline sodium citrate (1X= 0.15M NaCl, 0.015M sodium citrate)
TBS	Tris buffered saline (1X= 150 mM NaCl, 700 mM KCl, 25 mM Tris, pH 8
TCA	trichloroacetic acid
Tris	Tris-(hydroxymethyl)aminomethane
µCi	microcurie
µg	microgram
µl	microliter
µM	micromolar

CHAPTER 1: INTRODUCTION

Early development of the angiosperm takes place within tissues of the maternal plant from which it derives nourishment and protection. The product of this phase of development is the seed: a resilient vehicle containing a miniature, desiccated plant in a state of suspended animation. At maturity, the seed of most angiosperm species is metabolically quiescent and is capable of surviving harsh conditions for an extended period of time. When conditions become favourable the seed takes up water and, mobilizing food reserves stored during embryogenesis, germinates to produce the autotrophic seedling. This strategy permits the species to colonize environments which are periodically unfavourable. The qualities which make the seed so important to the plant, its high nutritional content and long storage life, are also highly valued by other species, including *Homo sapiens*.

Much is known about the morphological and biochemical processes occurring during seed development. However, the molecular mechanisms which regulate the formation of organs, and such processes as desiccation, dormancy and germination remain unclear. The present study was initiated in the hope of contributing towards the understanding of the regulation of embryogenesis.

This chapter will provide an overview of the processes which occur during angiosperm seed development. Emphasis will be placed on the expression of embryonic genes and the involvement of the phytohormone abscisic acid (ABA) in gene expression and the physiology of the embryo. Since various aspects of plant embryogenesis have been studied using different experimental systems it is often necessary to extrapolate data

from several species to obtain a general overview. In order to facilitate comparison of processes from different species I have compiled the published data in several tables found in this chapter or as Appendices.

1.1 Embryogenesis in Angiosperms

The life cycle of the flowering plant consists of an alternation of generations. The haploid gametophytes, the embryo sac and the pollen grain, produce the egg cell and the sperm cell which fuse to generate the diploid sporophyte. A unique characteristic of the angiosperm is the phenomenon called double fertilization. The embryo sac contains eight haploid cells, three of which are involved in fertilization. Two sperm cells are released from the pollen tube into the embryo sac. One fuses with the egg cell to form the zygote and the other fuses with the binucleate central cell to form the progenitor of the endosperm. The (usually) triploid endosperm is responsible for the synthesis and storage of food reserves which nourish the developing embryo and, in many species, the young seedling before it becomes autotrophic. During the first few days following fertilization the endosperm proliferates rapidly by a series of free nuclear divisions.

Like the embryo the endosperm undergoes development. Cells of the central endosperm become differentiated for the accumulation of food reserves. In maize and other grains, the cells of the aleurone, the outermost layer of endosperm cells, undergo a maturation process. These cells accumulate anthocyanin pigments, acquire desiccation tolerance, and remain viable in the dry seed. During germination, the aleurone plays a crucial role in the mobilization of food reserves from the central endosperm (For a review of maize endosperm development, see Kowles and Phillips 1988).

Once a sufficient mass of endosperm has accumulated the zygote commences growth. The first division is asymmetric. The larger of the two cells gives rise to the suspensor. This organ functions to anchor the small embryo during the liquid endosperm stage and to transfer nutrients from maternal tissue to the embryo until the cotyledon(s) is(are) developed. The suspensor is also the site of synthesis of gibberellic acid during early embryogenesis. This phytohormone stimulates the growth of the young embryo. The smaller cell of the first division gives rise to the embryo proper. It proliferates to form a spherical mass known as the globular proembryo. The elaboration of organs from this proembryo differs between monocotyledonous (monocots) and dicotyledonous (dicots) plants (for a review, see Wardlaw 1955). Various researchers have classified the stages of embryogenesis on the basis of age, mass, morphology or various physiological criteria. This diversity of classification schemes is further complicated by inherent differences in development between species, different rates of development between cultivars of the same species, and the effects of environmental variation. As a result it is often difficult to compare data generated by different researchers. In an attempt to impose order on a confusing literature I have adopted a simplified classification scheme similar to that described by Quatrano (1987). In this scheme embryogenesis is divided into three stages of similar duration termed early, mid and late. These stages are characterized by the rapid growth of the embryo by cell division and the differentiation of the embryo axis (early), the accumulation of storage materials (mid), and desiccation leading to metabolic quiescence (late). The classification schemes used to define the stages of development of some commonly studied seed plants are compared in Table 1 (see also Appendix 1). I

Table 1. Comparison of the Developmental Stages of some Commonly-Studied Angiosperm Species. This table redefines the embryogenesis of some commonly-studied angiosperms. Where possible, representative values of the age and mass of the embryos are included. Various other names have been assigned to these stages. For a more comprehensive treatment of this data, see Appendix 1.

FW, fresh weight; DAP, days after pollination;

1 Abbe and Stein 1954
2 Rogers and Quatrano 1983
3 Goldberg *et al.* 1989, Eisenberg and Mascarenhas 1985
4 Finkelstein *et al.* 1987, Comai and Harada 1990
5 Hocher *et al.* 1991
6 Xu *et al*, 1990
7 Dure 1985, Hughes and Galau 1991
8 Neill *et al.* 1987

Table 1. Comparison of the developmental stages of some commonly-studied angiosperm species.

SPECIES	STAGE	EMBRYO AGE (DAP)	EMBRYO MASS (mg FW)	DESCRIPTION
maize (1)	Early	0-14	<0.5	Up to appearance of coleoptilar tissue
	Mid	14-28	0.5-24	Formation of 1st to 3rd leaf primordia
	Late	28-60	24-50	formation of 4th to 6th leaf primordia
wheat (2)	Early	0-21		Completion of tissue differentiation
	Mid	21-31		Accumulation of storage reserves
	Late	31-50		Desiccation
soybean (3)	Early	0-30	0-150	Differentiation of axis and cotyledons
	Mid	30-90	150-300	Cell expansion; Accum. of storage proteins & lipids
	Late	90-12	400-100	Water loss; RNA and protein synthesis decrease
Brassica napus (4)	Early	0-25	0-1.8	Up to differentiation of axis and cotyledons
	Mid	25-45	1.8-5.8	Cell division ceases; Storage reserves accumulate
	Late	45-60	5.8-3.6	Desiccation; Loss of chlorophyll; Decline of metabolic activity
tomato (5)	Early	0-15	0-3.0	Up to torpedo stage
	Mid	15-30	3.0-6.5	Growth phase
	Late	30-60	6.5-6.5	Dehydration
alfalfa (6)	Early	0-15		Up to heart stage
	Mid	15-30		Elongation; Accumulation of storage reserves
	Late	30-36		Drying stage; Chlorophyll lost
cotton (7)	Early	0-27	0-40	Up to formation of cotyledon
	Mid	27-45	40-80	To end of abscission
	Late	>45	120-80	Desiccation

have used this table when designating the developmental stage at which an event occurs. Throughout this thesis I will refer to developmental stages as early, mid and late. It must be stressed that these are not necessarily the terms used by the investigators cited. It is possible that, due to the plastic nature of plant development, my designations may not all be accurate, particularly in cases where the event being considered occurs near the transition between stages.

A brief description of the development of a typical dicot and the monocot, maize, will serve to introduce the main events of embryogenesis in angiosperms.

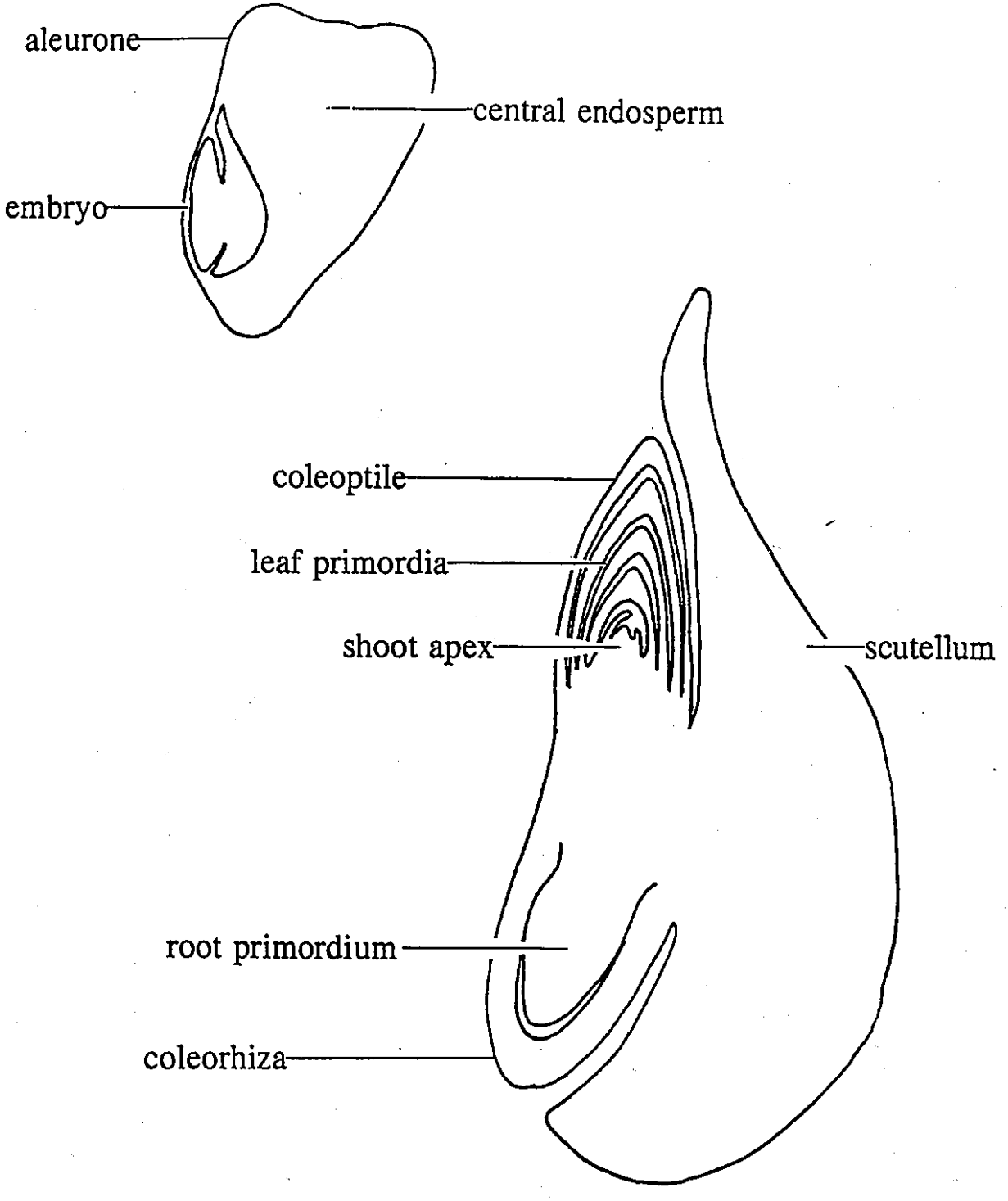
In many dicots the role of the endosperm is limited to the early stages of development. Amino acids, sugars and other nutrients are then transferred from the endosperm to the developing embryo where they are stored mostly in polymer form in the cotyledons. The endosperm then degenerates.

The embryonic development of a typical dicot, *Brassica napus*, is easily divided into three stages. During early embryogenesis, the embryo grows by cell division to form the main axis of primordial root and shoot, and two primordial seed leaves, the cotyledons. Mid embryogenesis begins when cell division stops. Further growth occurs by cell expansion as storage proteins, carbohydrates and lipids accumulate. Late embryogenesis begins when the water content of the embryo begins to decrease, metabolic activity declines, chlorophyll is lost, and the embryo enters a quiescent state (Finkelstein *et al.* 1987).

Most of the food reserves of the monocot seed are stored in the endosperm which continues to develop and comprises the largest part of the seed in many species. In maize,

Figure 1. Diagramatic Representation of a Mature Maize Kernel. Schematic drawings of a maize kernel and a mature embryo are shown in cross section.

MAIZE KERNEL



EMBRYO

a transition between cell division and accumulation of storage products in the endosperm occurs near the end of the early stage (Kowles and Phillips 1988, Marks *et al.* 1985). Enzyme activities for starch biosynthesis increase during this period as do the levels of mRNA for the major storage proteins of maize, the zeins (Marks *et al.* 1985). Carbohydrates and storage proteins continue to accumulate in the endosperm until the seed is fully mature and quiescent.

The mature monocot embryo is generally more developed than its dicot counterpart. The mature maize embryo, for example, has 5 or 6 primordial leaves ready to expand upon germination. The development of maize has been described in terms of the appearance of the major organs (Abbe and Stein 1954). During early embryogenesis the embryonic axis, which will develop into root and shoot, appears as a small protrusion on the side of the globular proembryo. Adjacent tissue then grows up over the shoot primordium to form the coleoptile, a sheath that will serve to protect the primordial leaves. The predominant part of the embryo becomes the scutellum, the singular cotyledon which serves to shuttle nutrients from the endosperm to the developing axis. At approximately the beginning of mid embryogenesis the first leaf primordium appears at the base of the shoot apex opposite the scutellum. Leaves then develop on alternating sides of the shoot meristem and are rolled up within the coleoptile. Late embryogenesis and desiccation begin when the embryo has approximately 4 leaves. The timing of the onset of desiccation and the final number of leaves depends on the growing conditions. A schematic representation of a mature maize kernel is shown in Figure 1.

Table 2. The developmental accumulation of mRNAs and/or proteins abundant during Early embryogenesis. The approximate time of the appearance and of maximum steady-state accumulation of the mRNA and/or protein product of genes expressed in the embryo during early embryogenesis. Data were obtained from the literature cited.

SPECIES	NAME OF GENE OR PROTEIN	LEVEL	TIME OF APPEAR-ANCE	TIME OF MAX. ACCUM.	STAGE OF MAX. ACCUM	REF
maize	HyPRP	m	12 DAP	18 DAP	Mid	1
	HPRG (Hydroxypro-line Rich GP)	m	12 DAP	20 DAP	Mid	2
	zeins	p	12 DAP	mature	Late	3
	19 kDa zein	m	10 DAP	16 DAP	Mid	4
oat	avenin	m	4 DAP	8 DAP	Early	5
barley	B11E	m	10 DAP	25 DAP	Mid	6
	CMa (α-amylase inhibitor)	m		15 DAF	Early	7
rice	Rice Lectin	m		10 DAP	Early	8
sunflower	helianthinin	b	12 DAP	20 DAP	?	9
B. napus	pAX58	m	18 DAP	23 DAP	Early	10
carrot	ECP31 *	b	Embryo-genic culture	Heart *	Early	11
pea	pCD7	b	16 DAP@	16 DAP@	Early	12

* this gene becomes active again during late embryogenesis (see Table 4); @ earliest time point examined; b, mRNA and protein; DAP, days after pollination; m, mRNA; p, protein

1 Josè-Estanyol *et al* 1992
2 Ruiz-Avila *et al*. 1991, 1992
3 Kodrzycki *et al*. 1989
4 Kriz *et al*. 1987
5 Chesnut *et al*. 1989
6 Olsen *et al*. 1990
7 Rasmussen and Johansson 1992
8 Wilkins and Raikhel, 1989
9 Goffner *et al*. 1990
10 Harada *et al*. 1988
11 Choi *et al*. 1987, Kiyosue *et al*. 1992a, 1992b

1.1.1 Genes Expressed during Early Embryogenesis

The differentiation of embryonic cells is accompanied by the expression of developmental and tissue-specific genes. For example, the transient expression of the carrot ECP31 gene during the earliest stages of somatic embryogenesis and during the induction of embryogenic competence in callus suggests this gene may be involved in establishing embryogenic identity (Kiyosue *et al.* 1992a). A partial list of genes expressed during early embryogenesis is shown in Table 2. Several of these gene products such as α-amylase inhibitor (Rasmussen and Johansson, 1992) and rice lectin (Wilkins and Raikhel, 1989) may serve to protect the embryo from predation or fungal attack. Other early genes encode cell wall proteins which may be involved in the differentiation of vascular (hydroxyproline-rich glycoprotein, Ruiz-Avila *et al.* 1991) and nonvascular tissue (HyPRP, Josè-Estanyol *et al* 1992).

Development of endosperm cells principally involves the elaboration of protein bodies from endoplasmic reticulum and the differentiation of amyloplasts from proplastids (see section 1.1.2). These events are accompanied by the expression of storage protein genes and genes involved in carbohydrate metabolism.

1.1.2 Accumulation of Macromolecules

Unlike the embryos of most lower plants which proceed directly into vegetative growth after early embryogenesis, most angiosperm embryos undergo a maturation process. In dicots, cell division ceases and storage reserves accumulate. In monocots, cell division continues during mid embryogenesis as the embryo enlarges and organs are elaborated. Monocot seeds accumulate food reserves both in the central endosperm and

Table 3. The Developmental Accumulation of mRNAs and/or Proteins Abundant during Mid Embryogenesis. The approximate time of the appearance and of maximum steady-state accumulation of the mRNA and/or protein product of genes expressed in the embryo during Mid embryogenesis. Data were obtained from the literature cited.

1 Williams and Tsang 1992, this thesis
2 Williams and Tsang 1991, this thesis
3 Belanger and Kriz 1989
4 Rivin and Grudt 1991
5 Vance and Huang, 1988
6 Qu *et al.* 1990
7 This thesis
8 Triplett and Quatrano 1982
9 Eisenberg and Mascarenhas 1985
10 Harada *et al.* 1989
11 Bray and Beachy 1985
12 Tierney *et al.* 1987
13 Walling *et al.* 1986
14 Jofuku and Goldberg, 1989
15 John and Long, 1990
16 Keddie *et al.* 1992
17 Finkelstein *et al.* 1985, 1987
18 Harada *et al.* 1988
19 Laroche-Raynal and Delseney 1986
20 Pang *et al.* 1988
21 Almoguera and Jordano, 1992

Table 3. The developmental accumulation of mRNAs and/or proteins abundant during Mid embryogenesis.

SPECIES	NAME OF GENE OR PROTEIN	LEVEL	TIME OF APPEAR-ANCE	STAGE OF APPEAR-ANCE	TIME OF MAX. ACCUM.	STAGE OF MAX. ACCUM	REF
maize	Emb5	m	21 DAP	Mid	27 DAP	M/L	1
	Emb564	m	21 DAP	Mid	27 DAP	M/L	2
	GLB1	p	21 DAP	Mid	27 DAP	Mid	3
	Glb1	m	18 DAP*	Mid	24 DAP	Mid	4
	GLB2	p	20 DAP	Mid	55 DAP	Late	3
	oleosin KD16	m	18 DAP*	Early?	25 DAP	Mid	5
	oleosin KD16	m	18 DAP*	Early?	40 DAP	Late	6
	oleosin KD18	m	18 DAP*	Early?	40 DAP	Late	6
	Emb604	m	14 DAP*	Early	21 DAP	Mid	7
wheat	WG Agglutinin	p	25 DAP	Mid	62 DAP	Late	8
soybean	glycinin	m	maturat	Mid			9
	α, ß-conglycinin	m	25 DAA	Early	77 DAA	Mid	10
	α, ß-conglycinin	p	19 DAA	Early	mature	Late	11
	ß, ß-conglycinin	m	35 DAA	Mid	80 DAA	Mid	10
	ß, ß-conglycinin	p	21 DAA	Early	mature	Late	11
	ß, ß-conglycinin	m	26 DAA	Early	35 DAA	Mid	12
	ß, ß-conglycinin	p	26 DAA	Early	mature	Late	12
	lectin		20 DAA	Early			13
	Kunitz trypsin inhibitor	m	25 DAP	Early	77 DAP	Mid	14
Phaseolus	arcelin 2	m	7 DAP	Early	15 DAP	Mid	15
B. napus	Oleosin	m	18 DAP	E/M	56 DAP	Late	16
	cruciferin	p	25 DAP	E/M	60 DAP	Late	17
	cruciferin	m	21 DAP	Early	40 DAP	Mid	17
	napin	p	21 DAP	Early	mature	Late	17
	napin	m	18 DAP	Early	28 DAP	Mid	17
	pAX92	m	23 DAP	Early	26 DAP	Mid	18
radish	cruciferin		25 DAP	E/M			19
Arabidop.	12S globulin	m	6 DAA	E/M	9 DAP	Mid	20
sunflower	Ha G3	m	10 DAP	?	12 DAP	?	21

*, earliest time point examined; DAA, days after anthesis; DAP, days after pollination; m, mRNA; p, protein; WGA, wheat germ agglutinin

in the embryo. During the transition from mid to late embryogenesis the embryo acquires desiccation tolerance (for a review of seed maturation see Bewley and Black 1985).

The mature seed contains food reserves used to nourish the young seedling during germination until it becomes fully autotrophic. Reduced nitrogen and sulfur in the form of seed storage proteins are accumulated in the endosperm of cereals and other seeds with large endosperms, in the cotyledons of legumes, lettuce, and many other dicots, and in the embryonic hypocotyl of some nuts (Bewley and Black 1985). Storage proteins are characterized by their relative abundance in mature seeds and their rapid disappearance during germination. As shown in Table 2, the storage proteins of the endosperm begin to accumulate during early embryogenesis. Embryo storage proteins generally appear during mid embryogenesis and reach maximum levels during late embryogenesis (Table 3). Storage protein transcripts also exhibit a characteristic developmental pattern of accumulation. They begin to accumulate several days before the protein can be detected, reach maximum accumulation during mid embryogenesis, then decline to relatively low levels in the mature seed.

The seed's reserves of reduced carbon are largely found in carbohydrates and lipids. Like most cereals and many other seeds, the predominant storage carbohydrates of maize endosperm are amylose and amylopectin (Soave and Salamini 1984). The most abundant carbohydrates in the mature maize embryo, on the other hand, are sucrose and raffinose (Koster and Leopold 1988). It is likely that these soluble sugars also play an important role in the desiccation tolerance of the embryo (see section 1.1.3). It has been argued that since storage carbohydrates are characterized by their abundance in mature

seeds and their rapid degradation during germination, mucilages and cell wall galactomannans which are abundant in the endosperm cells of some seeds should also be considered storage carbohydrates (Bewley and Black 1985).

Seed storage lipids such as triacylglycerol, glycolipids and phospholipids exhibit a temporal pattern of accumulation similar to that of the storage proteins. The embryo is the main storage site of lipids in the maize kernel. Up to 50% of the mass of the maize scutellum at maturity is triacylglycerol (Bewley and Black 1985). In maize and other oilseeds, storage lipids are packaged in small intracellular globules termed oleosomes. The unit membrane which surrounds each oil droplet is rich in small, alkaline proteins called oleosins which are believed to be involved in preventing coalition of the oleosomes. For a review of the structure of oil bodies, see Huang (1992).

Many of the remaining macronutrients required by the seedling are stored in the seed in the form of phytin. These salts of *myo*-inositol hexaphosphate represent a rich store of phosphorus, magnesium, potassium, calcium, and other inorganic cations which are mobilized by phytase during germination. Phytin accumulates during mid and late embryogenesis and is generally stored in globoids within protein bodies. (For a review of phytin synthesis and deposition, see Greenwood 1989).

Some proteins which accumulate in the maturing seed may serve other functions in addition to that of nutrient storage. Endosperm proteins from several cereals, for example, inhibit insect α-amylase and proteinase and may, therefore protect the seed against predation (Barber *et al.* 1986, Lyons *et al* 1987, for a review, see García-Olmedo *et al.* 1987).

Table 4. The developmental accumulation of late embryogenesis-abundant mRNAs and proteins. The approximate time of the appearance and of maximum steady-state accumulation of the mRNA and/or protein product of late embryogenesis abundant genes from a wide range of angiosperm species. Data were obtained from the literature cited.

1 Gómez *et al.* 1988
2 Goday *et al.* 1988, Pla *et al.* 1989
3 This thesis
4 Thomann *et al.* 1992
5 Triplett and Quatrano 1982
6 Litts *et al.* 1987
7 Mundy and Chua 1988
8 Espelund *et al.* 1992
9 Hughes and Galau 1991, Baker and Dure 1987
10 Choi *et al.* 1987, Kiyosue *et al.* 1992b
11 Barrat and Clark 1991
12 Cohen *et al.* 1991
13 Raynal *et al.* 1989
14 Harada *et al.* 1989
15 de Silva *et al.* 1990
16 Harada *et al.* 1988
17 Almoguera and Jordano, 1992

Table 4. The developmental accumulation of mRNAs and proteins abundant during Late embryogenesis.

SPECIES	NAME OF GENE OR PROTEIN	LEVEL	TIME OF APPEAR-ANCE (DAP)	STAGE OF APPEAR-ANCE	TIME OF MAX. ACCUM. (DAP)	REF
maize	pMAH9	m	30 DAP	M/L	40 DAP	1
	RAB-17	m	40 DAP	Late	60 DAP	2
	RAB-17	m	21 DAP	Mid	56 DAP	3
	MLG3	p	26 DAP	Mid	41 DAP	4
wheat	WGA	p	25 DAA	Mid	45 DAA	5
	Em	b			60 DAP	6
rice	RAB 21	p	10 DAP	Early	30 DAP	7
barley	B19.1	m	25 DAP	Mid	30 DAP	8
	B19.3	m	25 DAP	Mid	30 DAP	8
	B19.4	m	25 DAP	Mid	30 DAP	8
cotton	Lea D11	m	80 mg	Mid	120 mg	9
	LeaA D132	m	30 DAA	Mid	50 DAA	9
carrot	ECP31	m	28 DAA	M/L	mature	10
pea	ABR17	p	700 mg	Late	mature	11
	ABR18	p	700 mg	Late	600 mg*	11
tomato	pLE25	m	ST MG2	Mid	ST B	12
radish	p8B6	m	35 DAP	Late	60 DAP	13
B.napus	pLea76	m	37 DAP	Late	mature	14
	acyl carrier protein	m	20 DAP	Early	mature	15
	pCOT49	m	23 DAP	Mid	50 DAP	16
	pCOT4	m	23 DAP	Mid	45 DAP	16
	pCOT3	m	23 DAP	Mid	45 DAP	16
	pCOT46	m	23 DAP	Mid	45 DAP	16
sunflower	Ha ds10	m	10 DAP	?	mature	17
	Ha ds11	m	7 DAP	?	mature	17

* embryos just beginning to dehydrate (hence weight loss)
m, mRNA; p, protein; b, mRNA and protein; DAA, days after anthesis;

Transcripts or proteins which accumulate to high levels during late embryogenesis, after the peak in storage protein accumulation, have been identified in a wide range of plant species (Table 4). The predicted polypeptide products do not exhibit the characteristic amino acid composition or signal peptide of the storage proteins. Many of these genes appear to encode relatively small, highly hydrophilic proteins. The function of these late embryogenesis-abundant (Lea) genes is unknown. However, some Lea genes have been shown to be responsive to ABA and many can be induced in vegetative tissues by ABA, desiccation, salt, cold, or other stresses (for a review, see Skriver and Mundy 1990). Their expression in the embryo during desiccation and in vegetative tissue during stress suggests Lea genes may be involved in conferring tolerance to desiccation (see section 1.3.1).

1.1.3 Desiccation and Desiccation Tolerance in Seeds

The relative amount of water in the embryo (and endosperm of cereals) decreases almost linearly throughout embryonic development in maize (Neill *et al.* 1987), wheat, barley (Morris *et al.* 1991), and *Brassica napus* (Finkelstein *et al.* 1987). As the embryo enters the late stage, water is taken up at a reduced rate and is finally expelled from the seed. Although final water content may be as low as 4% (*B. napus*, Comai and Harada 1990), values of 20% (maize endosperm, Neill *et al.* 1987) to 30% (wheat and barley, Morris *et al.* 1991) are typical. The mechanism(s) involved in the removal of water from the mature seed is (are) unknown.

The ability of the mature seed to remain viable through severe cold, drought, or extended storage appears to depend upon its state of desiccation. The embryo must,

therefore, acquire the ability to survive water losses as great as 98% (Bartels *et al.* 1988). Desiccation tolerance is acquired during the transition to late embryogenesis, just before the onset of desiccation, in maize (Oishi and Bewley 1992), *Brassica napus* (Finkelstein *et al.* 1987), and other species (Bartels *et al.* 1988; see references in Kermode and Bewley 1987).

Dehydration can produce irreversible damage, primarily through disruption of cellular membranes and denaturation of enzymes. Plant cells appear to have evolved special mechanisms for dealing with seed desiccation. Unlike animal membranes, the membranes of seeds do not exhibit pronounced structural changes after desiccation (McKersie and Stinson 1980, Seewald *et al.* 1981, Priestley and de Kruijff 1982, Thompson and Platt-Aloia 1982). This suggests that desiccation tolerance may involve a reorganization of the cytoplasm and its components (Bruni and Leopold 1991). One mechanism which may serve to protect the desiccated cell is vitrification. Mature maize embryos having a water content less than 12% have been found to exist in a glassy state at temperatures below 25°C (Williams and Leopold 1988). A vitrified cytoplasm resists the formation of crystals due to high solute concentration or water freezing. Solutes such as sucrose and raffinose, which account for 20% of the dry weight of the maize embryo (Koster and Leopold 1988), are thought to be important in establishing the non-lipid component of the vitreous state (Williams and Leopold 1989). Sugars and other small solutes might also replace the water molecules bound to the surfaces of membranes and other structures, thus providing the hydrophilic interactions required to prevent denaturation of enzymes (Clegg 1986).

1.1.4 Quiescence and Dormancy

Metabolic activity declines as the water content of the seed declines. The rates of transcription of isolated *B. napus* nuclei decrease during maturation, reaching lowest values in the dry embryo (Comai and Harada 1990). There is little or no respiration in the dry seed. This is due, at least partly, to the absence of cytochrome C in mitochondrial membranes (Wilson and Bonner 1971, Nawa and Asahi 1973). In maize embryos, at least 35% water content is required for maximum mitochondrial activity as measured by oxygen consumption. This activity does not occur in seeds having less than 20% water content (Vertucci and Leopold 1986). It has been suggested that vitrification is responsible for the observed decrease in the rate of all diffusion-dependent processes, including metabolism, during seed quiescence (Burke 1986).

Many seeds germinate when the external environment becomes favourable to vegetative growth. Others remain dormant in a fully imbibed, metabolically competent state until some specific event or condition is met. Cold stratification, specific light irradiance, and after-ripening are common prerequisites for the germination of some dormant seeds. In species with true embryonic dormancy even the isolated embryo fails to germinate until the appropriate dormancy-breaking conditions are met. Both dormant and nondormant seeds have been shown to exhibit similar rates of water uptake and new protein synthesis during the first hours of imbibition (Bewley and Black 1985). Cell expansion and germination are then prevented in dormant seeds. The mechanism by which these processes are controlled is unknown.

1.1.5 Germination

Following imbibition, metabolic activity commences in the rehydrated seed. DNA repair (Zlatanova *et al.* 1987) and the translation of stored mRNA (Aspart *et al.* 1984, Delseny *et al.* 1977, Harada *et al.* 1988) occur during the first hours. Protein synthesis, but not transcription, is necessary for radicle protrusion in maize and tobacco (Kermode 1990, Arcila and Mohapatra 1992) suggesting that trancripts formed during embryogenesis are required for germination. As germination proceeds transcripts for nuclear-encoded chloroplast genes appear (Brusslan and Tobin 1992). Metabolism then becomes dominated by enzymes involved in the mobilization of stored food reserves. Malate synthase and isocitrate lyase, enzymes involved in the conversion of lipids to sugars, increase in activity during the first 3 days following imbibition (Comai and Harada 1990). In maize, carboxypeptidase activity is detected during the first 2 days, followed by numerous endoproteases (San Segundo *et al.* 1990). The protein bodies of maize are hydrolyzed progressively from their surface causing the sequential hydrolysis of first glutelins then zeins (Torrent *et al.* 1989). In dicots the protein bodies fuse with the vacuole where protein degradation occurs (Gifford and Bewley 1983). Ribonucleases are also released by aleurone tissue during germination and are presumably responsible for mobilization of endosperm RNA stores (Chrispeels and Varner 1967). Germination is largely under hormonal control. In cereals, gibberellic acid secreted by the embryo stimulates the production of hydrolytic enzymes in the endosperm (Jacobsen and Chandler 1987) and the transfer of lipase from protein bodies to oleosomes (Fernandez and Staehelin 1987).

The transcription of genes encoding storage proteins and Lea proteins is

inactivated during early germination. On the other hand, the genes for isocitrate lyase and malate synthase, enzymes which serve postgerminative functions, become active during late embryogenesis and continue to be transcribed during germination. Thus, the switch from embryogenic to germinative growth appears to occur during imbibition (Comai and Harada 1990).

The germinative process is considered to be completed upon radicle protrusion (Bewley and Black 1985). Post-germinative events such as elongation of radicle and plumule, which occur 48-72 hours after inbibition, require new rounds of replication and transcription (Arcila and Mohapatra 1992).

1.2 The Roles of Abscisic Acid During Vegetative Growth

The phytohormone ABA plays a central role in many processes during plant development. It is involved in such vegetative processes as leaf abscission, fruit drop, bud dormancy and senescence. Abscisic acid also appears to mediate the plant's response to such environmental stresses as cold, high salt, water stress, predation and wounding. During embryogenesis ABA is involved in storage protein synthesis, desiccation tolerance and dormancy.

Abscisic acid was first detected by early investigators searching for auxins in plant extracts. Growth-inhibiting substances, the "dormins", were detected in the peel of dormant, but not non-dormant potato (Hemberg 1949a) and the dormant terminal buds of *Fraxinus* (Hemberg 1949b). The predominant inhibitor was later identified as ABA (Millborrow 1967).

While characterizing the above "dormins" a growth inhibitor, later identified as ABA, was isolated from mature cotton fruit and shown to accelerate the abscission of immature fruit (for an early review, see Carnes 1966). Abscisic acid also stimulates the abscission of the fruit of lupin (Van Steveninck 1957, 1958a, b), and the senescing leaves of bean (Osborne 1958) and *Coleus* (Jacobs *et al.* 1962). It has since been demonstrated that ABA stimulates the synthesis of cellulases and pectinases in a thin layer of tissue across the stem or petiole resulting in degradation of the cells in this abscission zone (Sexton *et al.* 1980, Addicott 1982).

1.2.1 Abscisic Acid and Environmental Stresses

In response to soil drying stomata in the leaves close, restricting water loss

through transpiration. This response occurs before there is a change in the water potential or turgor of the leaf. Hence it is believed a chemical signal is generated by roots subjected to water stress (Stillwell *et al.* 1991). Recent experiments suggest that for maize and sunflower this chemical signal is ABA. Abscisic acid levels increase up to 50-fold in the xylem of water-stressed maize (Zhang *et al.* 1987, Zhang and Davies 1989, Zhang and Davies 1990). A brief osmotic stress to the roots elicits first a transient increase of ABA from the roots followed by a more sustained contribution from the leaves and other parts of the plant (Neales and McLeod 1991). The ABA secreted by water stressed cells is believed to derive partly from release by plastids and, possibly, other organelles (Zeevaart and Creelman 1988). The water stress-induced increase in ABA also involves *de novo* gene expression (Guerrero and Mullet 1986). It is possible, however, that a different chemical signal is transmitted by the roots of other species. The stomata of *Phaseolus* respond to soil drying before ABA levels in the xylem increase (Trejo and Davies 1991). Nonetheless, it is clear that stomatal closure is mediated by ABA from surrounding mesophyll cells.

High salt conditions produce an osmotic effect which results in a water stress to the plant and the concomitant increase in ABA. This increase in ABA is sufficient to account for the stimulation of some genes by ABA (Mundy and Chua 1988). The stimulation of the Em transcript by NaCl in rice suspension cells appears to result from both an increase in ABA as well as an increased sensitivity to ABA (Bostock and Quatrano 1992). The synergistic effects of NaCl and ABA on the expression of Em in these experiments suggest that NaCl may have an effect on components of the ABA

signal transduction pathway. However, at least some of the plant's responses to salt stress are unlikely to be mediated by ABA. The salt-stimulated increase in PEP carboxylase in dark-grown plants of *Mesembryanthemum crystallinum* is unaffected by inhibition of ABA biosynthesis (Thomas *et al.* 1992). Another example is the water stress-stimulated protein osmotin, which accumulates in tobacco cells incubated with NaCl. Although both ABA and NaCl stimulate accumulation of the osmotin transcript, the protein accumulates only in NaCl-treated cells and tissues. Furthermore, the accumulation of osmotin transcript in seedlings older than 2 months is sensitive to NaCl but insensitive to ABA (LaRosa *et al.* 1991). These results suggest that ABA and salt may stimulate gene expression *via* different signal transduction pathways.

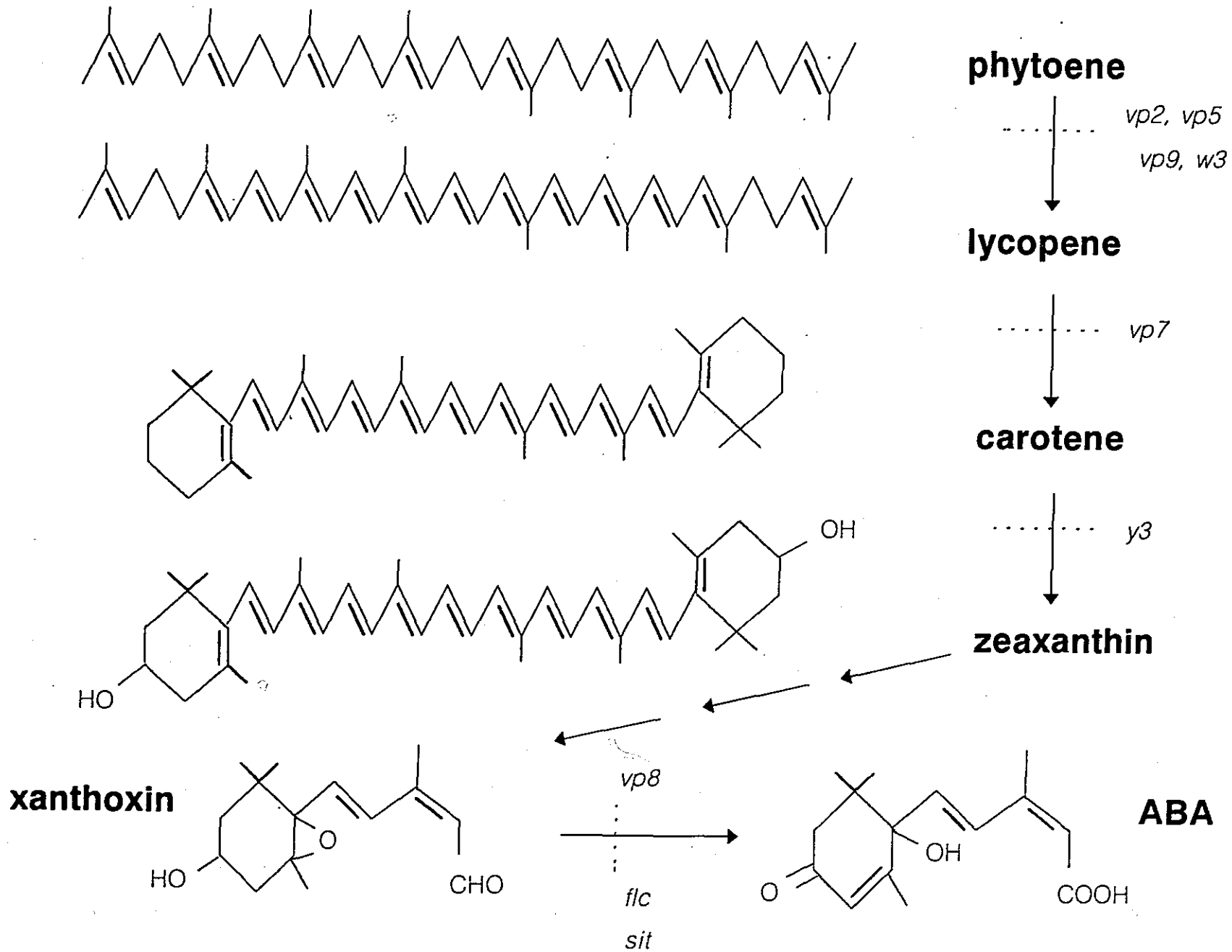
Abscisic acid has also been implicated in the plant's response to freezing stress. During cold-acclimation, endogenous ABA increases in the plant (Capell and Dörffling 1989) . The increase in ABA is greatest in cold-tolerant varieties. Furthermore, exogenous ABA has been shown to increase the survivability of non-acclimated plants to freezing (Irving and Lanphear 1968, Lee *et al.* 1992, Mohapatra *et al.* 1988, Tanino *et al.* 1990). When the ABA levels in *Brassica napus* suspension cells were manipulated using the ABA stimulator mefluidide, the ABA inhibitor fluridone, and exogenous ABA it was found that the resulting ABA levels in the cells correlated with the degree of freezing tolerance (Zhang *et al.* 1986, Ryu and Li 1989, Johnson-Flanagan *et al.* 1991). These experiments indicate that increases in ABA levels are necessary for the induction of freezing tolerance in *B. napus* suspension cells. Abscisic acid alone, however, appears unlikely to be the sole mediator of cold-acclimation. Abscisic acid-treated alfalfa does not

exhibit the survivability of cold-acclimated plants (Mohapatra *et al.* 1988). Furthermore, the set of proteins synthesized in ABA-treated plants is different from that of cold-acclimated plants (Mohapatra *et al.* 1987b, 1988) and several cold-acclimation specific genes are not responsive to ABA (Mohapatra *et al.* 1987a). Thus, although it is clearly involved in cold-acclimation, ABA alone may not be sufficient to elicit the full response to cold.

When damaged by insect predation or mechanical wounding the plant responds by the deposition of callose, lignin (Vance *et al.* 1980) and hydroxyproline-rich glycoproteins at the site of damage (for a general review, see Bowles 1990). Wounding also results in the systemic production of antimicrobial phytoalexins (Jahnen and Hahlbrock 1988), proteinase inhibitors (Green and Ryan 1972, Brown and Ryan 1984), chitinases (Legrand *et al.* 1987), glucanases (Kauffmann *et al.* 1987), aminopeptidase, and a threonine dehydratase (Hildmann *et al.* 1992). It is likely that oligosaccharides derived from the damaged cell wall function as the primary signal in the wound response. Endopolygalacturonidases purified from the fungus *Rhizopus stolonifer* and from tomato were found to release oligogalacturonides from the cell wall of tomato. These oligosaccharides elicit the accumulation of the wound-inducible proteinase inhibitors I and II (Bishop *et al.* 1981). Thus, the cell wall could be viewed as the receptor of the signal (wounding or hydrolases) and the resulting oligosaccharides would be the second messengers. Wounding, fungal elicitors (cell wall fragments), and infection cause the rapid transcriptional stimulation of phenylalanine ammonia lyase and chalcone synthase, key enzymes in the biosynthetic pathways of lignins and phytoalexins (Lawton and Lamb

1987). The rapidity of the response to elicitor (2-3 mins) indicates that either elicitor-receptor complexes interact directly with regulatory DNA sequences or that elicitor activates a short signal transduction cascade which modulates the activity of a transcriptional activator. A slower but more prolonged stimulation of hydroxyproline-rich glycoproteins is observed, indicating a second mechanism of regulation. Since oligogalacturonides have limited mobility within the plant it is likely that a secondary signal is involved in the systemic response. There exist several candidates for secondary signal molecules. These include salicylic acid (Ward *et al.* 1991), ABA and jasmonic acid. Upon wounding in tomato and potato, the endogenous concentration of ABA has been shown to increase both locally and systemically (Peña-Cortés *et al.* 1989). Abscisic acid stimulates the expression of proteinase II inhibitor (Peña-Cortés *et al.* 1989) which may be involved in the plant's defense against herbivorous attack. Wound-inducible transcripts also accumulate in non-wounded plants sprayed with 50 µM ABA. Furthermore, ABA-deficient mutants fail to accumulate these transcripts upon wounding, but do so when treated with exogenous ABA. Although exogenous methyl jasmonate also stimulates these genes, water stress does not (Hildmann *et al.* 1992). Thus it appears that ABA and jasmonic acid are part of a response cascade which results in the systemic response to infection or wounding.

Figure 2. Indirect Pathway of Abscisic Acid Synthesis. The theoretical biosynthetic pathway of ABA from carotenoid precursors in shown. Broken lines indicate possible points of lesion in maize viviparous mutants (After Smith *et al.* 1989).


phytoene
vp2, vp5
vp9, w3
lycopene
vp7
carotene
y3
zeaxanthin
OH
HO
xanthoxin
HO
O
CHO
vp8
flc
sit
OH
O
COOH
ABA

1.3 The Role(s) of Abscisic Acid During Embryogenesis

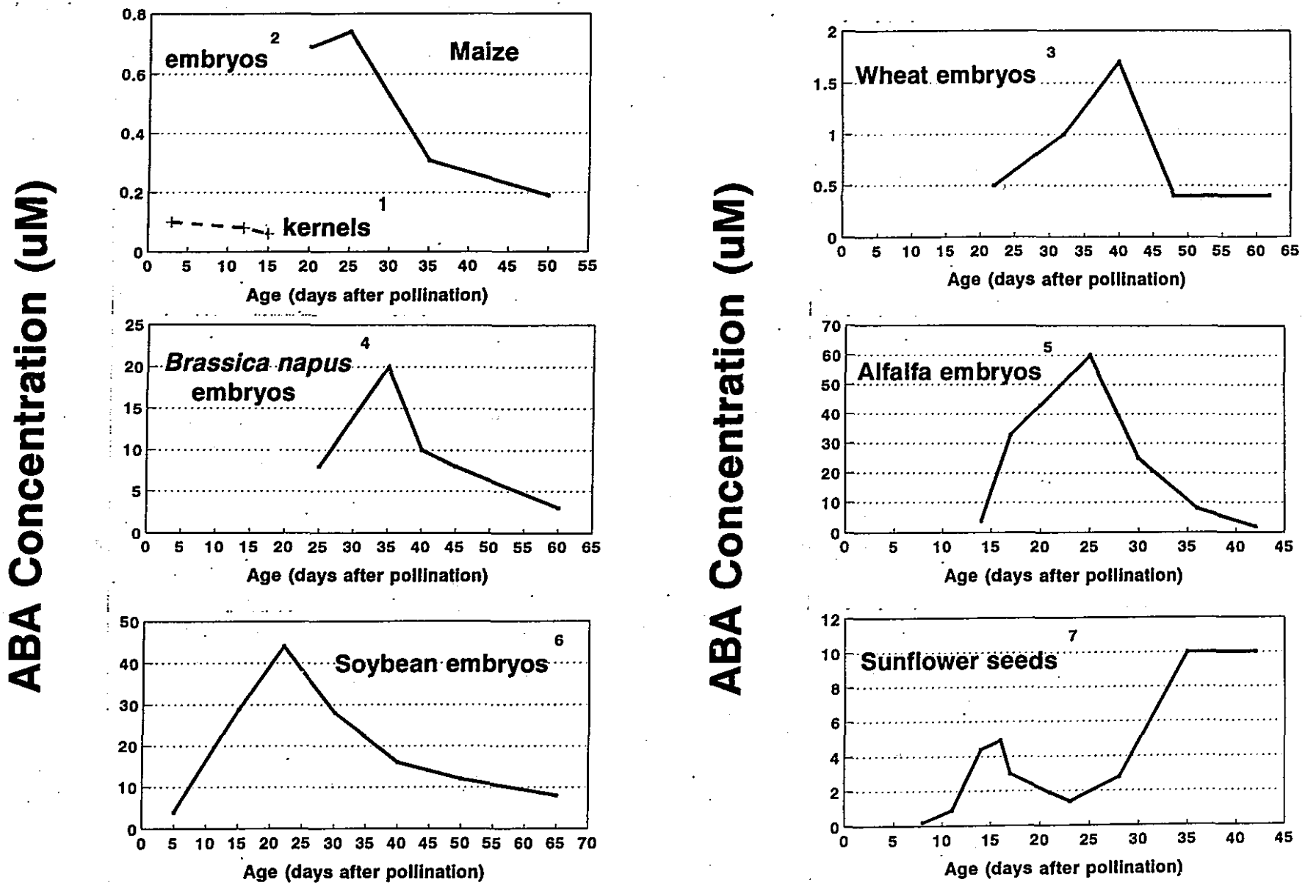
ABA is a sesquiterpene (Figure 2) and is, therefore, ultimately synthesized from mevalonate. Although the path(s) by which ABA is synthesized have not been demonstrated in higher plants, there is good evidence that it derives from cleavage of carotenoid precursors (for a review, see Zeevaart and Creelman 1988, Milborrow 1978). Study of carotenoid accumulation in ABA-deficient mutants suggests the biosynthetic pathway proceeds from phytoene through several intermediates (Smith *et al.* 1989) to xanthoxin (Parry *et al.* 1988) and finally to ABA. Since carotenoids are abundant in plants, the cleavage of a xanthophyll precursor is probably a key regulatory point in ABA synthesis.

The primary site of ABA synthesis within the cell is located in the cytoplasm (see references cited in Hartung and Slovik 1991) although isolated plasmids are also able to synthesize ABA (Milborrow 1974, Loveys 1977). Most or all tissues of the plant have the capacity to produce ABA (see section 1.2) including, in cereals, the cob (Hole *et al.* 1989), embryo and endosperm (Gage *et al.* 1989, Milborrow and Robinson 1973). Although the seed is an important source of ABA, it has been estimated that 50-70% of ABA in the embryo during mid-embryogenesis derives from maternal synthesis (Smith *et al.* 1989).

The high level of ABA accumulated during mid embryogenesis must be reduced prior to germination. Abscisic acid appears to be metabolized by the same pathways in the seed as elsewhere in the plant (for a review, see Zeevaart and Creelman 1988). Abscisic acid is degraded to phaseic acid and dihydrophaseic acid.

Figure 3. The Concentration of Embryonic ABA during Development for Several Species of Flowering Plants. Graphical representations of the ABA concentration of several angiosperm embryos during development. See Appendix 2 for a more comprehensive tabulation of data from these and other species.

1 Carnes and Wright 1988
2 Neill *et al.* 1987
3 Walker-Simmons 1987
4 Finkelstein *et al.* 1985, 1987
5 Xu *et al.* 1990
6 Ackerson 1984a, 1984b
7 Goffner *et al.* 1990


ABA Concentration (uM)
embryos
2
Maize
1
kernels
Age (days after pollination)
Brassica napus
embryos
4
Age (days after pollination)
Soybean embryos
6
Age (days after pollination)
ABA Concentration (uM)
Wheat embryos
3
Age (days after pollination)
Alfalfa embryos
5
Age (days after pollination)
Sunflower seeds
7
Age (days after pollination)

However, the decline in free ABA in seeds of *Phaseolus vulgaris* (Hsu 1979) and wheat (King 1976) coincides with the increase in the ABA glucosyl ester, suggesting that ABA in seeds is largely inactivated by conversion to the "bound" state.

The concentrations of ABA fluctuate during embryogenesis. Because published data on ABA content is given in various terms such as mass per seed, mass per wet weight and moles per dry weight it is often difficult to compare results from different researchers. In order to facilitate comparison of such data I have estimated the concentration of ABA during embryogenesis in several species of angiosperms. A detailed compilation of the published data and the assumptions used in my estimations can be found in Appendix 2. Figure 3 shows a graphical representation of my estimation of ABA concentrations in the embryos of several of these species. As can be seen in Figure 3, ABA content varies widely between species, with dicots exhibiting maximum concentrations between 10 µM and 150 µM and monocots between 0.6 and 2.5 µM. Even within a species ABA levels varies considerably between cultivars (Hocher *et al.* 1991).

Despite differences in magnitude, a general pattern of ABA accumulation is observed. The concentration increases rapidly during the transition from early embryogenesis to mid embryogenesis, reaches peak concentrations during mid embryogenesis, then declines during late embryogenesis. A second peak is observed in some dicots. In general, the increase in ABA parallels, more or less, the increase in dry and fresh weight of the embryo (Black 1983, Hocher *et al.* 1991). The subsequent decline of free ABA precedes by several days or coincides with net water loss. In other species, particularly in varieties with dormant seeds such as *Pyrus malus* (Le Page-Degivry and

Bulard, 1979), *Fraxinus* (Sondheimer *et al*, 1968), *Rosa* (Jackson, 1968), and *Arachis hypogaea* (Narasimhareddy and Swamy, 1979), the concentration of ABA remains high at maturity.

1.3.1 Abscisic Acid Stimulates Accumulation of Macromolecules

One important function of ABA is to regulate the flow of nutrients within the plant. It has been shown, for example, that photosynthates accumulate in roots (Karmoker and Van Steveninck 1979) and empty seed coats treated with ABA (Ross *et al.* 1987). These results suggest that ABA directs the site of photosynthate unloading. Since the effect is observed within 10 minutes of addition of ABA, it is likely ABA has a direct effect on the unloading system and does not change the amount or nature of the system (Ross *et al.* 1987). These results suggest that endogenous ABA may be involved in regulating the supply of biosynthetic precursors to the embryo during the rapid growth phase and to the root system during water stress.

The rate of accumulation of plant storage proteins is greatest during mid embryogenesis when ABA levels are high and decreases as ABA levels drop. Experimental evidence suggests that ABA regulates the accumulation of many storage proteins. Many of the embryonic genes stimulated by exogenous ABA are known to encode storage proteins (Table 5). Genetic evidence also supports a regulatory role for ABA. Embryo storage proteins do not accumulate in ABA-insensitive mutants of maize (Kriz *et al.* 1990) or *Arabidopsis* (Finkelstein and Somerville 1990) and ABA-deficient maize embryos exhibit reduced storage protein expression (Kriz *et al.* 1990). Interestingly, the ABA-deficient *aba* mutant of *Arabidopsis*, while exhibiting only 5% of the wild type

Table 5. Regulation of Seed Genes by ABA and Environmental Stresses. The effects of ABA and environmental stresses such as high salt, cold, desiccation and high osmoticum on the accumulation of mRNA (m), protein (p), or both (b) for various angiosperm seed genes. "+" indicates stimulation, "-" indicates no stimulation, a blank space indicates not determined.

1 Kriz *et al.* 1990, Rivin and Grudt 1991
2 Vance and Huang, 1988
3 Qu *et al.* 1990
4 This thesis
5 Thomann *et al.* 1992
6 Williams and Tsang 1992, this thesis
7 Williams and Tsang 1991, this thesis
8 Gómez *et al.* 1988
9 Pla *et al.* 1989, Vilardell *et al.* 1990, this thesis
10 Espelund *et al.* 1992
11 Close *et al.* 1989
12 Litts *et al.* 1987, Williamson and Quatrano 1988
13 Triplett and Quatrano 1982, Cammue *et al.* 1989, Mansfield and Raikhel 1990
14 Williamson and Quatrano 1988, Berge *et al.* 1989
15 Litts *et al.* 1992
16 Mundy and Chua 1988, Yamaguchi-Shinozaki *et al.* 1989
17 Mundy and Chua 1988
18 Kusano *et al.* 1992
19 Holbrook *et al.* 1992
20 Finkelstein *et al.* 1985, 1987, DeLisle and Crouch 1989, Taylor *et al.* 1990
21 Harada *et al.* 1989
22 Finkelstein and Somerville 1990
23 Raynal *et al.* 1989
24 Eisenberg and Mascarenhas 1985
25 Bray and Beachy 1985, 1987, Eisenberg and Mascarenhas 1985
26 Barrat and Clark 1991
27 Goffner *et al.* 1990
28 Almoguera and Jordano, 1992
29 Baker *et al.* 1988
30 Cohen *et al.* 1991
31 Choi *et al.* 1987, Kiyosue *et al.* 1992a, 1992b
32 Goldmark *et al.* 1992
33 Mohapatra *et al.* 1988
34 Singh *et al.* 1987, LaRosa *et al.* 1989

Table 5. Regulation of Seed Genes.

SPECIES	GENE NAME	LEVEL	RESPONSIVE E	V	I	C	AGENTS WHICH STIMATE ACCUMULATION: ABA	SALT	COLD	DES.	OSM.	REF	
maize	GLB-1	m	+		+		+					1	
	oleosinKD16	m			+		+					2	
	oleosinKD18	m			+		+					3	
	oleosinKD18	m	+	-			+			-		4	
	MLG3	p	+	+			+			+	+	5	
	Emb5	m	+	-			+	-		-		6	x
	Emb564	b	+	-			+	-		-		7	x
	pMAH9	m	+	+			+			+		8	
	Rab-17	b	+	+		+	+			+		9	y
barley	B19.1	m	+				+	+	-		+	10	x
	B19.3; 19.4	m	+				+	-	-		+	10	x
	pHVA39	m		+						+		11	y
wheat	7s globulin	m			+		+					12	
	WGA	p	+	+	+		+				+	13	
	Em	m	+	+			+			+		14	x
rice	Emp1	m			+		+					15	x
	rab16A; B; C	m	+	+		+	+	+				16	y
	rab16D	m	-	+		+	+	+				17	y
	RAB 21	b	+	+		+	+	+		+		17	y
	rab25	m		+		+	+				+	18	y
B. napus	oleosinKD20	p	+				+					19	
	napin	b	+				+					20	
	cruciferin	b	+				+				+	20	
	pLea76	m	+s	+			+			+		21	
Arabidop.	cruciferin	m	+				+					22	
radish	p8B6	m	+							+		23	x
soybean	glycinin	m	+				+					24	
	conglycinin	b	+				+				+	25	
pea	ABR17	b	+	+			+			+		26	
	ABR18	b	+				+					26	
sunflower	helianthinin	b	+				+				+	27	
	Ha ds10;ds11	m	+	+			+				+	28	
cotton	Lea4D19	m	+				+				+	29	x
	LeaAD132	m	+				+				-	29	x
	Lea D11	m	+				+				+	29	y
tomato	pLE25	m	+	+			+	+	-	+	-	30	
carrot	ECP31	m	+				+					31	
Bromus secalinas	pBS128	m	+				+					32	
alfalfa	pSM1409	m		+			+		+	+		33	
tobacco	osmotin	p		+r		+	+	+				34	

b, mRNA and protein; C, cultured cells; DES., desiccation; E, embryo; I, imbibed mature embryos; m, mRNA; OSM., high osmoticum; p, protein; r, roots; s, somatic embryos; V, vegetative tissue; WGA, wheat germ agglutinin; x, y, members of 2 homologous groups

level of ABA, exhibits no reduction in accumulated cruciferin (Karssen *et al.* 1983). This may imply that ABA accumulation in *Arabidopsis* seeds exceeds levels necessary for cruciferin stimulation. However, the endosperm storage proteins, which appear during early embryogenesis, are not responsive to ABA.

Abscisic acid appears to affect the nature of seed storage lipids. Abscisic acid stimulates the precocious accumulation of eicosenoic acid (20:1) and erucic acid (22:1) in zygotic (Finkelstein and Somerville 1989) and microspore-derived embryos (Holbrook *et al.* 1992) of *Brassica napus*. It has also been shown that the seeds of the ABA-insensitive *abi3* mutant of *Arabidopsis* accumulate reduced levels of eicosenoic acid. It appears that ABA regulates fatty acid elongation in these seeds since elevated levels of precursor 18:1 and alternate product 18:2 are found. The difference is detected as early as 5 DAP (Finkelstein and Somerville 1990) indicating that the pathway(s) affected by the *abi3* lesion are active prior to the rise in embryonic ABA levels.

Exogenous ABA stimulates the precocious accumulation of many Lea transcripts. As can be seen in Table 5, some Lea genes can also be induced in vegetative tissues by ABA, water stress, high salinity and cold. A few are responsive only during embryogenesis. The accumulation of some LEA proteins during embryogenesis coincides with desiccation tolerance (Bartels *et al.* 1988). This correlation and the expression of LEA proteins in vegetative tissues during water stress suggests that the role of these proteins during embryogenesis may be related to the process of desiccation tolerance.

While numerous genes involved in embryogenesis are stimulated by ABA, gibberellic acid-stimulated genes are inhibited by ABA. Thus, many of the genes

expressed during germination are inhibited by ABA. The most intensively studied of these are the α-amylase genes of barley aleurone (for a review, see Jacobsen and Chandler 1987). Other ABA-inhibited genes include the chlorophyll a/b binding protein genes of soybean (Chang and Walling 1991) and several barley genes which accumulate during germination (Liu *et al.* 1992).

1.3.2 Abscisic Acid and Desiccation Tolerance

Several lines of evidence suggest ABA is involved in establishing desiccation tolerance. First, desiccation tolerance is acquired when ABA levels are high, prior to the onset of maturation drying (Bartels *et al.* 1988, Finkelstein *et al.* 1987, Kermode and Bewley 1987, Oishi and Bewley 1990). Second, exogenous ABA induces desiccation tolerance prematurely in embryos of maize (Bochicchio *et al.* 1988, 1991), barley (Bartels *et al.* 1988), white spruce seeds (Attree *et al.* 1991) and the somatic embryos of alfalfa (Senaratna *et al.* 1989). Furthermore, the ABA-deficient, ABA-insensitive double mutant (*aba*, *abi3*) of *Arabidopsis* fails to acquire desiccation tolerance unless the roots of the mother plant are incubated in ABA (Koorneef *et al.* 1989) or the developing seeds are treated with exogenous ABA prior to desiccation treatment (Meurs *et al.* 1992).

Although the mechanism of desiccation tolerance is unknown it has been suggested that soluble sugars and proteins are involved. Sucrose, for example, protects membranes from rehydration damage (Crowe *et al.* 1989) and is unloaded in tissues treated with ABA (see section 1.3.1). It has also been suggested that the ABA-induced soluble proteins such as the desiccation-related proteins in *Craterostigma plantagineum* provide protection from dehydration damage (Bartels *et al.* 1990).

Abscisic acid may also be involved in the regulation of seed drying. The *aba, abi3* double mutant of *Arabidopsis* fails to desiccate unless supplied with exogenous or maternal ABA (Koorneef *et al.* 1989). Exogenous ABA has also been reported to induce dehydration of wheat grain (King *et al.* 1976).

1.3.3 The Role of Abscisic Acid in the Inhibition of Precocious Germination

Although embryos of many species become competent to germinate during mid embryogenesis, this event does not normally take place until after the seed has completed the maturation process and has undergone a period of storage. Precocious germination does occur naturally in the mangrove, in viviparous mutants of several species, and can be induced in seeds by various manipulations.

The immature embryos of cotton (Ihle and Dure 1972), *Phaseolus vulgaris* (Sussex and Dale 1979), *Brassica* (Crouch and Sussex 1981, Finkelstein *et al.* 1985), alfalfa (Xu *et al.* 1990), maize (Neill *et al.* 1987), wheat (Triplett and Quatrano 1982, Morris *et al.* 1988), and barley (Morris *et al.* 1988) germinate precociously when excised from the seed during mid embryogenesis and kept moist. This suggests that the tissues surrounding the embryo somehow inhibit germination. When incubated in the presence of surrounding structures, excised embryos do not germinate, suggesting that diffusible factors are involved (Xu *et al.* 1990). Evidence suggests that endogenous ABA is the diffusible factor involved in the inhibition of precocious germination. There is a strong correlation between the concentration of ABA at the time of excision and the lag before isolated embryos of *Brassica napus* germinate on basal medium (Finkelstein *et al.* 1985). Furthermore, large decreases in the endogenous concentration of ABA of isolated embryos of wheat and

Table 6. The inhibitory effect of ABA on precocious germination of isolated embryos. A more detailed tabulation of data can be found in Appendix 3

SPECIES	EMBRYO AGE	[ABA] (μM)	GERMINATION (%)
maize (1)	15-25 DAP	10	0
	27-35 DAP	0	0
	40 DAP	10	10
	42 DAP	50	0
	45 DAP	10	20
	50 DAP	10	40
	55 DAP	10	100
wheat (2)	25-35 DAP	5	0
	48 DAP	5	25
	60 DAP	5	75
alfalfa (3)	Stage III	0	85
	Stage VI	0	20
	Stage IX	0	100
	St.III-VII	10	0
lettuce (4)	mature	3	0
Brassica napus (5)	0-40 DPA	1	0
soybean (6)	50-100 mg	1	0
	150-200 mg	1	15
	450-500 mg	1	40
	all stages	10	0
Haplopappus gracilis (7)	seeds	10	0

1 Neill *et al.* 1987; Rivin and Grudt 1991
2 Walker-Simmons 1987
3 Xu *et al.* 1990
4 Black *et al.* 1974
5 Finkelstein *et al.* 1985, 1987
6 Eisenberg and Mascarenhas 1985
7 Galli *et al.* 1979

barley (Morris *et al.* 1988), and of *Brassica napus* (Finkelstein *et al.* 1985) are observed during precocious germination in vitro. These losses appear to be due to leaching of ABA into the surrounding medium.

As shown in Table 6 and Appendix 3, physiological concentrations of exogenous ABA prevent the precocious germination of early and mid stage embryos. This appears to be due to diffusion of ABA down its concentration gradient since the endogenous concentration of ABA in soybean embryos reflects exogenous ABA levels (Chang and Walling 1991). Exogenous ABA has been shown to be taken up and metabolized by isolated embryos of maize (Robichaud and Sussex 1987). Moreover, exogenous ABA appears to affect isolated embryos in a manner similar to the natural environment of the seed. In wheat (Triplett and Quatrano 1982), barley (Bartels *et al.* 1988), and maize (Fong *et al.* 1983) abscisic acid allows the accumulation of maturation-specific polypeptides while inhibiting the accumulation of germination-specific polypeptides. Interestingly, the embryo exhibits increasingly reduced sensitivity to ABA during late embryogenesis (see Table 6; Appendix 3).

The embryo's sensitivity to ABA during late embryogenesis decreases as the water content decreases. Several lines of evidence suggest ABA and the osmotic potential of the embryo may act together to prevent precocious germination. The inhibition of germination in muskmelon seeds by ABA and osmotic potential is additive during mid embryogenesis and the sensitivity to both agents declines during late embryogenesis (Welbaum *et al.* 1990). It has been suggested that the water status of the seed inhibits germination during the final stages of seed development (Finkelstein and Crouch 1986, Morris *et al.* 1991).

The reduced sensitivity of the muskmelon seed to ABA and water potential during development suggests a common control point.

While ABA and high osmoticum both inhibit precocious germination, neither alone maintains the normal pattern of gene expression in excised embryos at all stages (Finkelstein *et al.* 1987, Morris *et al.* 1988, Rivin and Grundt 1991, Xu *et al.* 1990). These results may be due to the changing sensitivities of embryonic genes to these agents during development. For example, whereas genes responsive to ABA during mid embryogensis do not respond during late embryogenesis (Rivin and Grundt 1991, Williams and Tsang 1991), several new proteins unique to ABA-treated embryos can be stimulated during late embryogenesis (Xu. *et al.* 1990).

The available data suggest that ABA inhibits precocious germination during mid embryogenesis. As solutes accumulate and water is removed the increasing osmotic potential of the cytoplasm gradually replaces the role of ABA. In mature, non-dormant seeds germination is prevented by the low water content.

A number of hormonal mutants are available which provide excellent materials for investigating the role of ABA in precocious germination. Seed vivipary is the condition characterized by the progression from embryogenesis directly into germination with little or no desiccation or cessation of growth. Vivipary occurs naturally in *Rhizophora mangle*. The embryos of this species are insensitive to exogenous ABA (Sussex 1975). Similarly, the viviparous mutants of *Zea mays* are either insensitive to ABA and/or deficient in ABA biosynthesis. These mutants are all recessive and exhibit pleiotropic effects. The seeds fail to acquire desiccation tolerance, exhibit little water loss and germinate precociously on

the cob (Robertson 1955). Three classes have been defined.

The single class I mutant, *vp1*, exhibits a general failure of seed maturation which culminates in precocious germination of the embryo. The mutant also lacks the purple pigment anthocyanin and exhibits numerous unrelated enzyme deficiencies (Dooner 1985). Embryos of *vp1* exhibit normal levels of ABA (Neill *et al.* 1987). They are, however, insensitive to this hormone during embryogenesis (Robichaud *et al.* 1980, Robichaud and Sussex 1986). The decrease in sensitivity of this mutant to ABA does not result from defects in uptake or metabolism of ABA (Robichaud and Sussex 1987). The *Vp1* gene has been cloned by transposon tagging (McCarty *et al.* 1989) and shown to encode a 2.5 kb seed-specific transcript. Neither the *Vp1* transcript nor the C1 transcript, which encodes a transcriptional activator required for the light-stimulated synthesis of anthocyanins in the scutellum and aleurone (Goff *et al.* 1991), are detected in *vp1* homozygous seeds. While the exact role of VP1 has not yet been determined, it appears to be required for several signal transduction pathways in the seed and may regulate key elements such as an ABA receptor and the C1 protein (McCarty *et al.* 1989). Alternatively, *Vp1* could encode a factor common to both pathways (Hattori *et al.* 1992).

The class II viviparous mutants- represented by *vp2*, *vp5*, *vp7*, *vp9*, *w3*, *y3* and *y9*- exhibit reduced levels of both carotenoids and ABA (Robertson 1955, Neill *et al.* 1987) but are responsive to exogenous ABA (Robichaud *et al.* 1980). All class II mutants except *y3* and *y9* (the *y3* mutant is temperature sensitive while *y9* is leaky) produce lethal, albino seedlings due to the photooxidation of chlorophylls in the absence of carotenoids (Robertson 1975). The study of carotenoid accumulation in homozygous mutant seed has

allowed a tentative identification of the lesions of the class II mutants all of which appear to lie after the synthesis of phytoene (Smith *et al.* 1989, see Figure 2).

Class III mutants- represented by *vp8* and *vp10*- have normal carotenoids but reduced ABA. These mutants appear to accumulate a weak growth inhibitor, possibly xanthoxin, and hence are believed to be blocked in the pathway between ABA and its unidentified precursor (Smith *et al.* 1989).

Evidence suggests that the precocious germination of viviparous mutants is different from normal germination. For example, high levels of the maize storage protein GLB1 and its transcript, which disappear during normal germination, persist during germination of viviparous mutants (Kriz *et al.* 1990). The pattern of proteins synthesized by germinating maize embryos during chemically-induced vivipary is also different from that seen during normal germination (Oishi and Bewley 1992). Since prematurely desiccated wild-type embryos, when germinated, exhibit a pattern of protein synthesis characteristic of normal germination, the transition from embryonic development to normal germination apparently requires more than the removal of the germination inhibitor ABA.

Arabidopsis mutants exhibiting either reduced levels of ABA (*aba*) or reduced sensitivity to ABA (*abi*) have been isolated (Koorneef *et al.* 1984). The *abi* mutants were selected by their ability to germinate on exogenous ABA. The *abi1* and *abi2* mutants are most severely affected during vegetative growth, being prone to wilting and exhibiting reduced ABA-stimulated accumulation of proline. The *abi3* mutant is affected more during embryogenesis, exhibiting reduced accumulation of cruciferin mRNA and protein,

and reduced eicosenoic acid accumulation. None of the single mutants displays vivipary. This is thought to be due to the leaky nature of the mutations (Koorneef *et al.* 1989). On the other hand, seeds of the double mutant (*aba*, *abi3*) exhibit reduced water loss, the absence of brown pigmentation, inhibition of storage protein accumulation, the absence of desiccation tolerance, and germinate precociously inside the silique. Seed development in the *aba*, *abi3* double mutant is normal, however, except for the lack of dormancy, if the roots of the mother plant are incubated in ABA or if the mother plant is *Aba/aba* heterozygous. These results suggest that while maternal ABA may be involved in regulating many events during maturation, embryonic ABA is essential for the prevention of precocious germination.

Mutants lacking normal responses to water stress have been isolated from several species. The X-ray induced tomato mutants *flacca*, *notabilis*, and *sitiens* are ABA deficient (Tal and Nevo 1973, Neill and Horgan 1985). Furthermore, ABA applied to the leaves restores turgor of water-stressed *flacca* plants by permitting stomatal closure and increasing hydraulic conductance (Bradford 1983). Seeds of the *sitiens* mutant germinate precociously (Groot and Karssen 1992). Several of these mutants are blocked just after or just before xanthoxin, suggesting that at least in tomato ABA is synthesized from xanthoxin (Parry *et al.* 1988).

The *droopy* mutant of potato (Quarrie 1982) and the *wilty* mutant of pea (Wang *et al.* 1984) accumulate reduced levels of ABA during water stress. The *droopy* mutant also produces seeds which germinate prematurely (Quarrie 1982). These phenotypes, particularly of the tomato *sitiens* and the potato *droopy* mutants, demonstrate a strong

correlation between precocious germination and reduced endogenous ABA.

Vivipary can be induced in plants by premature drying and by treatments with chemicals such as fluridone which inhibit ABA biosynthesis. Although both drying and fluridone have been shown to result in similar reductions of the ABA content of immature maize kernels (Oishi and Bewley 1992), prematurely dried kernels germinate earlier than fluridone treated kernels (Oishi and Bewley 1990). Moreover, artificial drying of immature muskmelon seeds results in decreased sensitivity to both ABA and osmotic potential (Welbaum *et al.* 1990). These results suggest that desiccation "shuts down" the ABA-dependent inhibition of germination by reducing the levels of ABA and reducing the embryo's sensitivity to ABA. This is likely an important step in the transition from embryonic to vegetative development.

Molecular evidence also suggests that drying may be required to effect the transition from embryonic to vegetative gene expression. The proteins synthesized during the germination of prematurely dried embryos are similar to those synthesized during normal germination. Fluridone-induced viviparous embryos, on the other hand, synthesize some embryonic and some germinative proteins. When these embryos are isolated and incubated on basal medium for 24 hours their protein synthesis switches to the germinative set. Moreover, the ABA content of prematurely-dried embryos is greater than that of the fluridone-treated embryos (Oishi and Bewley 1992). These results suggest that something other than ABA is also involved in the regulation of the switch from embryonic to germinative development. It has been suggested that drying acts as the switch that turns off embryo-specific genes and potentiates germination-specific genes

(Misra *et al.* 1985). If this is the case, it would provide a mechanism by which the developmental program of the seed could optimize embryo growth in response to its environment.

1.3.4 Abscisic Acid and Seed Dormancy

Mutational analyses suggest that normal levels of ABA and a functional ABA response system are required for the induction of dormancy. The ABA-deficient (*aba*) or ABA-insensitive (*abi*) mutants of *Arabidopsis* exhibit reduced, delayed, or no dormancy (Koorneef *et al.* 1989). Furthermore, exogenous ABA prevents germination of the ABA-deficient seeds (Karssen *et al.* 1983). It has long been noted that seeds which contain little ABA at maturity exhibit little or no dormancy (for a review, see Black 1983). However, no good correlation can be made between ABA content and dormancy. In some species, sensitivity to ABA is more important in determining dormancy than is ABA concentration (Walker-Simmons 1987).

Experimental evidence supporting a role for ABA in the maintenance of dormancy is inconclusive. It is clear that diffusible inhibitors are involved in maintaining dormancy in *Rosa arvensis* (Jackson 1968), *Taxus baccata* (LePage-Degivry 1968, 1970) and *Acer pseudoplatanus* (Webb and Wareing 1972). Although embryo extracts inhibit germination of these washed embryos, the concentration of exogenous ABA required to inhibit germination often exceeds the physiological range (Webb and Wareing 1972). These results suggest that ABA alone is unlikely to be the endogenous inhibitor in these seeds.

The mechanisms by which ABA inhibits germination are uncertain. Abscisic acid reduces overall RNA and protein synthesis in imbibed mature embryos of wheat (Chen

and Osborne 1970) and lettuce (Fountain and Bewley 1976). It seems likely that much of this reduction is due to the specific inhibition of germination-specific transcripts. Abscisic acid specifically inhibits the accumulation of enzymes associated with germination (Dure 1975). In particular, many of the enzymes involved in mobilization of stored food reserves during germination are stimulated by gibberellic acid and inhibited by ABA (Jacobsen and Chandler 1987). These include carboxypeptidases and cysteine-proteases (San Segundo *et al.* 1990) and α-amylases (Chrispeels and Varner 1966, Chandler *et al.* 1984, Nolan and Ho 1988, O'Neill *et al.* 1990). Regulation of α-amylase activity by gibberellic acid and ABA occurs at least partly at the transcriptional level (Jacobsen and Beach 1985). Maximum α-amylase activity in maize aleurone depends both on the absence of inhibition by ABA and the competence of the tissue to respond to gibberellic acid. Seed drying results in both (Oishi and Bewley 1990). A second mechanism of control of reserve mobilization by ABA has been found in the ABA-stimulated accumulation of inhibitors of α-amylase and trypsin (Barber *et al.* 1986, Leah and Mundy 1989).

It is unknown whether the inhibitory effect of ABA on seed germination is the result of altered gene expression or due to some other mechanism. It has been suggested that ABA inhibits germination by restricting the uptake of water (Dure 1975, Walbot 1978) possibly by modulating the activity of membrane pumps (Ballarin-Denti and Cocucci 1979).

1.4 Mechanism of Abscisic Acid Action

Despite the fact that plants are known to respond to numerous environmental stimuli, little is known about how plants sense primary signals. At the present time, phytochrome is the only well-characterized plant receptor. Researchers have examined plants for the elements known to be involved in signal transduction in animals. In animals, hormones act by one of two general mechanisms. Steroid hormones bind directly to transcriptional factors to regulate gene expression (Beato 1989). Other hormones interact with receptors on the plasmalemma with the result that a second messenger is generated within the target cell (Deutsch *et al.* 1988). The available evidence suggests that Ca^{2+}, inositol phosphates and diacylglycerol may act as second messengers in plants. Although Ca^{2+} is clearly involved in some ABA responses (see section 1.4.1) the ABA signal transduction pathway remains largely unclear.

There appear to be two classes of response to ABA. The rapid response occurs within 5 minutes, involves changes in K^+ ion flux, and is specific to the normally occurring (+)-enantiomer. The best known example of a rapid response is stomatal closure (for a review, see Van Steveninck and Van Steveninck 1983). The slow response generally occurs after 30 minutes, appears to require changes in gene expression, and is elicited by either (+)- or (-)-ABA. Examples include inhibition of seedling growth and the induction of seed storage proteins (for a review, see Zeevaart and Creelman 1988). It should be noted, however, that both slow and rapid responses appear to have elements in common. Both the *abi1* and *abi2* mutants of *Arabidopsis* exhibit deficiencies in the ABA-dependent inhibition of both seedling growth and stomatal closure (Finkelstein and

Somerville 1990).

Abscisic acid-deficient and ABA-insensitive mutants provide a promising approach to the study of the ABA-response pathway. Although the functions of the affected genes have not been elucidated yet, it is likely that the identification of the wild-type loci using transposon tagging and the eventual use of complementation will help elucidate elements of the pathway(s). The first molecular analysis of a plant hormone response mutant was the cloning, via transposon tagging, of the maize *Vp1* gene (McCarty *et al.* 1989). As reviewed in section 1.3.3, mutants at the *Vp1* locus exhibit seed-specific pleiotropic effects due to a reduced sensitivity to ABA. In wild-type embryos the *Vp1* transcript is abundant in early embryogenesis prior to the increase in ABA (McCarty *et al.* 1989). Since both ABA and VP1 are required for the expression of the regulatory gene *C1*, it has been suggested that VP1 interacts with a set of subordinate regulatory genes, such as *C1*, which control elements of signal transduction pathways for light and ABA (McCarty *et al.* 1989). Alternatively, the *Vp1* gene product might be a tissue-specific component of signal transduction common to both pathways. It has also been hypothesized that ABA binds to VP1 and the resulting complex stimulates transcription of target genes (Smith *et al.* 1989).

1.4.1 Abscisic Acid Receptors and Second Messengers

The first step of ABA action must involve the interaction of ABA with specific receptors. The naturally occurring cis(+)ABA and physiologically active homologs have been shown to bind to the plasmalemma (Hornberg and Weiler 1984). This binding appears to be mediated by a protein since it is prevented if the protoplasts are first treated

mildly with trypsin. Abscisic acid-binding proteins have been identified on the surface of guard cell plasmalemma by ultraviolet crosslinking studies (Hartung 1983, Hocking *et al* 1978, Hornberg and Weiler 1984). Although the estimated dissociation constants (3-4 nM, 35 nM) are consistent with the physiological concentrations of ABA required for response, conclusive evidence that any of these proteins functions as part of an ABA signal transduction system is lacking.

The C1' carbon of the ABA molecule has 4 different constituents and is enantiomeric. Only the naturally-occurring (+)-enantiomer is active in the closure of barley stomata (Cummins and Sondheimer 1973). However, both enantiomers inhibit the gibberellic acid-induced growth of wheat embryos and production of α-amylase (Walton 1983). The configuration of the constituents about the double bonds at C2 and C4 define 4 ABA isomers. Only the C2-*cis*, C4-*trans* isomer of ABA activates stomatal closure (Milborrow 1978). The stringent requirement of the guard cell response for the (+)-cis,trans isomer is clearly indicative of a specific receptor for ABA. The ability of both (-)- and (+)-ABA to inhibit plant growth, however, suggests that different receptors may be used by cells other than guard cells. At this time, evidence suggests Ca^{2+} is a second messenger for the ABA response. None of the other classical second messengers have been shown to be involved.

Calcium plays an important role in regulating growth and development in plants (For reviews, see Hepler and Wayne 1985, Trewavas 1986, Poovaiah and Reddy 1987). Increases in the level of calcium are involved in cell division (Wolniak *et al.* 1983), phytochrome-mediated events (Roux *et al.* 1986), secretion (Jones and Carbonell 1984,

Akazawa and Mitsui 1985) and tropic responses (Evans 1985, Pickard 1985). Cytoplasmic Ca^{2+} increases within one minute of addition of exogenous ABA to intact guard cells of *Commelina communis* (McAinsh *et al.* 1990, 1992) and maize coleoptiles (Gehring *et al.* 1990). Since cytoplasmic Ca^{2+} modulates the activity of anion and cation channels involved in regulating stomatal turgor (Schroeder and Hagiwara 1989) Ca^{2+} may be acting as a second messenger in guard cells. It is unknown whether the Ca^{2+} originates from apoplastic and/or intracellular stores. Evidence for both an apoplastic site (Schroeder and Hagiwara 1990) and an intracellular site (Gilroy *et al.* 1991) have been reported.

Circumstantial evidence indicates that higher plants utilize a transmembrane signaling system involving the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) similar to that of animal cells. The light-induction of pulvinus folding in *S. saman* is accompanied by an increase in IP_3 and diacylglycerol (DAG) and a decrease in PIP_2 (Morse *et al.* 1987, 1989). Similar changes in PIP_2, DAG and IP_3 were observed in auxin-treated *C. roseus* cultured cells (Ettlinger and Lehle 1988). Exogenous IP_3, but not IP or IP_2, causes the release of Ca^{2+} from maize coleoptile microsomes (Reddy and Poovaiah 1987) and *Avena* root vacuoles (Schumaker and Sze 1987). Guard cells are also responsive to IP_3 (Blatt *et al.* 1990, Gilroy *et al.* 1990) and DAG (Lee and Assman 1991). These findings suggest that DAG and IP_3 may act as second messengers in plant cells.

Other elements of a phospholipid signalling system also appear to exist in plant cells. Plant plasmalemma proteins which react with G-protein-specific antibodies also bind GTP and GTPγS, but not other NTPs (Blum *et al.* 1988) suggesting they may be plant G-proteins. Furthermore, PIP_2-specific, Ca^{2+}-stimulated (Einspahr *et al.* 1989, McMurray

and Irvine 1988, Melin *et al.* 1987) and guanine nucleotide-stimulated phospholipase C activity are exhibited by plant membranes (Dillenschneider *et al.* 1986, Einspahr *et al.* 1989). Higher plants also appear to have protein kinases requiring Ca^{2+}, phospholipid and DAG for maximum activity (Elliot and Kokke 1987, Elliot *et al.* 1987). Thus, with the exception of membrane-bound receptors, all the components of an inositol phosphate signaling system have been detected in plants. What remains to be demonstrated is the interaction of these elements to transduce an external signal into a cellular response.

There is little evidence at the present time for a cyclic AMP (cAMP) second messenger systems in plants. Neither adenylate cyclase (Yunghaus and Morré 1977) nor cAMP-dependent protein kinase have been found in plants (Brown and Newton 1981, Salimath and Marmé 1983). Although cAMP has been detected in higher plants (Brown and Newton 1981) it has not been shown to be essential for any physiological response (Hepler and Wayne 1985). It is interesting to note, however, that the central motif of the cAMP response element (CACGTG) is shared by the ABA-response element and occurs in numerous other plant promoters. Furthermore, the proteins which interact with these response elements also share structural similarities with cAMP response element binding proteins (see section 1.4.2), suggesting that elements of the two pathways may have evolved from an common ancestral pathway.

1.4.2 Abscisic Acid and Gene Regulation

Abscisic acid has an inhibitory effect on the accumulation of RNA in general. Specific transcripts, however, have been shown to accumulate in response to ABA. Modulation of mRNAs could be effected by changes in the rates of transcription,

transcript stability, or a combination of both.

Abscisic acid has been demonstrated to reduce RNA synthesis in embryos of pear (Bex 1972a) and bean (Walbot *et al.* 1975, Walton *et al.* 1970), leaves of barley (Poulson and Beevers 1970) and radish (Wareing and Phillips 1970), and maize coleoptiles (Bex 1972b). It should be borne in mind that ABA induces senescence in leaves and inhibits the growth of embryos and seedlings (see sections 1.2, 1.3.3, 1.3.4). It is therefore possible that the reduced transcription seen in some of these processes is a secondary effect of ABA on general metabolism.

Nuclear run-on experiments indicate that the ABA-inhibition of gibberellic acid-stimulated α-amylase gene expression occurs, at least partly, at the transcriptional level (Jacobsen and Beach 1985). Deletion analysis of an α-amylase promoter indicates that the sequence(s) responsive to ABA and gibberellic acid appear to coincide with a major responsive element mapped to the same region (Jacobsen and Close 1991). Abscisic acid has also been reported to inhibit the gibberellic acid-enhanced transcription of several other mRNAs (Zwar and Jacobsen 1972, Ho and Varner 1974).

Abscisic acid does not have a direct effect on *in vitro* transcription of isolated chromatin. However, when ABA is included in the homogenization buffer, isolated chromatin exhibits reduced transcriptional activity (Pearson and Wareing 1969). These results suggest that ABA interacts with a cellular factor in affecting transcription *in vitro.* The nature of this factor(s) and its relevance to transcription *in vivo* are unknown.

Early evidence that ABA might act at the transcriptional level was provided by Sacher and Davies (1974) who showed that the ABA-stimulated increase in ribonuclease

activity was inhibited by inhibitors of RNA synthesis. Nuclear run-off experiments have since demonstrated that ABA stimulates the rates of transcription of cruciferin and napin genes in *Brassica napus* (DeLisle and Crouch 1989). Abscisic acid has also been shown to stimulate the transcription of the wheat Em gene (Marcotte *et al.* 1989), the rice Rab21 gene (Mundy and Chua 1988), and the α' subunit of ß-conglycinin (Chen *et al.* 1986). These results indicate that the modulation of transcription rates is an important control point for ABA-responsive genes.

Transcription has been found to be regulated by the interaction of protein factors with specific nucleic acid sequences within or in the vicinity of the regulated gene. Nucleic acid sequences 5' to the wheat Em gene confer ABA expression to a reporter gene in rice protoplasts and confer both ABA- and seed-specific expression in trangenic tobacco. Deletion analysis revealed that a 50 bp sequence (-152 to -103) confers ABA responsiveness in either orientation (Marcotte *et al.* 1989). The 5' regions of several ABA-responsive genes including the α' subunit of ß-conglycinin (Chen *et al.* 1986) and the rice Rab21 (Mundy and Chua 1988) contain sequences similar to an 8 bp motif (CGAGCAGG) identified in the ABA responsive element (ABRE) of Em. The significance of this motif is, at present, unknown. The gibberellic acid response element (GARE) of barley α-amylase (TAACAAA) directs the gibberellic acid-stimulated transcription of reporter genes. Abscisic acid-inhibition of transcriptional stimulation also requires this element (Gubler and Jacobsen 1992).

Transgenic experiments indicate that multiple copies of hormone responsive elements may be required to confer hormone responsiveness on minimal promoters

(Skriver *et al.* 1991). Yet these elements naturally occur singly. Single copies of the barley α-amylase GARE and the wheat Em ABRE require the presence of a second element to mediate hormone-responsive transcription. Recently an element from the barley amylase promoter was identified which confers ABA- or gibberellic acid-responsiveness to a reporter gene driven by a minimal promoter with an ABRE or a GARE, respectively. This O2S element (TTGACCATCATT) is similar to the DNA sequence recognized by the endosperm-specific transcriptional regulator Opaque-2 (Rogers and Rogers 1992). Thus, the promoter regions of hormonally-regulated genes will likely prove to consist of a complex set of elements defining tissue-, spatial- and temporal-specificity, conferring hormonal regulation, and establishing or modifying the quantitative level of transcriptional activity.

At the present time little is known about the trans-factors which interact with plant hormone-responsive gene promoters. Guiltinan *et al.* (1990) have isolated a cDNA encoding a protein (EmBP-1) which binds specifically to the wheat Em ABRE. The predicted amino acid sequence specifies a protein having a leucine zipper domain and a basic domain, suggesting it may act as a transcription factor like bZip proteins in yeast and animals (for reviews, see Busch and Sassone-Corsi 1990, Ziff 1990). Methylation interference studies indicate this protein binds to the Em(1a) sequence (GACACGTGGC) within the ABRE (Guiltinan *et al.* 1990). Like numerous other plant DNA sequences believed to bind transcription factors, Em(1a) contains the palindromic G-box motif (CACGTG). A recent study has shown the dinucleotides flanking the G-box motif determine the class of plant protein which can bind and the relative affinity of the

interaction (Williams *et al.* 1992). These results suggest the plethora of cis- and trans-elements used by modern eukaryotes may have evolved from a few ancestral prototypes.

While ABA regulates gene activity primarily at the level of transcription, post-transcriptional modulation may also be important. For example, ABA inhibits translation in general but not transcription in barley coleoptiles (Bonnafous *et al.* 1973). The stability of the Em transcript in cultured wheat embryos has been shown to be ABA-dependent (Williamson and Quatrano 1988). In this case, however, the role of ABA appears to be indirect, inhibiting the accumulation of germination-specific genes required for the destabilization of the Em transcript. An element (80 bp) within the 5'-untranslated region of the Em transcript enhances the ABA-response 10-fold (Marcotte *et al.* 1989). It is possible that the observed enhancement of expression by this element is partly a result of increased transcript stability. However, this has not been demonstrated.

1.4.3 Models of Abscisic Acid Action

The rapid changes in membrane function observed during the fast response to ABA could be caused by the reversible binding of ABA with membrane pores (Van Steveninck and Van Steveninck 1983), proton- or Na^+ pumps (Ballarin-Denti and Cocucci 1979, Maslowski *et al.* 1974), and/or lipid components of the plasmalemma (Lea and Collins 1979). Alternatively, the interaction of ABA with membrane receptors could result in dramatic changes in the conformation, and function, of the plasmalemma lipid-phase (Purohit *et al.* 1992a).

Koorneef *et al.* (1989) have suggested that the effects of ABA on processes during seed development are the result of a simple cascade. In their model, the primary action

of ABA is the inhibition of the uptake of water via its effects on membrane components. In turn, the ensuing water loss prevents precocious germination and stimulates expression of genes encoding storage proteins and proteins involved in desiccation tolerance.

The ABA-stimulation of transcripts homologous to a serine/threonine protein kinase in wheat (Anderberg and Walker-Simmons 1992), the possible involvement of Ca^{2+} as second messenger in stomatal closure (see section 1.4.1), and the identification of transcriptional factors similar to those involved in the cyclic AMP response (see section 1.4.2) indicate that some components of the signal cascades operating in animal cells and lower eukaryotes may play a role in both the fast and slow responses to ABA.

1.5 Research Strategy

In order to learn something about the molecular basis of angiosperm embryo development, I chose to examine the expression of embryo-specific genes from *Zea mays* L. This species was chosen for several reasons besides its great agronomic value. The separation of male from female flowers facilitates controlled pollination, allowing for the synchronization of development. A single cob can bear several hundred seeds, and the embryos are relatively large compared with other monocots. The absence of well-developed glumes enclosing the karyopsis allows isolation of intact embryos. Moreover, maize is genetically the best characterized plant and numerous mutants are available for study.

I chose to isolate genes expressed during mid- to late-embryogenesis since it is during this period when many of the important events of maturation occur: acquisition of cold- and desiccation-tolerance, inhibition of germination, accumulation of food reserves, desiccation, and others. In order to understand the factors regulating embryogenesis it is necessary to study the activity of specific genes during development and in response to experimental perturbations. I chose, therefore, to examine the steady-state concentrations of five mRNAs exhibiting different patterns of accumulation during embryogenesis. I also examined the effects of exogenous ABA and water stress on the expression of these genes. The sequences of two of the embryo-specific maize cDNAs were determined in hopes of identifying the potential functions of the predicted polypeptides. The accumulation of the final polypeptide product of one of the characterized genes was also examined using antibodies raised against a fusion protein synthesized in *Escherichia coli*.

CHAPTER 2. MATERIALS AND METHODS

2.1 Culture of *Zea mays*

Wild-type (Co255) kernels were a gift from Dr. R. Hamilton, Plant Research Center, Agriculture Canada, Ottawa. *Viviparous-1* (*vp1*) and *vp5* mutant W22 kernels were a gift from Dr. B. Burr, Brookhaven National Laboratories., Long Island, New York. Maize suspension cells (Ketchum *et al.* 1989) were provided by Dr. K. Ketchum, McGill University. Mutant and wild-type plants of *Zea mays* L. were grown under field conditions at the Macdonald College Seed Farm, St. Anne-de-Bellevue, Quebec, or under controlled conditions in the McGill University Phytotron. In the Phytotron, the plants were grown in 12-inch diameter pots filled with Promix (Premier Brands, Inc.), nourished with Hoagland's Modified Solution, and maintained at 29°C:19°C on a light:dark cycle of 16h:8h at light fluences of 1100 μE/m2. Ear shoots and tassels were bagged before the emergence of silks and hand pollinated within two days after the silks were cut. Homozygous *vp1* kernels were harvested at the time of radicle elongation, surface-sterilized with 2.5% sodium hypochlorite and grown until the root system was well established on 0.9% agar containing N6 medium (Chu *et al.* 1975) before transferring to soil. Seedlings were grown on autoclaved vermiculite in the dark at 25°C.

2.2 Isolation of RNA

Tissue (0.05 to 2.0 g) was ground to a powder in liquid nitrogen using mortar and pestle. Total RNA was extracted as described (Theologis *et al.* 1985) except that the CsCl fractionation step was omitted. Briefly: freshly powdered plant tissue was homogenized with a minimum of 5 volumes of 50 mM Tris.HCl (pH 7.5), 150 mM NaCl, 5 mM

EDTA, 5% SDS at top speed in a blender for 2 to 3 minutes. Proteinase K (Boehringer Mannheim) was added to 50 µg/ml and the suspension was stirred for 20 minutes at room temperature. One half volumes each of phenol and chloroform were added and, after stirring for 20 minutes, the aqueous and organic phases were separated by centrifugation. The aqueous phase was reextraced with phenol/chloroform twice more, adjusted to 0.4 M NaCl and nucleic acids were precipitated with 2.5 volumes of ethanol at -20°C overnight. The nucleic acids were collected by centrifugation at 10,000 x g for 30 minutes, briefly air dried, resuspended in sterile distilled water, then reprecipitated.

Poly(A)$^+$RNA was isolated by affinity chromatography using oligo(dT)-cellulose (Pharmacia). Poly(A)$^+$RNA was precipitated from ethanol, resuspended to 1 µg/µl in DEP-treated water, and stored at -70°C. The concentrations of nucleic acids were determined spectrophotometrically.

2.3 Construction and Screening of the cDNA Library

A cDNA library was constructed using poly(A)$^+$RNA extracted from Stage 6 maize embryos primed with oligo(dT) as described (Maniatis *et al.* 1982). Blunt-ended, double-stranded cDNA was d(C)-tailed, annealed to d(G)-tailed PstI-pBR322 (Bethesda Research Laboratories), and used to transform *Escherichia coli* DH1 cells (Hanahan 1985). Ampicillin-sensitive, tetracyclin-resistant recombinants were grown in microtiter dishes, 1 clone/well, then transferred to nitrocellulose filters (BA85, Schleicher and Schuell) as described (Maniatis et al. 1982). Poly(A)$^+$RNA was hydrolyzed to an average size of 500 nt by incubation at 90°C for 30 minutes in 50 mM Tris (pH 9.5), 5 mM glycine, 100 µM spermidine, 10 µM EDTA prior to end-labelling with [γ-^{32}P]ATP catalyzed by T4

polynucleotide kinase. Triplicate filters were challenged with radiolabelled RNA from maturation phase maize embryos or seedling roots or shoots. The membranes were prehybridized at 42°C for 2 hours in 50% formamide, 5x SSC, 1% SDS, 2x Denhardt's, 250 µg/ml sheared salmon sperm DNA, 30 µg/ml poly(A). Hybridization with radiolabelled RNA was performed for 36 hours at 42°C in the same solution used for prehybridization. Membranes were washed briefly in 2x SSC, 0.1% SDS at room temperature, then twice for 30 minutes in 0.1x SSC, 0.1% SDS at 68°C. Differential hybridization was detected by exposing the membranes to Kodak X-omat film at -70°C between intensifying screens.

2.4 Isolation of DNA

Plasmid DNA was isolated by the alkaline lysis method and purified either by CsCl gradient centrifugation or by repeated phenol/chloroform extraction and RNase digestion followed by precipitation from ethanol (Maniatis *et al.* 1982).

Nuclei were isolated from etiolated seedling leaves as described (Luthe and Quatrano 1980) with slight modifications. Briefly, tissue (20g) was ground to a powder in liquid nitrogen using mortar and pestle then homogenized in a blender with 200 ml 0.44 M sucrose, 2.5% Ficoll (molecular weight 400,000), 5% Dextran 40, 25 mM Tris.HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM ß-mercaptoethanol, 0.5% (v/v) Triton X-100. The slurry was filtered through 3 layers of cheesecloth, one layer of Miracloth, and two layers of Nytex (80 and 61 µm mesh). Nuclei were pelleted at 2000 x g at 4°C, lysed by resuspension in 20 ml 20 mM EDTA, 50 mM Tris.HCl (pH 8), 1% sarkosyl and DNA was purified by CsCl gradient centrifugation (Maniatis *et al.* 1982).

2.5 Solution Hybridization

Radiolabelled, single-stranded cDNA (18 ng) complementary to embryonic poly(A)$^+$RNA was incubated in 25 µl of 0.41 M sodium phosphate buffer (pH 7.4), 0.2% SDS with poly(A)$^+$RNA (10 µg) from mature embryos, seedling roots and hypocotyl, or seedling roots, hypocotyl and leaves. Aliquots of 5 µl were sealed in capillary tubes, heated to 100°C for 5 minutes, then incubated at 68°C. The fraction of cDNA hybridized was estimated as follows. The unhybridized cDNA was digested with S1 nuclease (ca. 10 U/µg nucleic acid for 40 minutes at 37°C). Half the digestion mixture was placed on each of two Whatman DE81 filters. One was dried for estimation of total cDNA. The other was washed 6x with 0.5M Na_2HPO_4, 2x with distilled water, then 2x with 95% ethanol to remove all but duplex nucleic acids. The filters were then dried, placed in scintillant, and radioactivity was estimated by scintillation counter.

2.6 Treatment with ABA and Desiccation

Embryos were excised from surface-sterilized seeds. One set of embryos was plunged immediately into liquid nitrogen for storage. Another set was placed axis side down on 0.9% agar containing N6 medium in the presence or absence of 1 µM ABA (racemic mixture; Sigma) or other supplement. Following incubation in the dark for 16 h at 27°C, the treated embryos were rinsed with 10 mM $CaCl_2$ and then stored in liquid nitrogen. Harvested seedlings free of vermiculite were either processed immediately or their roots were immersed in 100 µM ABA in 10 mM $CaCl_2$ for 16 hours at 25°C in the dark. Other seedlings were cleaned of vermiculite, air dried at 25°C for 1 hour, then placed in plastic bags at 25°C for 16 hours.

2.7 Northern Blot Analysis of RNA

Ten micrograms of total RNA was denatured in formamide and formaldehyde and size-fractionated by electrophoresis through 1.5% agarose/formaldehyde gel (Maniatis *et al.* 1982). Acridine orange was used to visualize the RNA (McMaster and Carmichael 1977) and ensure that equivalent amounts of non-degraded RNA had been used. RNA was transferred passively onto Nytran membranes (Schleicher & Schuell) according to manufacturer's recommendations using 10x SSC. DNA fragments for probes were isolated by size fractionation in agarose gels followed by electroelution into dialysis tubing (Maniatis *et al.* 1982). Radiolabelled probes were generated either by nick-translation (Bethesda Research Laboratories kit) using [α-^{32}P]dCTP (ICN, 3,000 Ci/mmol) or by random primer extension (BRL kit). The nick-translated probes had a specific activity of 2-8 x 10^7 c.p.m./µg of DNA. The probes obtained by random primer extension had a specific activity of 3-10 x 10^8 c.p.m./µg DNA. Hybridizations were carried out in 50% formamide, 5x SSC, 1% SDS, 5x Denhardt's, 100 µg/ml salmon sperm DNA at 42°C and high stringency washes were performed in 0.1x SSC, 0.1% SDS at 68°C. The blots were exposed to Kodak X-Omat films with intensifying screens, at -70°C.

Blots were reused following removal of the previous probe by boiling for 20 minutes in 0.01x SSPE (1x = 0.18M NaCl, 0.01M NaH_2PO_2, 0.001M EDTA, pH7.4), 1% SDS. Removal was confirmed by autoradiography.

Densitometry of the autoradiographs was performed using a densitometer (E.C. Apparatus Corp., St. Petersburg, Florida) and recorded using a chart recorder. Baselines were determined visually and the peaks were cut out and weighed to estimate relative

areas.

2.8 DNA Blot Analysis

Ten micrograms of genomic DNA were digested with restriction endonucleases (Bethesda Research Laboratories) according to the supplier's directions, size fractionated by electrophoresis in a 0.6% agarose gel, denatured, and transferred by capillary action to a Nytran membrane (Schleicher and Schuell) using 10x SSC (Maniatis *et al.* 1982). Hybridization conditions were essentially the same as for Northern blots (see section 2.5).

2.9 Sequencing

Double-stranded DNA sequencing of pBR322 subclones was performed using synthetic oligonucleotide primers complementary to sequences located on either strand 5' to the *Pst*I site. (Hattori and Sakaki 1986). Complementary DNA was subcloned into Bluescript phagemid vectors (Stratagene) for isolation of single-stranded templates (Viera and Messing 1987). DNA sequencing was carried out using the dideoxy method (Sanger *et al.* 1977) with the Klenow fragment of DNA polymerase I (Stambaugh and Blakesley 1988) or using Taq polymerase (Innis *et al.* 1988). Sequencing reactions were size fractionated on 6% polyacrylamide gels containing 8M urea. Sequence data was analyzed using PC/GENE (Intelligenetics, Mountain View, Calif) computer software. Searches for sequence homology employed FASTA (Pearson and Lipman 1988) comparisons with the GenBank (Bilofsky and Burks 1988) data bank and the data banks at NRC (Ottawa).

2.10 Production of ß-Galactosidase-Emb564 Fusion Protein.

In order to generate large quantities of Emb564 antigen for injection into rabbits, the entire coding region of Emb564 was inserted into the polylinker of the pWR590-1

expression vector (Guo *et al.* 1984). This vector includes the *E. coli* lac promoter and a truncated portion of the *lac*Z gene coding for the first 590 amino acids. The polylinker at the 3' end of the truncated *lac*Z gene allows for cloning and the creation of gene fusion. (For details of construction of pWR590:Emb564 fusion, see Appendix 4).

The Bluescript subclone containing the Emb564 cDNA inserted into the *Pst*I site was digested with *Bam*HI and *Eco*RI, sites which flank *Pst*I. The fragment was isolated and further digested with *Dra*II which cuts 35 nucleotides 5' of the translation start site. The sticky ends were blunted and ligated to *Eco*RI linkers. Following digestion with *Eco*RI and *Pst*I, the fragment was size fractionated on a 10% polyacrylamide gel, eluted by passive diffusion into 0.5M ammonium acetate, 1 mM EDTA overnight, and cloned into Bluescript. The *Eco*RI end was sequenced to verify its structure.

The *Eco*RI, *Pst*I fragment was subcloned into the pWR590-1 vector and used to transform *E. coli* DH5αF'. Putative transformants were screened for the production of fusion protein. The construct was predicted to yield a fusion protein containing the truncated ß-galactosidase gene, plus 1 amino acid (Gly) introduced by the linker, plus 12 amino acids corresponding to the 5' noncoding region of Emb564, plus the entire open reading frame of Emb564. Analysis of the plasmid DNA from 24 transformants yielded 2 copies of the desired construct.

2.11 Production of EMB564-Specific Polyclonal Antibodies

Fusion protein was isolated from *E. coli* by size fractionation through polyacrylamide gels followed by electroelution into dialysis tubing. The protein was injected into rabbits for the production of antibodies. An emulsion was made containing

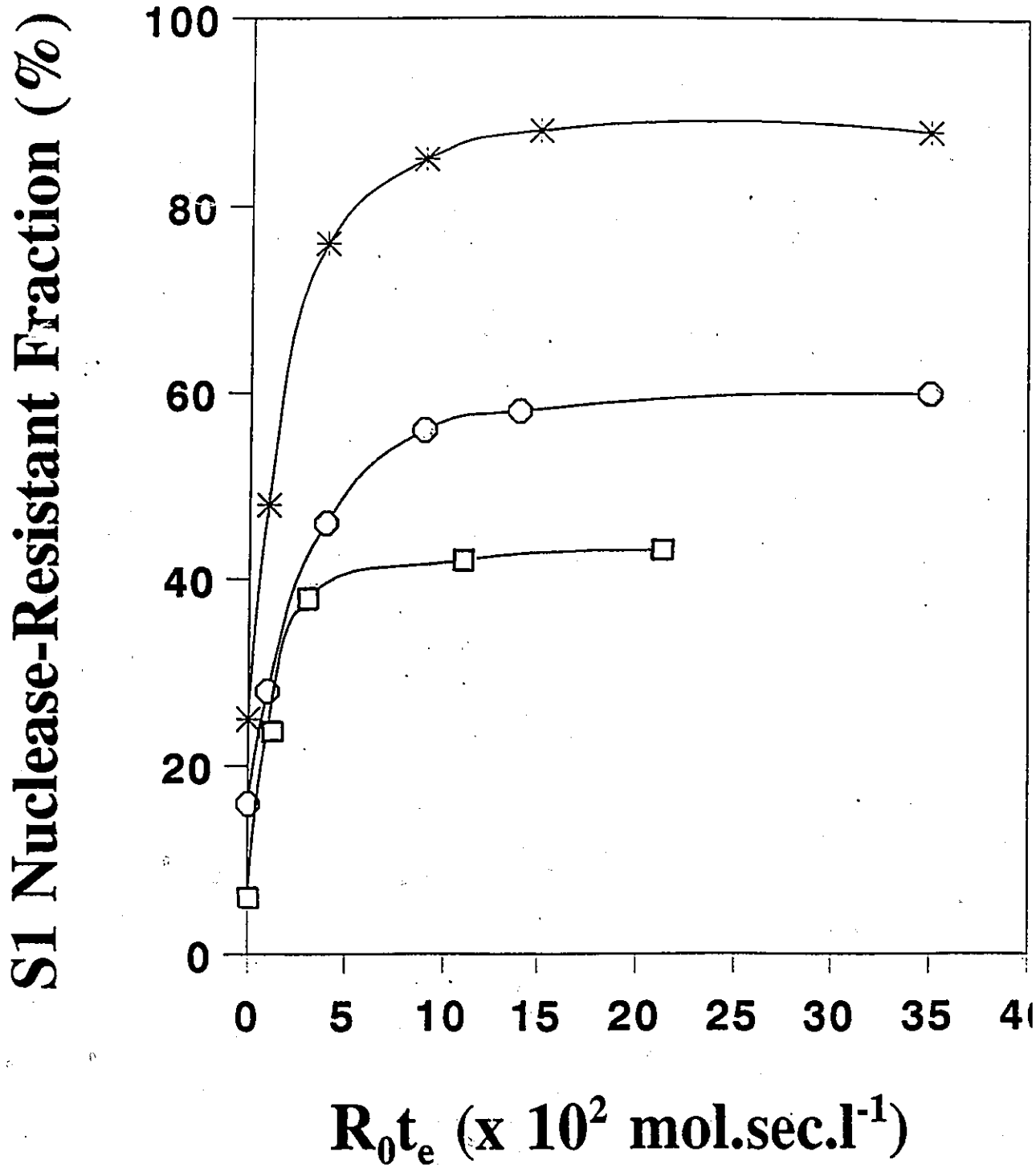
500 µg of partially purified fusion protein in 0.5 ml plus 0.5 ml of RIBI adjuvant (Cedarlane Labs, Ltd., Ont.). The emulsion was injected subcutaneously into 4 different sites in New Zealand white female rabbits. A booster shot containing 100 µg of partially purified *lac*Z:EMB564 fusion protein emulsified with Freund's incomplete adjuvant was given 4 weeks later. Ten days after the booster shot, blood was collected by heart puncture (Animal Centre, McGill University). The blood was allowed to clot and the serum was collected. The Emb564-specific antibodies were partially purified by passing the total serum first over an affinity column containing total proteins from an *E. coli* strain expressing the pWR590 ß-galactosidase covalently linked to Affigel (Biorad) beads to remove antibodies to bacterial proteins. The unbound serum was then passed over an affinity column containing β-galactosidase:EMB564 fusion protein covalently linked to Affigel (Biorad) beads. The bound antibodies were removed at low pH, neutralized with Tris pH8 and diluted as detailed below.

2.12 Immunoblot Analysis of Proteins.

Protein samples were extracted from tissue freshly ground in liquid nitrogen by boiling 3 minutes in SDS sample buffer (Laemmli 1970) followed by centrifugation to remove insoluble debris. Concentration was estimated as described (Esen 1978). Ten micrograms of each sample were separated electrophoretically on 15% polyacrylamide SDS gels (Maniatis *et al.* 1982), then electrophoretically transferred to Immobilon P membranes (Millipore) according to the manufacturer's directions. The membranes were blocked in 5% (w/v) skim milk powder (Carnation), 0.5% Tween 20, 1X TBS (150 mM NaCl, 700 mM KCl, 25 mM Tris, pH 8) for 2 hours at room temperature (Harlow and

Lane, 1988). The blots were then incubated with a 1:1000 dilution of either preimmune serum or Emb564 affinity purified anti-sera in 5% milk powder, 0.5% Tween 20, 1X TBS for 16 hours at 4°C. The blots were washed a minimum of 4 times for 15 minutes each in 0.5% Tween 20 in 1X TBS, then challenged with a 1:1000 dilution of ^{125}I-conjugated goat anti-rabbit IgG secondary antibody as described for the primary antibody for 2 hours at room temperature. The secondary antibody was removed by washing the blots a minumum of 4 times, 15 minutes each with 0.5% Tween 20 in 1X TBS. Proteins bound by antibodies were detected by exposure of X-ray film at -70°C with intensifying screens.

Figure 4. Solution Hybridization of Maize Embryo cDNA to Poly(A)$^+$RNA from Embryonic and Seedling Tissues. Radiolabelled, single-stranded cDNA (18 ng) complementary to embryonic poly(A)$^+$RNA was incubated with poly(A)$^+$RNA (10 µg) from mature embryos (*), seedling roots and hypocotyl (□), or seedling roots, hypocotyl and leaves (o) in 25 µl of 0.41 M sodium phosphate buffer (pH 7.4), 0.2% SDS. Aliquots (5 µl) were removed at various times, sealed in capillary tubes, and heated to 100°C for 5 minutes, then incubated at 68°C. The fraction of cDNA hybridized was estimated as described in Materials and Methods and was plotted against R_0t_e.

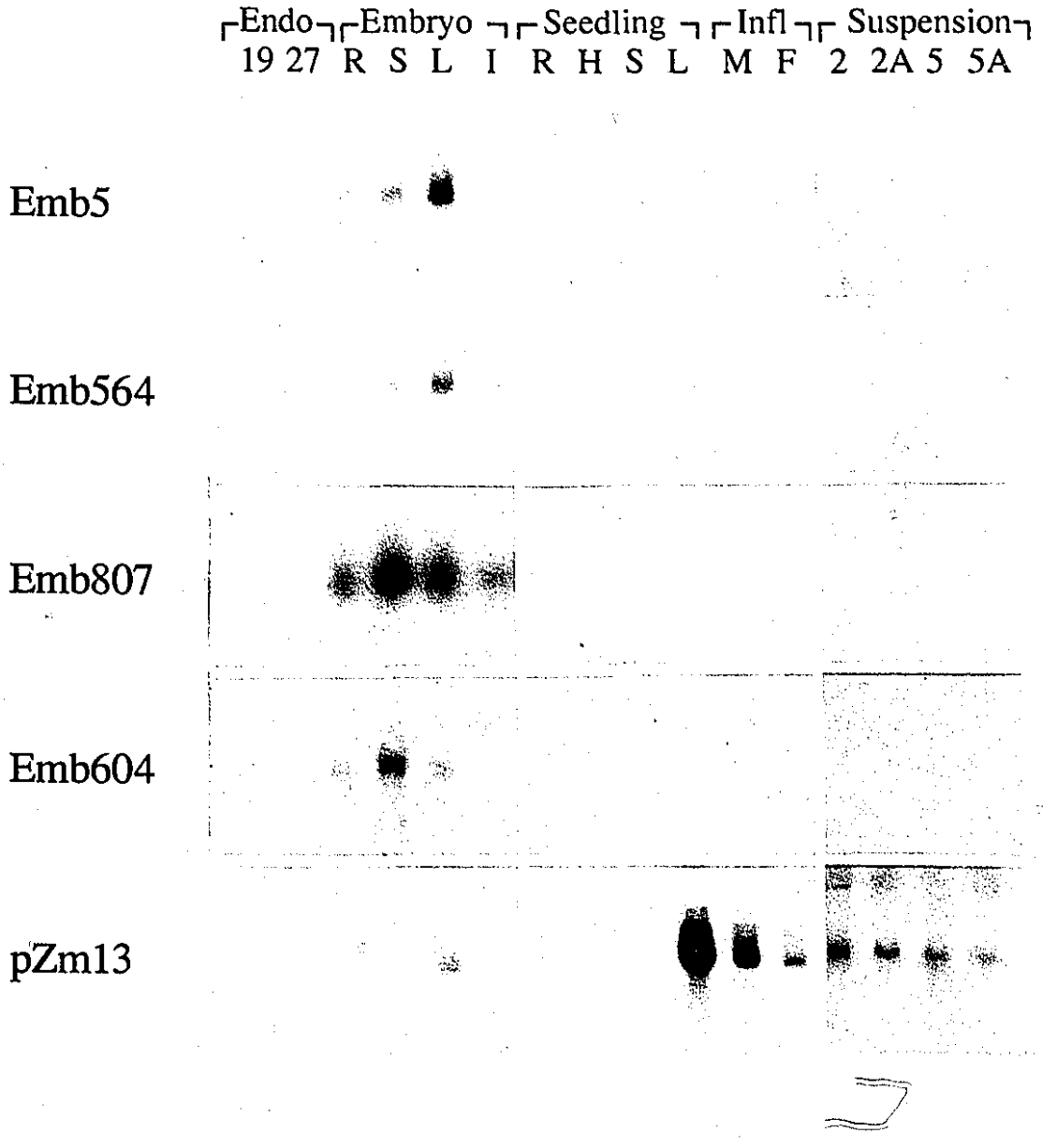

S1 Nuclease-Resistant Fraction (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
R_0t_e (x 10^2 mol.sec.l^{-1})

CHAPTER 3. RESULTS.

3.1 Identification of Maize Embryo-Specific cDNA Clones

Since the primary objective of my research was to identify and characterize plant embryo-specific genes I first examined the relative abundance of embryo-specific transcripts in the mature maize embryo. Complementary DNA to maize embryonic transcripts was hybridized to an excess of poly(A)$^+$RNA from embryonic or seedling tissues to an equivalent R_0t of 4000 mol sec l^{-1} when the reaction had approached saturation. The results of these experiments indicated that of the transcripts abundant in the mature maize embryo half represent sequences found in abundance in seedling roots and shoots, two thirds represent sequences found in abundance in seedling roots, shoots and leaves, and approximately 30% represented transcripts found in low abundance in seedling tissues (Fig. 4). These results suggested a cDNA library rich in embryo-specific sequences could be obtained with embryonic mRNAs as template. A cDNA library was constructed. The size of inserts were estimated to range from 0.2 kb to 2.0 kb with a mean of 0.5 kb. One thousand cDNA clones were screened by differential hybridization to radiolabelled RNA isolated from mature maize embryos, seedling roots or seedling shoots. Fifty three clones hybridized strongly to embryo RNA but not to seedling RNA. Nineteen embryo-specific clones were analyzed by RNA blot hybridization. Four clones, Emb5, Emb564, Emb604, and Emb807, exhibiting a single size-class of transcripts were chosen for detailed analysis. A fifth clone exhibiting expression in embryo and seedling was used as a control.

Figure 5. Accumulation of Transcripts in Embryonic and Vegetative Tissues, and in Suspension Cells. Total cellular RNA was isolated from developing central endosperm 19 DAP (19), central endosperm 27 DAP (27), the primordial roots of 46-mg embryos (R), the scutella of 46-mg embryos (S), the apical shoots of 46-mg embryos (L), the embryos from dry, stored seed after 16 hours of imbibition (I), 5-day seedling roots (R), 5-day seedling hypocotyls (H), 5-day seedling scutella (S), 5-day seedling leaves (L), male inflorescence (M), female inflorescence (F), maize suspension cells 2 days after transfer without (2) and with (2A) 10 µM ABA, and maize suspension cells 5 days after transfer without (5) and with (5A) 10 µM ABA. Ten micrograms of the cellular RNA was examined by Northern blotting with radiolabelled DNA from Emb5, Emb564, Emb604, Emb807, and pZm13 as probes.


Endo
Embryo
Seedling
Infl
Suspension
19 27 R S L I R H S L M F 2 2A 5 5A
Emb5
Emb564
Emb807
Emb604
pZm13

3.2 Tissue-Specific Expression of Maize Genes

Figure 5 shows the levels of transcripts complementary to the five cDNAs in various embryonic, vegetative and reproductive tissues. The transcripts of Emb5, Emb564, Emb604 and Emb807 were detected only in embryonic organs and were not found in endosperm, seedling tissues, inflorescences, and suspension cells. With the exception of Emb807, levels of each transcript differed between the different tissues. The levels of Emb5 and Emb564 transcripts were highest in embryonic leaves. The Emb604 transcript attained the highest level of accumulation in the scutellum. The level of Emb807 mRNA did not differ greatly between scutellum, embryonic roots and embryonic leaves.

In contrast to the other four transcripts, the 1.1 kb pZm13 RNA exhibited greatest accumulation in seedling leaves followed by male inflorescence and female inflorescence. In the mature embryo it was found to accumulate predominantly in the shoot region, followed by the scutellum, and at low levels in the embryonic root. Unlike the other three mRNA populations, the 1.3 kb pZm13 RNA, but not the 1.1 kb RNA species, was detected in suspension cells whether incubated with ABA or not.

3.3 Differential Expression of Five Maize Transcripts During Development.

The patterns of accumulation of the cloned cDNAs during development were examined by RNA blot hybridization. Figure 6 shows the developmental profile of mRNAs complementary to the cloned cDNAs. Figure 7 shows the densitometric estimation of relative transcript levels. The 1.2 kb transcript complementary to Emb604 can be described as early-to-mid embryogenesis abundant. It was moderately accumulated

Figure 6. Developmental Accumulation of mRNA Complementary to Emb5, Emb564, Emb604, Emb807 and pZm13. Total cellular RNA was isolated from embryos of the following stages: 0.5 mg (14 days after pollination (DAP)), 1.0 mg (15 DAP), 3.0 mg (17 DAP), 10 mg (21 DAP), 14 mg (23 DAP), 20 mg (27 DAP), 24 mg (28 DAP), 30 mg (33 DAP), 34 mg (37 DAP), 40 mg (42 DAP), 46 mg (56 DAP), and 48 mg (65 DAP), and from mature embryos extracted from seed which had been imbibed for 16 hours (Imb). RNA was size fractionated on formaldehyde-agar, blotted to Nytran, and hybridized with radiolabelled probes as described in Material and Methods.

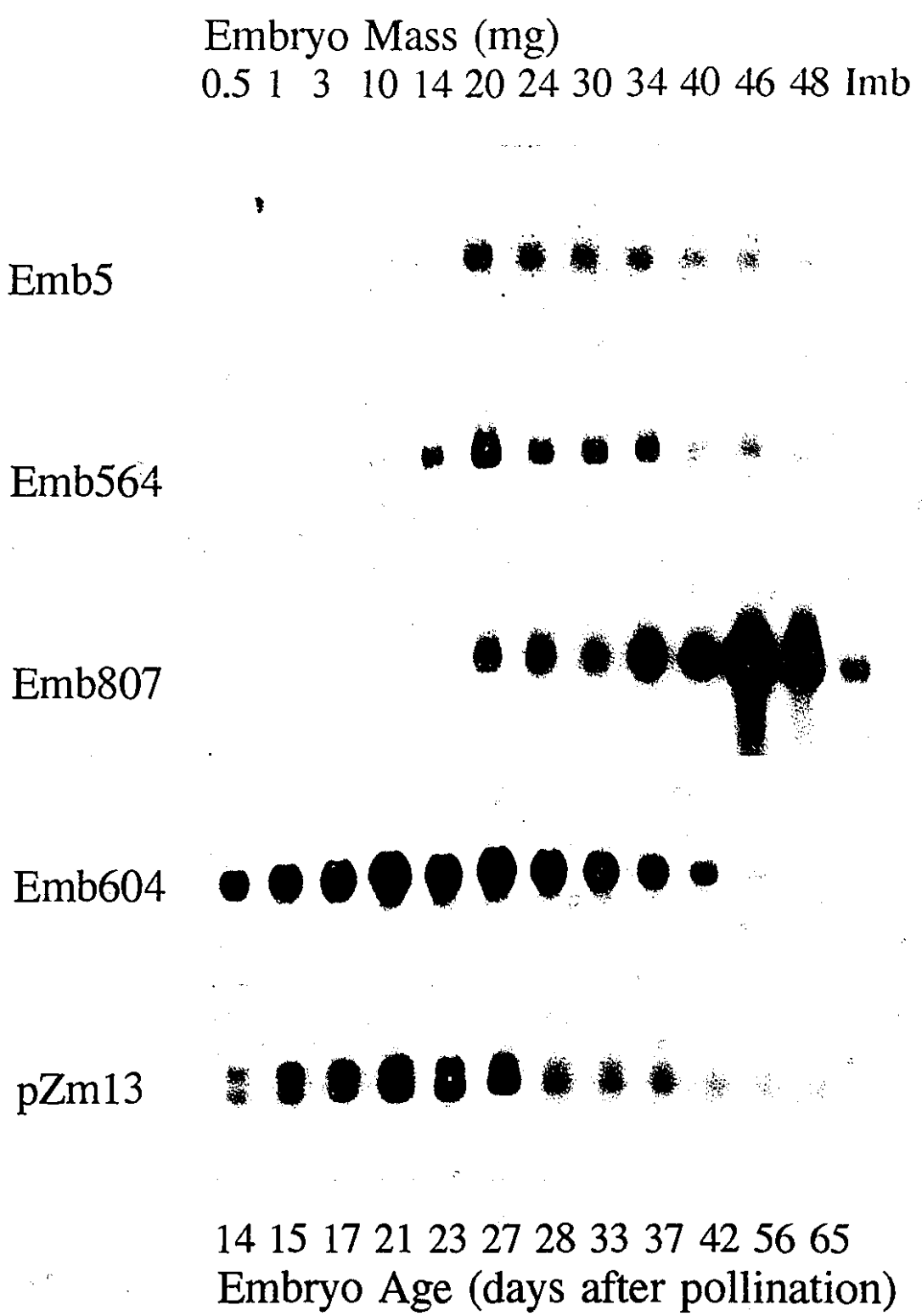
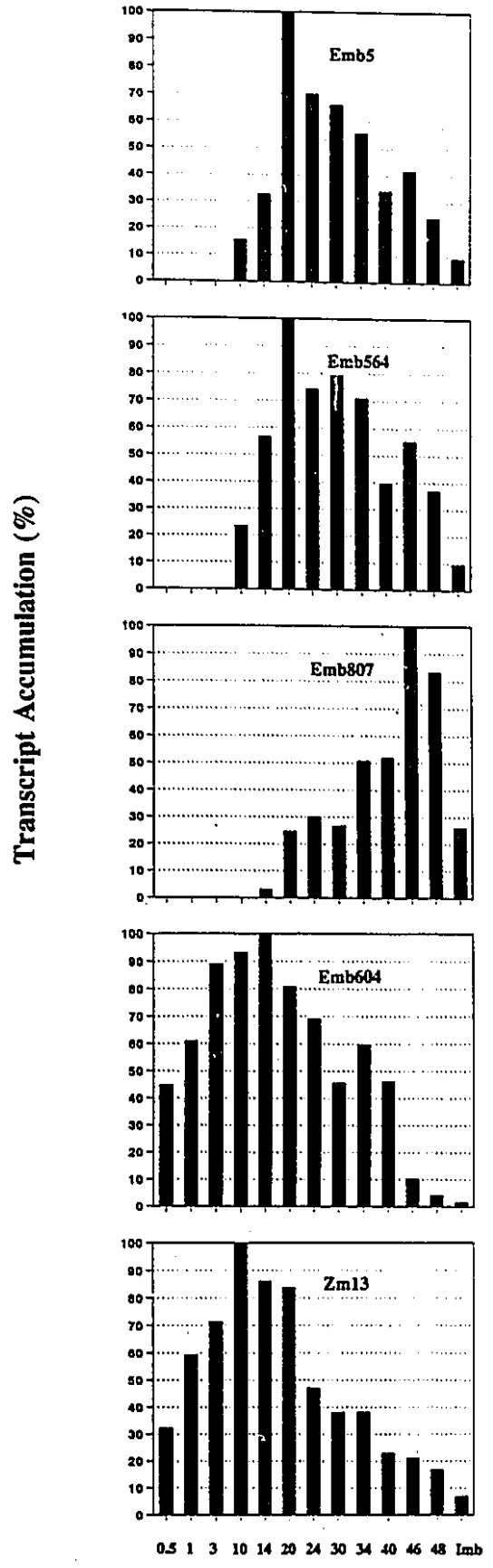

Embryo Mass (mg)
0.5 1 3 10 14 20 24 30 34 40 46 48 Imb
Emb5
Emb564
Emb807
Emb604
pZm13
14 15 17 21 23 27 28 33 37 42 56 65
Embryo Age (days after pollination)

Figure 7. Densitometric Estimation of Relative Accumulation of Transcript during Development. The relative intensities of signals from autoradiographs shown in Fig. 6 were estimated by densitometry and graphically displayed.


Transcript Accumulation (%)
Emb5
Emb564
Emb807
Emb604
Zm13
0.5 1 3 10 14 20 24 30 34 40 46 48 Imb
Embryo Mass (mg)

in 0.5-mg embryos (14 DAP), reached maximum accumulation in embryos 10 mg to 20 mg in mass (21-27 DAP), then decreased gradually to a very low level in mature, 48-mg embryos (65 DAP). The Emb604 transcript was barely detectable in the embryo after 16 hours of seed imbibition.

The mid embryogenesis abundant cDNAs Emb5 and Emb564 exhibited identical developmental profiles. In contrast to Emb604, the 0.8 kb transcripts complementary to Emb5 and Emb564 were barely detectable before the embryo had attained a mass of 10 mg (21 DAP). These RNAs reached maximum accumulation in 20-mg embryos (27 DAP), and then decreased gradually during maturation. The levels of the two transcripts were much reduced in the embryo after 16 hours of seed imbibition.

The Emb807 transcript can best be described as late embryogenesis abundant. Similar to Emb5 and Emb564 the 1.1 kb transcript detected with Emb807 first appeared in 10-mg embryos. Steady-state levels of Emb807 transcript then gradually increased until the embryo reached maturity. Emb807 transcript was clearly detectable even after 16 hours of seed imbibition.

Although expressed throughout development, at least in some tissues (see section 3.2), the steady-state levels of pZm13 RNA also changed during embryogenesis. Two RNA species, 1.1 kb and 1.3 kb, were detected early in embryogenesis, reaching maximum steady-state levels in 10 mg embryos (21 DAP), then declined to low levels in the mature embryo.

Figure 8. Stimulation of Transcript Accumulation in Embryos by ABA. Immature embryos having the masses shown were excised from the seed and incubated on nutrient agar in the absence (N) or presence of 1 μM ABA (A) for 16 h. Total cellular RNA from these and from untreated embryos (C) was size-fractionated by electrophoresis through formaldehyde-agarose, blotted to Nytran, and hybridized with radiolabelled probes complementary to Emb5, Emb564, Emb604, Emb807 or pZm13.

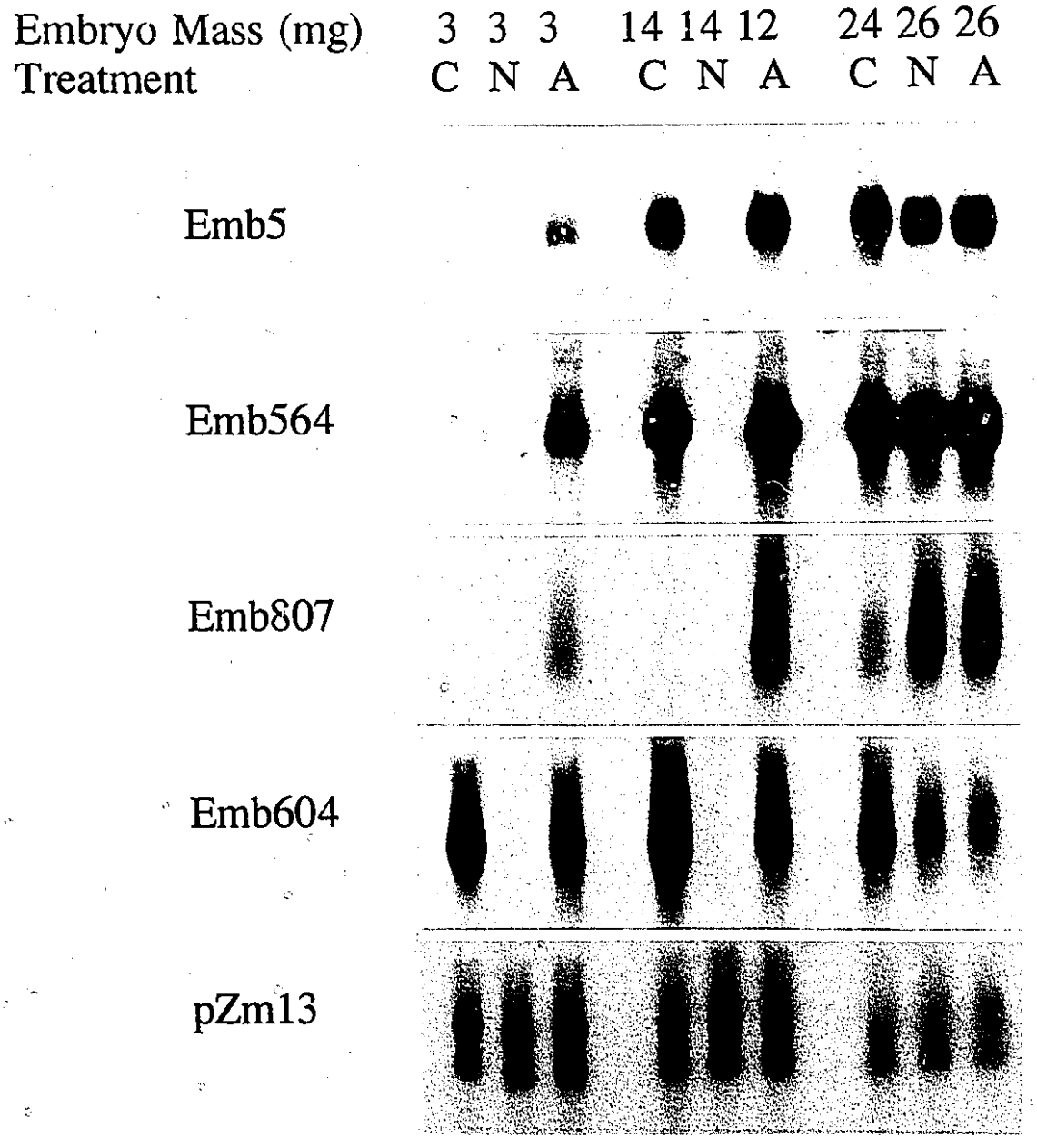
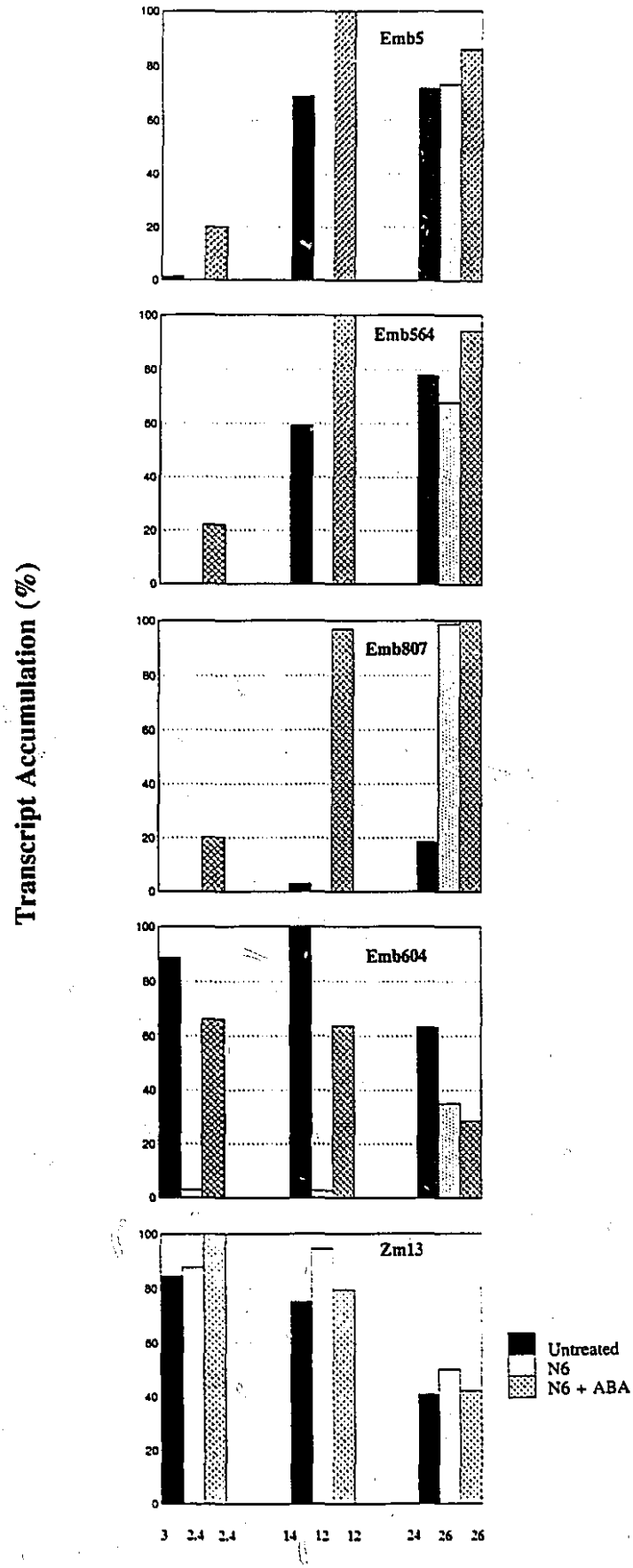

Embryo Mass (mg)
3 3 3 14 14 12 24 26 26
Treatment
C N A C N A C N A
Emb5
Emb564
Emb807
Emb604
pZm13

Figure 9. Densitometric Estimation of Relative Accumulation of Transcript in Embryos Incubated with and without ABA. The relative intensities of signals from autoradiographs shown in Fig. 8 were estimated by densitometry and graphically displayed. The greatest value measured on each autoradiograph was considered 100%.


Transcript Accumulation (%)
Emb5
Emb564
Emb807
Emb604
Zm13
Untreated
N6
N6 + ABA
3 2.4 2.4 14 12 12 24 26 26
Embryo Mass (mg)

3.4 Steady-State Levels of Emb5, Emb564 and Emb807 Transcripts in the Embryo are Stimulated by Abscisic Acid

It was noted that the first appearance of Emb5, Emb564 and Emb807 transcripts occurred approximately 21 days after pollination (Fig. 6), a time when the phytohormone ABA was accumulating within the maize seed (see section 1.3). Furthermore, Emb5 and Emb564 transcripts attained maximum steady-state concentrations during the period when ABA levels were reported to be high (27 - 33 DAP) then decreased as the concentration of ABA in the seed decreased. Several genes expressed in the plant embryo during the mid-to-late stages of embryogenesis have been found to be stimulated by exogenous ABA (see section 1.3.1). These observations suggested that Emb5, Emb564 and Emb807 might be regulated by endogenous ABA during embryogenesis. To test this possibility, immature embryos of various ages were incubated in the presence or absence of ABA. The results of RNA blot analysis are shown in Figure 8 and densitometric analysis of these autoradiograms is shown in Fig. 9.

Emb5 and Emb564 transcripts were barely detectable in untreated 3-mg embryos, undetectable in 3-mg embryos incubated without ABA, and accumulated to a modest level in 3-mg embryos incubated with ABA. There was a large accumulation of Emb5 and Emb564 transcripts in 14-mg embryos and in 12-mg embryos incubated in the presence of ABA. However, no transcript was detected in 12-mg embryos incubated without ABA. There was no appreciable difference in the high steady-state levels of Emb5 and Emb564 in the control 24-mg embryos or in 26-mg embryos incubated with or without ABA.

Transcript complementary to Emb807 was not detectable in 3-mg embryos, barely

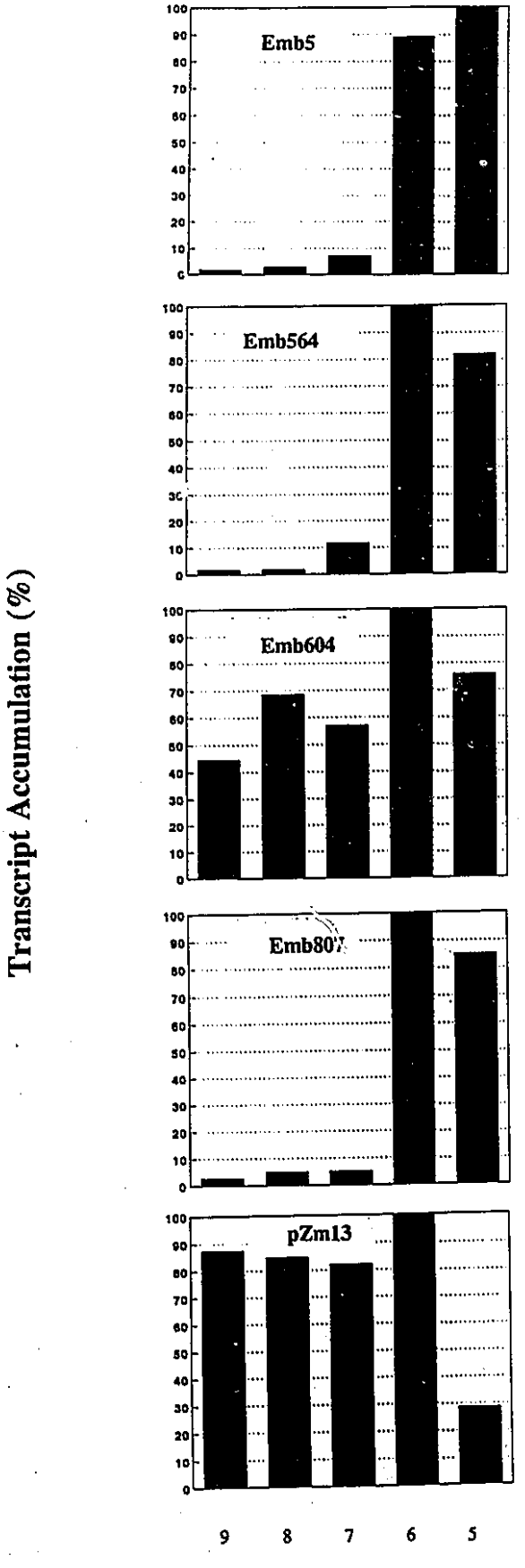
detectable in 14-mg embryos, and was only modestly accumulated in 24-mg embryos. When incubated with ABA, 3-mg embryos expressed a moderate level of the Emb807 transcript while 12-mg embryos exhibited a large steady-state accumulation of Emb807. Neither 3-mg embryos nor 12-mg embryos accumulated transcript in the absence of ABA. Twenty-six-mg embryos incubated without ABA or with ABA exhibited similar levels of Emb807 transcript. It is uncertain whether the difference in the levels of Emb807 transcript in untreated 24-mg embryos and treated 26-mg embryos was a response to the treatments or reflects differences between the physiological state of embryos on two different ears. Transcripts for Emb604 were present at a high level in untreated 3-mg embryos, untreated 14-mg embryos and untreated 24-mg embryos. However, 3-mg embryos and 12-mg embryos incubated without ABA showed a great reduction of Emb604 transcript. Treatment with ABA maintained Emb604 transcript in 3-mg and 12-mg embryos. On the other hand, 26-mg embryos incubated without ABA exhibited similar levels of Emb604 transcript as embryos incubated with ABA.

The pZm13 transcript levels did not differ substantially between untreated embryos and embryos incubated in the presence or absence of ABA.

3.5 Concentration Dependence of Abscisic Acid-Stimulation

We examined the effects of different ABA concentrations on the accumulation of transcripts in young embryos. As can be seen in Fig. 10 (see also Appendix 5), maximum steady-state levels of Emb5, Emb564, Emb604, and Emb807 were found in embryos treated with 1 μM to 10 μM ABA. Accumulation of transcripts complementary to Emb5,

Figure 10. Densitometric Estimation of Relative Accumulation of Transcript in Embryos Incubated with Different Concentrations of ABA. The relative intensities of signals from autoradiographs shown in Appendix 5 were estimated by densitometry and graphically displayed. Examination of the stained gel indicated that lane 2 (10^{-8} M ABA) was underloaded. Densitometric values for this lane obtained from all autoradiographs were therefore adjusted by a factor which increased the Zm13 estimation to a value intermediate to those obtained from lanes 1 and 3. The greatest value measured on each autoradiograph was considered 100%.

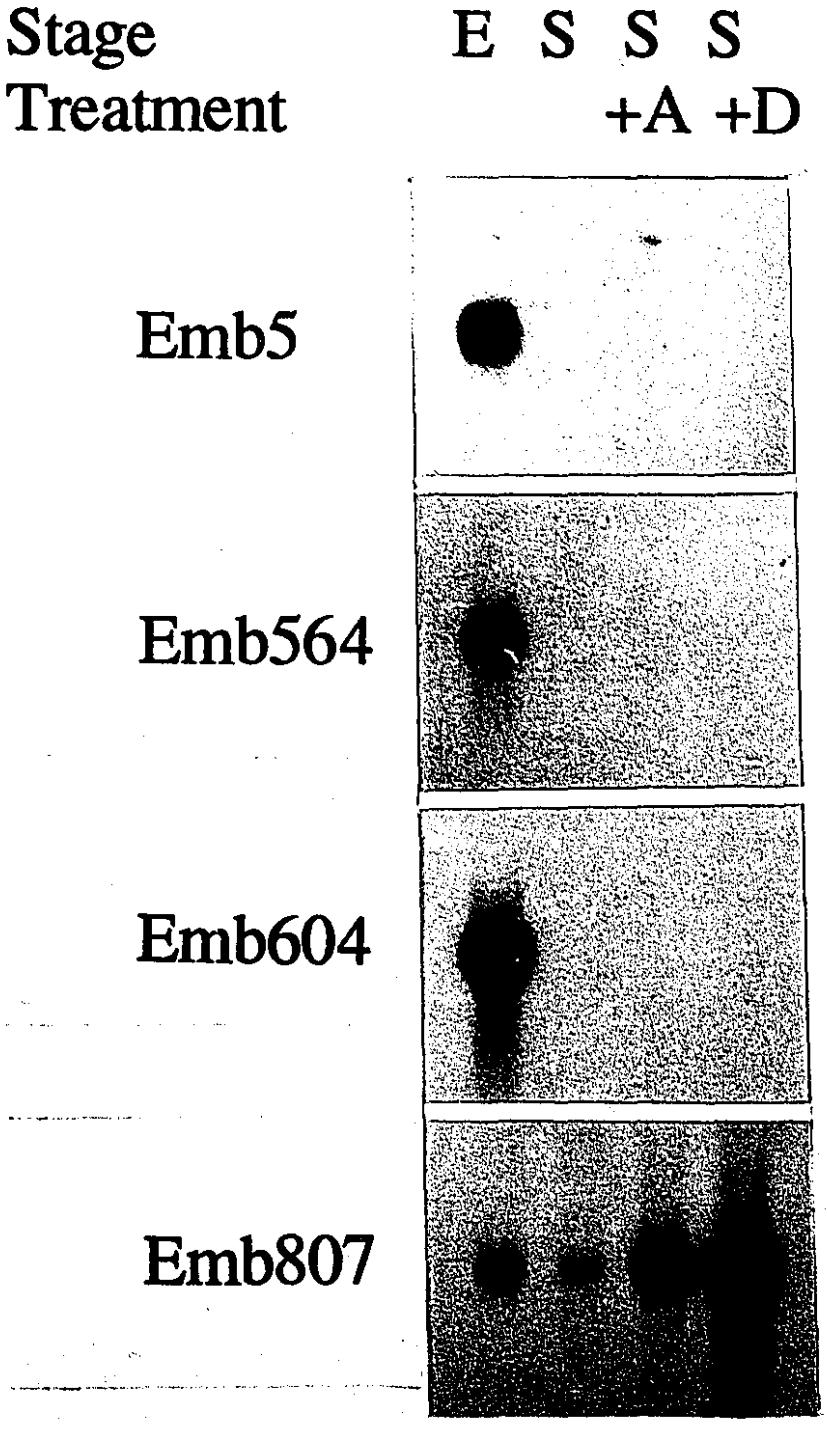

Emb5
Emb564
Emb604
Emb80?
pZm13
Transcript Accumulation (%)
100
90
80
70
60
50
40
30
20
10
0
9
8
7
6
5
-log [ABA]

Emb564 and Emb807 were similarly affected by ABA concentrations. Transcripts were barely detectable in embryos treated with 1-10 nM ABA and increased to 5-10% maximum values in embryos treated with 100 nM ABA. On the other hand, the accumulation of Emb604 transcript in embryos incubated with 1 nM and 100 nM ABA was approximately 45% and 55% of maximum, respectively. The Zm13 RNA species accumulated to greater than 80% maximum levels in the 1 nM and 100 nM ABA treatments. The reduced level of Zm13 and Emb604 transcript observed in the 10 nM ABA lane shown in Appendix 5 was the result of sample underloading. The reduced (30% of maximum) amount of Zm13 RNA detected in embryos treated with 10 µM ABA, however, appears to reflect a genuine decrease since a reduction of similar magnitude is not observed in the other transcripts. Incubation of embryos with 100 µM ABA resulted in the apparent degradation of all RNA species (data not shown) possibly due to toxicity.

3.6 Induction of Gene Expression in Seedlings

The concentration of ABA has been shown to increase in drought-stressed plants where it stimulates the accumulation of specific transcripts and proteins (Skriver and Mundy 1990). We tested the ability of seven day old seedlings to express Emb5, Emb564, Emb604 and Emb807 when subjected to water stress or exogenous ABA. We found no accumulation of Emb5, Emb564 and Emb604 transcripts in untreated, ABA-treated, or water-stressed seedlings (Fig. 11). Emb807, on the other hand, was expressed at high levels in seedlings subjected to water stress or whose roots were immersed 16 hours in 100 µM ABA, in agreement with previous reports (Close *et al.* 1989, Vilardell *et al.*

Figure 11. Stimulation of Transcript Accumulation in Water-Stressed and ABA-Treated Seedlings. RNA was extracted from seedlings whose roots were placed in 10 mM CaCl2 containing 100 µM ABA for 16 hours (S+A); from seedlings which were shaken free of vermiculite, air dried at 25°C for 1 hour until they had lost 40% of their fresh weight then stored 16 hours at 25°C in a plastic bag (S+D); or from whole, untreated 7 day old maize seedlings (S) . RNA from 20 mg embryos was included as a control (E). Total cellular RNA was analyzed by Northern blotting with radiolabelled probes of Emb5, Emb564, Emb604 and Emb807. Densitometric estimation of transcript quantities can be seen in Appendix 6.


Stage
Treatment
E
S
S +A
S +D
Emb5
Emb564
Emb604
Emb807

1990). The low level of this transcript detected in untreated seedling tissue here but not in Figure 5 or in other studies (Close *et al.* 1989, Vilardell *et al.* 1990) suggests these plants may have been subjected to a mild stress prior to harvest.

3.7 The Effects of Abscisic Acid on Transcript Accumulation in Abscisic Acid-Deficient and Abscisic Acid-Insensitive Viviparous Mutants

To further characterize the involvement of ABA in the expression of embryonic genes, we examined the levels of transcripts and the effects of exogenous ABA on their accumulation in the ABA-deficient *vp5* mutant and the ABA-insensitive *vp1* mutant (Robichaud and Sussex 1986, Robichaud *et al.* 1980). As can be seen in Fig. 12 and Appendix 6 the steady-state levels of Emb5, Emb564 and Emb807 transcripts in untreated 16-mg and 40-mg *vp5* embryos were very low compared to that seen in wild-type embryos of comparable age. This low level of expression can be explained by the presence of reduced levels of ABA in *vp5* embryos (Neill *et al.* 1986). Figure 12 shows that exogenous ABA stimulated the accumulation of transcripts for Emb5, Emb564 and Emb807 in 16-mg *vp5* embryos. Although exogenous ABA did not stimulate the accumulation of Emb5 or Emb564 transcripts in 40-mg *vp5* embryos, a low level of stimulation of Emb807 transcript was observed. A large accumulation of Emb604 transcript, on the other hand, was found in untreated 16-mg *vp5* embryos which appeared to be unaffected by exogenous ABA. A much smaller accumulation was found in untreated 40-mg embryos *vp5* embryos. The expression of Emb604 also differed from the other genes in the ABA-insensitive *vp1* mutant embryos. Whereas no transcripts complementary to Emb5, Emb564 or Emb807 could be detected in untreated or ABA-

Figure 12. Effects of ABA on the Accumulation of Emb5, Emb564, Emb604, Emb807 and pZm13 Transcripts in Wild-Type and *Viviparous* Embryos. One set of embryos was frozen immediately and kept as untreated controls (C). The second set of embryos was incubated on N6 medium supplemented with 1 μM ABA for 16 h (A). Total cellular RNA extracted from wild-type embryos and mutant embryos was examined by RNA blot hybridization. Densitometric estimations of transcript levels can be found in Appendix 6.

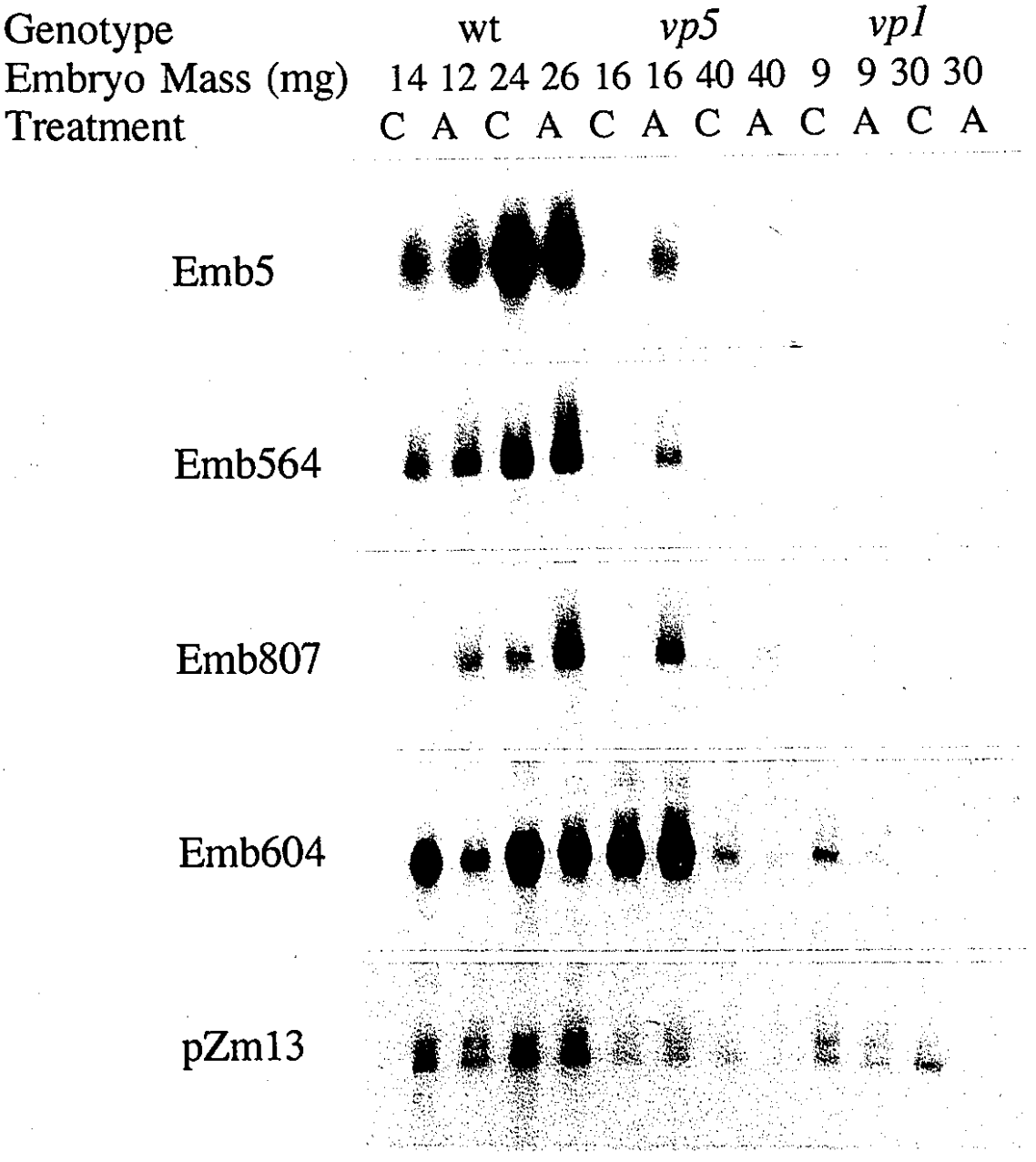

Genotype wt vp5 vp1
Embryo Mass (mg) 14 12 24 26 16 16 40 40 9 9 30 30
Treatment C A C A C A C A C A C A
Emb5
Emb564
Emb807
Emb604
pZm13

Figure 13. Nucleotide Sequence of Emb5.

The DNA and predicted amino acid sequence are numbered from the first nucleotide in the clone and the first methionine, respectively. The asterisks indicate the termination codon. TAG trinucleotides in the noncoding region are underlined. Arrows indicate imperfect tandem repeats.

```
                                    Met Ala Ser Gly Gln Glu Ser Arg   8
AGCTTTGCTTGTTAGTTTGGGGACGGCG ATG GCG TCC GGT CAG GAA AGC AGG   52

Glu Glu Leu Ala Arg Met Ala Glu Glu Gly Gln Thr Val Val Ala    23
GAG GAG CTG GCG CGC ATG GCC GAG GAG GGG CAG ACC GTC GTC GCG    97

Arg Gly Gly Lys Thr Leu Glu Ala Gln Glu His Leu Ala Glu Gly    38
CGC GGC GGC AAG ACC CTC GAG GCG CAG GAG CAC CTC GCC GAA GGG   142

                  ____________________________________________

Arg Ser His Gly Gly Gln Thr Arg Ser Glu Gln Leu Gly His Glu    53
CGC AGT CAC GGC GGG CAG ACC CGG AGT GAG CAG CTG GGC CAT GAG   187

_____________________________________________> ______________

Gly Tyr Ser Glu Met Gly Ser Lys Gly Gly Gln Thr Arg Lys Glu    68
GGG TAC AGC GAG ATG GGC AGC AAG GGC GGG CAG ACC CGC AAA GAG   232

_____________________________________________________________>

Gln Leu Gly His Glu Gly Tyr Ser Glu Met Gly Arg Lys Gly Gly    83
CAG CTG GGC CAC GAA GGG TAC AGC GAG ATG GGG AGG AAG GGC GGC   277

Leu Ser Thr Met Gln Glu Ser Gly Glu Arg Ala Ala Arg Glu Gly    98
CTG AGC ACC ATG CAG GAG TCC GGC GAG CGC GCC GCC CGG GAG GGC   322

Ile Glu Ile Asp Glu Ser Lys Phe Arg Thr Lys Ser ***           110
ATC GAG ATC GAC GAG TCC AAG TTC AGG ACC AAG TCC TAGATCTGATGC 370

GTGCCCCCGCTTAGCATGTAGTACGTAGCTGCAGGAATGTAGTGTCGTCGTAGTAGTAGCC 431
```

treated *vp1* embryos, transcript for Emb604 accumulated slightly in 9-mg *vp1* embryos and was faintly detectable in 30-mg *vp1* embryos. Incubation of *vp1* embryos with ABA did not result in increased accumulation of Emb604 transcripts. pZm13, which was previously found to be unresponsive to ABA (Fig. 8), was detected in *vp5* and *vp1* embryos at levels somewhat lower than in wild-type embryos of similar age. Exogenous ABA did not appear to affect the accumulation of pZm13 RNA in viviparous embryos.

3.8 Nucleotide Sequences of Two Embryo-Specific cDNAs, Emb5 and Emb564.

To further characterize the clones, the complete sequence of Emb5 and Emb564, and partial sequences of Emb604, Emb807 and pZm13 were determined.

Appendix 7 describes the strategy used to sequence both strands of Emb5. The sequence of Emb5 (Fig. 13) exhibited a single open reading frame of 330 bp. The open reading frame is G + C rich (69.7%), particularly in the third codon position (92.6%). A 60 bp tandem repeat is evident within the ORF (see section 3.9). The 3' noncoding region consists of 74 bp in which the trinucleotide TAG occurs 8 times. No consensus polyadenylation signal sequences are evident.

The Emb564 sequence resulted from an attempt to obtain a longer Emb5 cDNA clone. The Emb5 sequence was used to screen the cDNA library and identified a clone, pZm56, bearing a 0.7 bp insert. This insert was found to be similar but not identical to Emb5. The first 57 nucleotides of pZm56 were subsequently found to be identical to the last 57 nucleotides of a small insert, pZm7, which had been found to hybridize to a 0.8 kb transcript exhibiting the same developmental pattern of accumulation as Emb5. I have, therefore, concluded these two clones represent overlapping cDNAs of the same transcript,

Figure 14. Nucleotide Sequence of Emb564.

The DNA and predicted amino acid sequence are numbered from the first nucleotide in the clone and the first methionine, respectively. The asterisks indicate the termination codon. ATG trinucleotides in the noncoding regions are underlined. Three putative polyadenylation signal sequences are shown in bold. The inverted repeats are marked with arrows and dots (.) indicate mismatches.

```
CAGACAACACTCACCGATAGCAAGTAACGCCGCCGACGTTTCGAGAGGAGAGTATCCAA  59

                              <————————.++.————————>
GCTAGCCAAGCGCGCACCTCGGTGACCTAGCTAGCTACCTAGCTAGTTCAGGCGACGAT 118

MET Ala Ser Gly Gln Glu Ser Arg Lys Glu Leu Asp Arg Lys Ala  15
ATG GCG TCT GGT CAG GAG AGC AGG AAG GAG CTG GAC CGC AAG GCT 163

Arg Glu Gly Glu Thr Val Val Pro Gly Gly Thr Gly Gly Lys Ser  30
CGC GAG GGC GAG ACC GTC GTC CCC GGC GGG ACC GGC GGC AAG AGC 208

Val Glu Ala Gln Glu His Leu Ala Glu Gly Arg Ser Arg Gly Gly  45
GTC GAG GCC CAG GAG CAC CTC GCC GAA GGG CGC AGC CGC GGA GGC 253

Gln Thr Arg Arg Glu Gln Leu Gly Gln Gln Gly Tyr Ser Glu MET  60
CAG ACT CGC AGG GAG CAG CTG GGG CAG CAG GGG TAC AGC GAG ATG 298

Gly Lys Lys Gly Gly Leu Ser Thr Thr Asp Glu Ser Gly Gly Glu  75
GGG AAG AAG GGC GGT CTG AGC ACC ACG GAC GAG TCC GGC GGC GAG 343

Arg Ala Ala Arg Glu Gly Val Thr Ile Asp Glu Ser Lys Phe Thr  90
CGC GCC GCC AGG GAG GGC GTC ACG ATC GAC GAG TCC AAG TTC ACC 388

Lys***                                                      91
AAGTAGTCCTATCGCGTGCGTGCGTACGCAGCGTTCGTAGCTGTCTGCGTAGTGTACCT 447

TGGCCGTAGCTAGTGCTACTCTCTACTTTGGAGTAGCTGAATGATAATAATGTAGCTAG 506

CAGCATGCATGTAGGCGATCATACATACGTACGTACACAGGTACGTGTGTGTGGATCAT 565

GTATGCTTCCAGAAGGGTATCCTAGCTTAATCAGTCACGTCGTCAAGTCGTCAATATGG 624

CAGGTTCGCGTCAGGGCTTTGTAGTTTGATAGCCTGTTTCACGACTCCTCAATAATCAA 683

GTAGTGTAATGAAAGTTTGGCGGTTCCTTGTTATTATCTGCTTGTTGTATCTCCTGCTC 742

TGCACTGGTTAATTTCTCCCATATTGTGGTAAAAAAAAAAAAAAAAAAAAAAAAA     797
```

Figure 15. Alignment of Emb564 Inverted Repeat with Similar Sequences.

The 10 bp inverted repeat located in the 5'-untranslated region of Emb564 is shown aligned with similar sequences from the maize Rubisco small subunit (Lebrun *et al.* 1987; Matsuoka *et al.* 1987) and soybean chalcone synthase (Wingender *et al.* 1989).

```
Emb564      TGACCTAGCTAGCTACCTAGCTAGTTCA
RBCS        CCGG...........G.........C.T
RBCss       CCCG.CG........G.........C.C
X16185      AA.AT..........G...T....GATG
```

which I call Emb564. Considering the high G+C content of the open reading frame it is unlikely pZm7 was primed by oligo(dT). One possible mechanism of its origin involves self-priming by sequences at the 3' terminus of a partially degraded transcript. A likely candidate for such an event is the octanucleotide CCTGCTCT 28 nucleotides upstream of the polyadenylation site which is complementary to the sequence AGAGCAGG at nt 135, just 3' to the end of pZm7. The strategy used to sequence Emb564 is shown in Appendix 8. Figure 14 shows the nucleic acid sequence of the Emb564 cDNA (Williams and Tsang 1991). The cDNA appears to contain the entire 3' non-coding region since it includes part of the poly(A) tail and three putative polyadenylation sites. Most plant genes contain two or more polyadenylation signals (Heidecker and Messing 1986). The last of these is generally found 15-23 bp from the polyadenylation site and tends to diverge from the eukaryotic consensus, (A/G)ATAA(A/T). In Emb564 the sequence AATAAT is found twice, 100 bp and 285 bp downstream from the translation stop codon. The final putative polyadenylation signal, GTTAAT, is found 18 bp upstream of the polyadenylation site. A single open reading frame encoding 91 amino acids was observed. Upstream from the translation initiation codon is a 10-bp inverted repeat sequence: $(CTAG)_2CTAC(CTAG)_2$. A similar sequence was found in the 3' untranslated region of several maize ribulose-1,5-bisphosphate carboxylase small subunit mRNAs (Lebrun *et al.* 1987, Matsuoka *et al.* 1987) and immediately upstream of the translation start codon of the soybean chalcone synthetase gene (Wingender *et al.* 1989). These sequences are shown aligned in Fig. 15. Sequences showing similarity to the Emb564 inverted repeat were also found in the soybean proline-rich cell wall protein gene (Hong *et al.* 1987), and the *C. elegans* glp-1

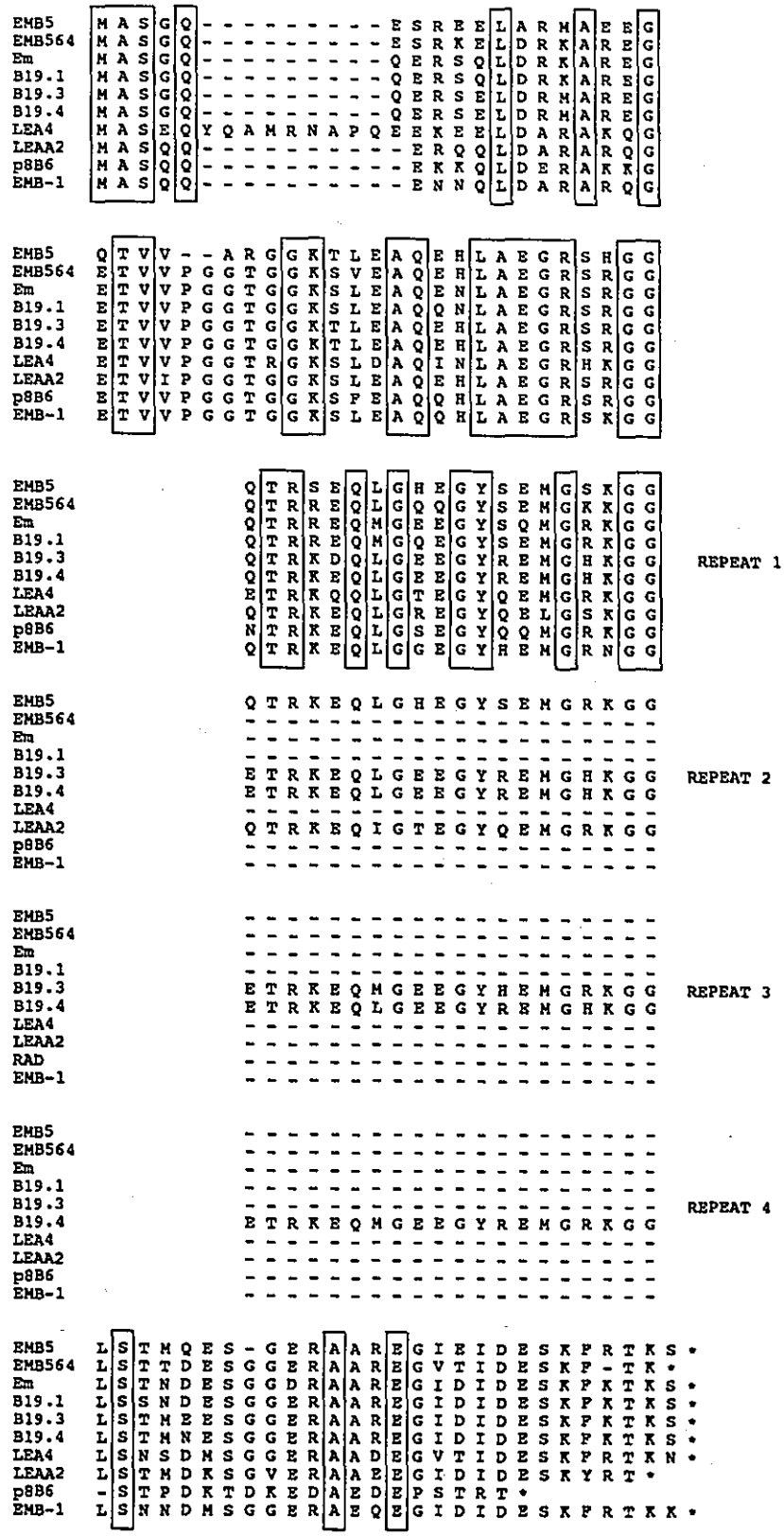
	MOLE PERCENT									
AMINO ACID	EMB5	EMB564	Em	B19.1	B19.3	B19.4	Lea4	LeaA2	EMB-1	p8B6
GLYCINE	17.3	18.7	18.3	18.3	20.3	20.9	14.7	18.2	18.5	15.7
ARGININE	9.1	9.9	10.8	10.8	9.8	10.5	7.8	10.0	7.6	7.2
GLUTAMATE	17.3	14.3	12.9	12.9	18.8	19.6	12.7	13.6	12.0	12.0
SERINE	10.0	8.8	9.7	10.8	5.3	4.6	5.9	6.4	6.5	7.2
GLUTAMINE	7.3	6.6	7.5	8.6	5.3	5.2	7.8	10.9	9.8	9.6
VALINE	1.8	4.4	2.2	2.2	1.5	1.3	2.9	1.8	2.2	2.4
ALANINE	7.3	6.6	6.5	6.4	4.5	3.9	8.8	6.4	6.5	6.0
LYSINE	5.4	7.7	6.5	6.4	7.5	7.8	7.8	6.4	6.5	10.8
THREONINE	5.4	7.7	5.4	4.3	6.0	5.9	5.9	6.4	4.3	8.4
LEUCINE	5.4	4.4	4.3	4.3	4.5	4.6	4.9	5.5	5.4	3.6
ASPARTATE	0.9	3.3	5.4	4.3	3.0	2.0	4.9	3.6	4.3	6.0
METHIONINE	4.5	2.2	3.2	3.2	5.3	5.2	3.9	2.7	3.3	2.4
PROLINE	0	1.1	1.1	1.1	0.8	0.7	2.0	0.9	1.1	3.6
HISTIDINE	3.6	1.1	0	0	3.0	2.6	1.0	0.9	2.2	1.2
ASPARAGINE	0	0	2.2	2.2	0	0.7	3.9	0	5.4	1.2
ISOLEUCINE	1.8	1.1	2.2	2.2	1.5	1.3	2.0	3.6	2.2	0
CYSTEINE	0	0	0	0	0	0	0	0	0	0
PHENYLALANINE	0.9	1.1	1.1	1.1	0.8	0.7	1.0	0	1.1	1.2
TYROSINE	1.8	1.1	1.1	1.1	2.2	2.6	2.0	2.7	1.1	1.2
TRYPTOPHAN	0	0	0	0	0	0	0	0	0	0

Table 7. Comparison of Amino Acid Content of Emb5, Emb564 and Homologs.

The amino acid content (mole %) of Emb5 (Williams and Tsang, 1992), Emb564 (Williams and Tsang, 1992), the wheat Em (Litts *et al.* 1987), the barley B19 genes (Espelund *et al.* 1992), the cotton Lea4 and LeaA2 (Galau *et al.* 1992), the radish p8B6 gene (Raynal *et al.* 1989), and the carrot EMB-1 (Ulrich *et al.* 1990) are shown.

Figure 16. Comparison of the Amino Acid Sequence of Emb5 and Homologs.

The predicted amino acid sequences of Emb5 (Williams and Tsang, 1992), Emb564 (Williams and Tsang, 1991), the wheat Em (Litts *et al.* 1987), the barley B19 genes (Espelund *et al.* 1992), the cotton Lea4 and LeaA2 (Galau *et al.* 1992), a radish Lea gene (Raynal *et al.* 89), and the carrot EMB-1 (Ulrich *et al.* 1990) are aligned.


EMB5
EMB564
Em
B19.1
B19.3
B19.4
LEA4
LEAA2
p8B6
EMB-1
REPEAT 1
REPEAT 2
REPEAT 3
REPEAT 4
RAD

gene (Yochem and Greenwald 1989).

Emb5 and Emb564 are most similar in their open reading frames, sharing 84% similarity over 273 nucleotides. Significant similarity is retained in their 3' sequences also. The 65 or 66 nucleotide sequence immediately following the translation stop codon is N_v-GCGTGC-N_w-CGTAGCTG-N_x-GTAGTGT-N_y-CGTAG-N_z-TAG (where N_i represents i nucleotides sharing little homology). The values of v, w, x, y, and z are, respectively 7, 19, 7, 3 and 0 for Emb5 and 7, 16, 5, 8 and 1 for Emb564.

3.9 Emb5 and Emb564 are Homologous to Proteins from Other Species.

The predicted amino acid sequences suggest that Emb5 and Emb564 encode small (12 kD and 10 kD, respectively) hydrophilic polypeptides. The predicted polypeptides are rich in hydrophilic amino acids and poor in aromatic and sulfur-containing amino acids. The amino acid composition of Emb5, Emb564 and homologs is shown in Table 7. No distinct secondary structure is predicted for either polypeptide (see section 4.1).

The predicted amino acid sequence of Emb564 was compared against the Genbank (Bilofsky and Burks 1988) data bank for sequence similarity. Emb564 was found to share 81.7% identity with the wheat Em gene (Litts *et al.* 1987). Extensive homology was also found between Emb564 and the radish p8B6 gene (Raynal *et al.* 1989), the cotton LEA4 and LeaA2 proteins (Baker *et al.* 1988, Galau *et al.* 1992), the carrot EMB-1 gene (Ulrich *et al.* 1990), the rice Emp1 gene (Litts *et al.* 1992) and the barley B19 genes (Espelund *et al.* 1992). Figure 16 shows an alignment of the homologous sequences. Of interest is the 20 amino acid motif which occurs once in Emb564, Em, EMB-1, B19-1, Lea4, and the p8B6 gene, but occurs as a tandem duplication in Emb5 and LeaA2, and occurs in

Figure 17A. Alignment of Emb604 with the Oleosin KD-18 Gene.

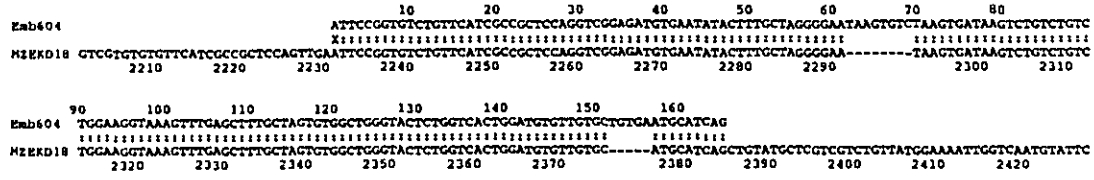
The partial sequence of Emb604 is aligned with the corresponding sequence of the maize Oleosin KD-18 gene. (Qu and Huang 1990)

Figure 17B. Alignment of Emb807 with the Rab-17 Gene.

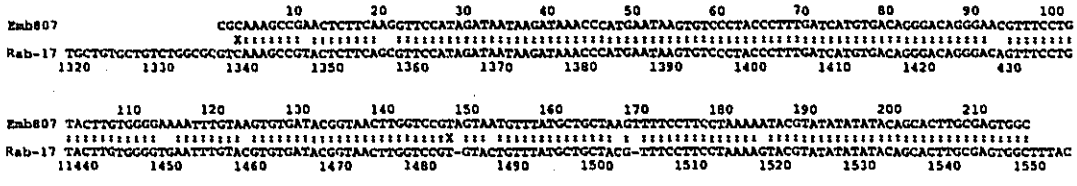
The partial sequence of Emb807 is aligned with the corresponding sequence of the maize Rab-17 gene. (Vilardell *et al.* 1990)

Figure 17C. Alignment of Zm13 with Maize Chloroplast 23S rRNA. The partial sequence of Zm13 is aligned with the corresponding sequence of the maize chloroplast 23S rRNA. (Edwards and Kössel 1981)

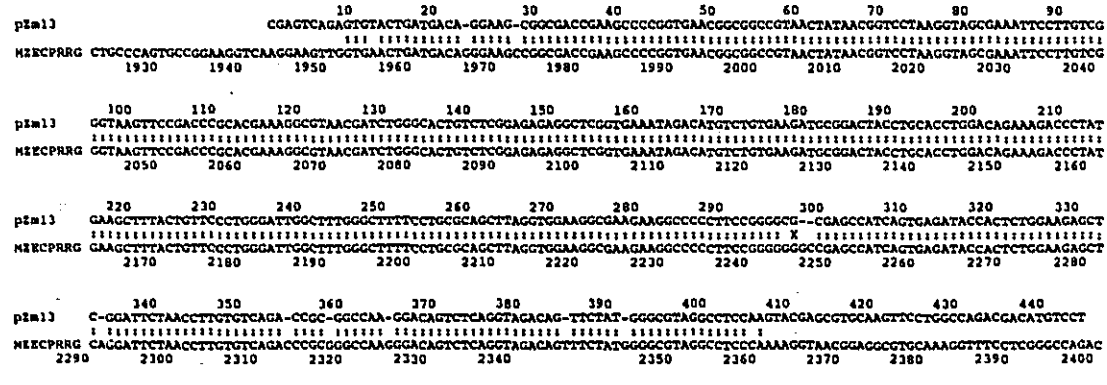
A



B



C



three and four tandem copies in B19-3 and B19-4, respectively.

3.10 Partial Sequences of Three Other cDNAs and Their Identification as Rab-17, Oleosin KD18 and Chloroplast 23S rRNA

Parts of the Emb604, Emb807 and pZm13 cDNAs were sequenced and compared with the Genbank library. Significant similarity was found to Oleosin KD18 (Qu and Huang 1990), Rab-17 (Vilardell *et al.* 1990), and chloroplast 23S rRNA (Edwards and Kössel 1981), respectively. Figure 17 shows the alignment of these partial sequences with the corresponding region of the previously reported sequences. The partial sequence of Emb604 was found to have 91% identity to nucleotides 840-991 in the 3' untranslated region of the Oleosin KD18 cDNA (Qu and Huang 1990). The partial sequence of Emb807 was found to share 94% identity with nucleotides 780-990 in the 3' untranslated region of the RAB-17 gene (Vilardell *et al.* 1990). The partial sequence of pZm13 was found to share 97% identity with nucleotides 1953-2360 at the 3' end of the maize chloroplast 23S rRNA gene (Edwards and Kössel 1981). Since these sequences have been characterized before, we did not extend our sequence analysis. It is uncertain whether the differences between the sequences reported here and the published sequences are the result of allelic differences or are due to sequencing errors. The sequences of pZm13, Emb604 and Emb807 must be considered as tentative, as the sequence of the complementary strand was not determined.

3.11 Genomic Organization of Genes Emb5, Emb564, Emb604 and Emb807.

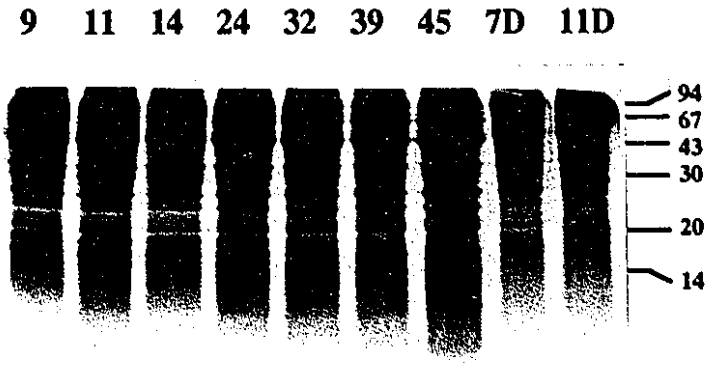
The genomic organization of the Emb5, Emb564, Emb604 and Emb807 genes was examined. DNA blot hybridization (Fig. 18) indicated that each probe hybridized strongly

Figure 18. Genomic Organization of Emb5, Emb564, Emb604 and Emb807. Nuclear DNA was extracted from young seedlings, digested with *Bgl* II, *Bam* H1, *Dra* I, *Eco* R1, *Hin*d III, *Hin*f I, *Msp* I or *Pvu* II then size-fractionated on agarose gel and blotted to Nytran. Probes complementary to Emb5, Emb604 and Emb807 were hybridized successively and washed at high stringency. Numbers to the right represent DNA molecular weight markers (kb).

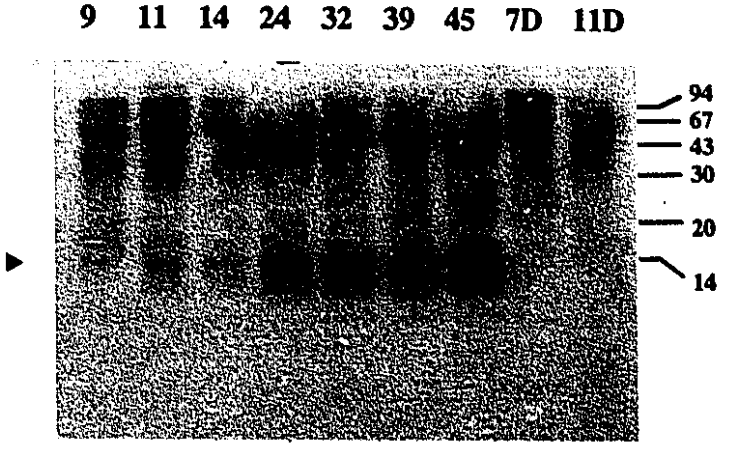
Emb5
Emb564
Emb604
Emb807
Bgl II
Bam HI
Dra I
EcoR I
Hind III
Hinf I
Msp I
Pvu II
23
9.3
6.6
4.4
2.3
2.0

Figure 19. Developmental Accumulation of Proteins Recognized by Anti-Emb564 Antiserum. SDS-solubilized proteins were isolated from embryos of the following stages: 9 mg (9), 11 mg (11), 14 mg (14), 24 mg (24), 32 mg (32), 39 mg (39), 45 mg (45), and from seedlings 7 days (7D) and 11 days (11D) after imbibition. Proteins were size-fractionated on SDS-polyacrylamide gels and either stained with Coomassie Blue (A), or electroblotted to membranes and incubated with anti-EMB564 serum (B) or pre-immune serum (C). Bound antibodies were detected with radiolabelled goat anti-rabbit antibodies as described in Material and Methods. Arrowhead indicates position of putative EMB564 protein. Numbers to the right indicate protein molecular weight markers (kD).

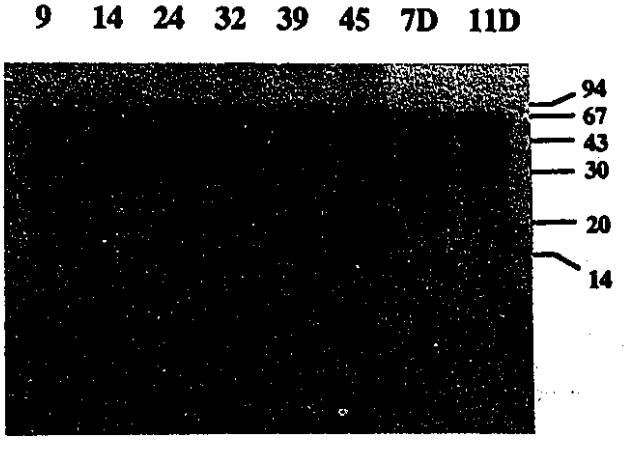
A


9 11 14 24 32 39 45 7D 11D
94
67
43
30
20
14

B


9 11 14 24 32 39 45 7D 11D
94
67
43
30
20
14

C


9 14 24 32 39 45 7D 11D
94
67
43
30
20
14

to a single DNA size class in each digest. In several digests probed with Emb5, secondary, weaker bands were seen. These bands can be attributed to cross-reactivity of the Emb5 probe to the homologous Emb564 gene (Williams and Tsang 1991). These results suggest that each of the sequences reported here is represented by only one or very few closely-linked genes.

3.12 Expression of the Emb564 Polypeptide During Embryogenesis and Germination.

To examine the expression of the EMB564 protein, antibodies were generated in rabbits against purified ß-galactosidase-EMB564 fusion protein and used to probe protein blots. The antiserum reacted with a protein slightly smaller than 14 kD in maize embryo extracts (Fig. 19). This protein reached detectable levels in 11-mg embryos, coincident with the appearance of the Emb5 and Emb564 transcripts. The steady-state level of this protein increased during the remaining period of seed maturation, then decreased to undetectable levels during the first week of germination. Larger molecular weight proteins detected by the anti-EMB564 serum were also detected by preimmune serum and were considered to be non-specific for anti-EMB564 antibodies.

3.13 Anti-EMB564 Antibodies Recognize Small Proteins in Other Species.

The seeds of some common agronomic plant species were examined for proteins similar to EMB564. Western blot analysis (Fig. 20) indicated that anti-EMB564 antibodies recognize relatively small proteins (9kD-25kD) in all species examined.

3.14 The Steady-state Level of EMB564 Protein Decreases in the Absence of ABA.

To examine the effect of ABA on the level of EMB564 in immature embryos, 11-mg maize embryos were incubated with or without exogenous ABA. As shown in Figure

Figure 20. Accumulation of Emb564-like Proteins in Other Species. SDS-solubilized proteins were extracted from maize embryos, wheat germ, and the seeds of tomato, carrot, radish, pea, parsley, lettuce and cucumber. Following size fractionation on 15% polyacrylamide gel, the proteins were either stained with Coomassie Brilliant Blue (A) or electroblotted to an Immobilon P membrane and reacted with anti-EMB564 serum (B) or preimmune serum (C). Arrowhead indicates position of putative EMB564 protein. Numbers to the right indicate molecular weight markers (kD).

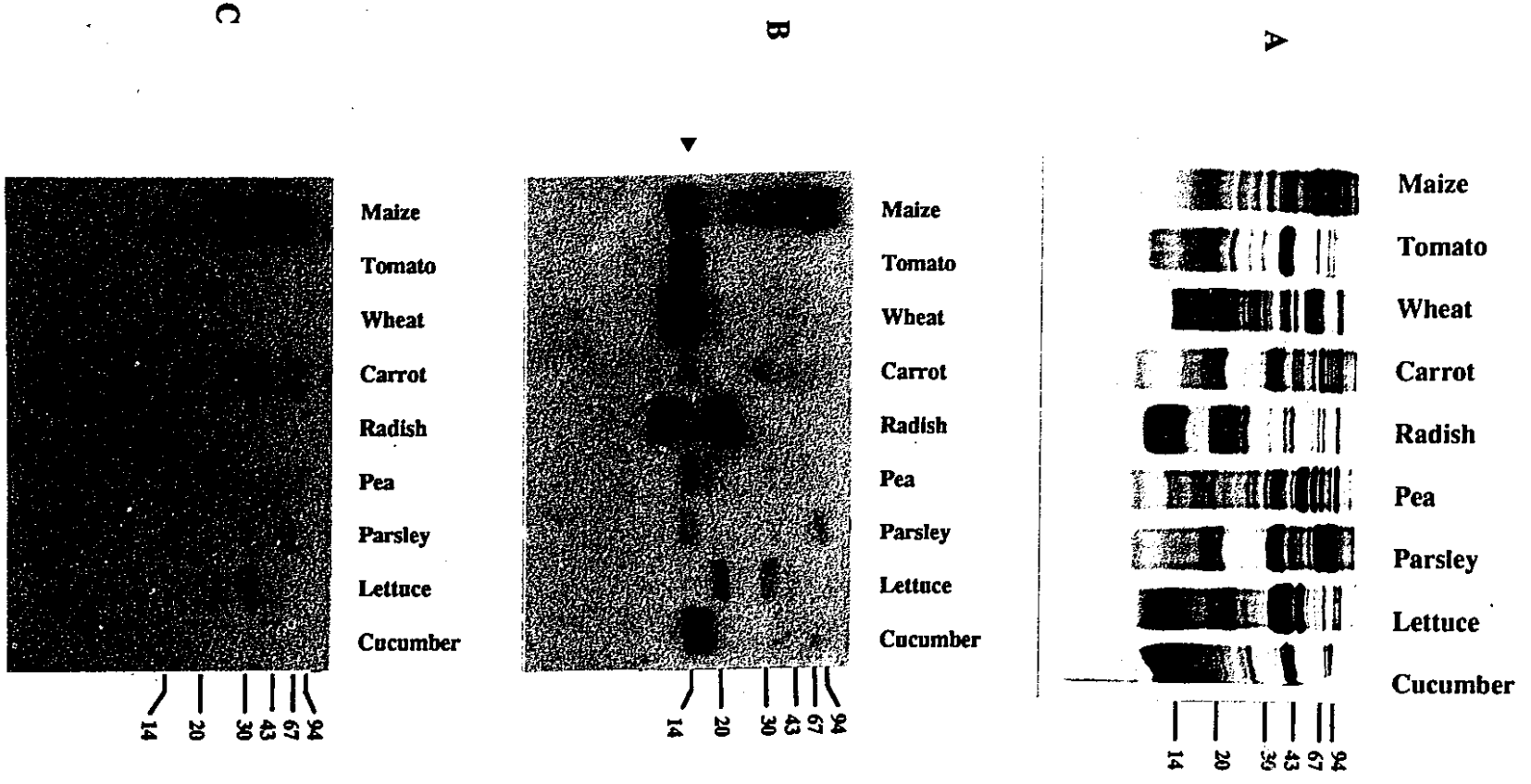
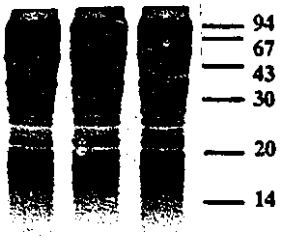
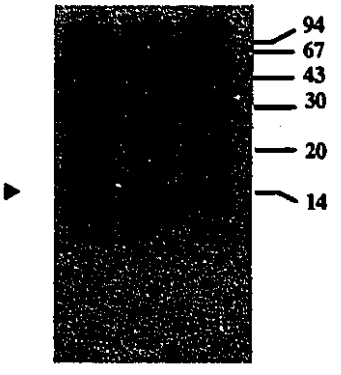

C
B
A
Maize
Tomato
Wheat
Carrot
Radish
Pea
Parsley
Lettuce
Cucumber
14
20
30
43
67
94
35

Figure 21. The Effect of ABA on Emb564 Protein Accumulation. Proteins were extracted from 11-mg embryos (11), and 11-mg embryos incubated 16 h with (11A) or without (11N) 1 µM ABA. After fractionation by electrophoresis through 15% polyacrylamide, proteins were either stained with Coomassie Brilliant Blue (A) or electroblotted to Immobilon P membranes and reacted with anti-EMB564 serum (B) or preimmune serum (C). Bound antibodies were detected with radiolabelled goat anti-rabbit antibodies as described in Material and Methods. Arrowhead indicates position of putative EMB564 protein. Numbers to the right indicate molecular weight markers (kD).

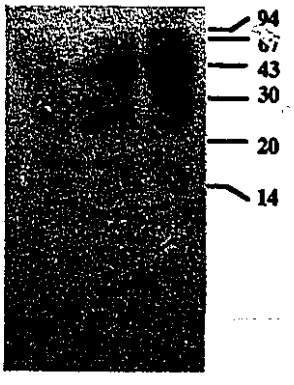
A


11 11A 11N
94
67
43
30
20
14

B


11 11A 11N
94
67
43
30
20
14

C


11 11A 11N
94
67
43
30
20
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21, exogenous ABA increased the steady-state level of the putative EMB564 protein. This protein dropped to an undetectable level in embryos incubated without ABA. These treatments did not result in a noticeable change in the concentrations of any other proteins detected with Coomassie Blue or antisera.

CHAPTER 4. DISCUSSION

As reviewed in Chapter 1, ABA acts as a signal for a variety of processes. During vegetative growth ABA is involved in the plant's response to various stresses (see section 1.2.1) and appears to play an important role in fruit maturation and senescence (see section 1.2). Its primary role during embryogenesis appears to be one of maintaining the maturation pathway (see sections 1.3.1, 1.3.2) and preventing germination (section 1.3.3).

For almost all plant species characterized the concentrations of ABA fluctuate during embryogenesis. In maize the level of ABA increases from less than 0.1 μM (Carnes and Wright 1988) early in embryogenesis (0-15 DAP) to about 0.7 μM (Neill *et al.* 1987) during mid embryogenesis (25 DAP). This temporally modulated increase in the concentration of a small signal molecule provides a simple means of regulating the developmental program of the embryo. Sets of genes having different sensitivities to ABA could be stimulated sequentially as the ABA concentration increased. Such a scheme would introduce some degree of flexibility into the timing of developmental events. For example, unfavourable conditions such as drought or cold might cause the ABA concentration of the embryo to rise earlier or at a greater rate than during more favourable conditions. As a result, the whole developmental program would be shifted and tolerance to desiccation and cold would be attained earlier. In this thesis I describe three classes of ABA-responsive genes. Class I genes are responsive to nM ABA and, as a result, are expressed early when ABA levels are low. Class II and Class III genes are responsive to μM ABA and are consequently expressed during mid embryogenesis during peak ABA levels. The role of ABA in the regulation of gene expression during late embryogenesis

is uncertain. However, the inhibitory effect of ABA on the expression of genes associated with germination suggests the growth regulator plays a similar, if inverse, role during this phase of seed development.

To understand the mechanism by which ABA regulates development it is necessary to characterize the elements of the signal transduction pathway affected by ABA. At the present time little is known about this pathway. Although ABA binding proteins in the plasma membrane have been identified, no ABA receptor has been characterized. Although Ca^{2+} levels have been shown to be affected by exogenous ABA in some tissues (see section 1.4.1) the relationship between ABA and this second messenger is undetermined. While the early steps in ABA signal tranduction are obscure, the effect of ABA on gene expression is clear. Dozens of genes have been shown to be regulated by ABA, particularly vegetative stress-induced genes (for a review, see Skriver and Mundy 1990). Some embryonic genes have been found to respond to ABA, particularly a group of Lea genes. Recent reports have identified promoter elements essential for transcriptional stimulation by ABA (see section 1.4.2). In addition, a gene encoding a protein which binds to the ABA-responsive element (Guiltinan *et al.* 1990) has been cloned. At the present time, it is uncertain whether these ABA-responsive, embryonic genes are regulated by one or several mechanisms.

I have examined the steady-state transcript levels of 4 ABA-responsive genes during embryogenesis. Three patterns of developmental accumulation were observed. Differences in the response to exogenous ABA during embryogenesis and to water stress during vegetative growth suggest these genes may be regulated by different mechanisms.

I have also characterized the accumulation of the polypeptide product of one of these genes. The conclusions based on these experiments are presented here.

4.1 Emb5 and Emb564 are Similar to a Group of Highly Conserved Seed Proteins.

The sequences of two of the cDNAs characterized in this study, Emb5 (Fig. 13) and Emb564 (Fig. 14), share 66% identity at the nucleotide level, indicating they are members of a gene family. The greatest similarity at the nucleic acid level is seen in the open reading frames (84% over 273 nucleotides). The predicted amino acid sequences of Emb5 and Emb564 share considerable similarity with embryonic sequences identified in a wide range of angiosperm species, both monocot and dicot. Emb564 was found to share amino acid sequence identity with Emb5 (76%), the wheat Em protein (83%), barley B19.1 (84%), B19.3 (82%), B19.4 (83%), cotton Lea4 (71%), LeaA2 (72%), carrot EMB-1 (70%), and the radish p8B6 protein (67%) (see Fig. 16 and Table 8). At 41 of 93 positions the amino acid was found to be invariant between the different genes. A further 11 positions exhibited only conservative changes in amino acid. These polypeptides are rich in glycine, basic, acidic, and hydroxylic amino acids (see Table 7). They are poor in aromatic and sulfur-containing amino acid, and proline. Computer analysis using the methods of Garnier *et al.* (1978) and Kyte and Doolittle (1982) indicated the structures of the predicted polypeptides are very similar. Overall, these proteins were predicted to be very hydrophilic. The amino terminal 43-54 amino acids contains two short sequences with potential to form α-helical structures. The remaining polypeptide was predicted to exist in a predominantly random coil configuration as it is comprised of very short segments of ß-extended structure and turns interspersed with coiled structure. A central

Table 8. Sequence Identity between Emb5, Emb564 and Homologs

	Predicted Amino Acid Sequence Identity (%)	
Gene Name	Emb5	Emb564
Emb5 (maize)	-	76.4
Emb564 (maize)	76.4	-
Em (wheat)	72.0	83.1
B19.1 (barley)	72.0	84.3
B19.3 (barley)	78.8	82.0
B19.4 (barley)	79.6	83.1
Lea4 (cotton)	62.4	70.8
LeaA2 (cotton)	70.6	72.4
EMB-1 (carrot)	62.2	70.1
p8B6 (radish)	50.6	66.7

region consists of one or more copies of a 20 amino acid repeat motif rich in charged amino acids. The 19 to 28 amino acids at the carboxy terminus contains the highest density of charge with acidic residues predominating.

The high content of acidic, basic, and amide-containing amino acids in these polypeptides suggests their hydration potential is especially high. In addition, the random coil configuration would be expected to expose more hydrophilic sites to the surrounding medium (see the discussion in McCubbin *et al.* 1985). It is unknown whether proteins with multiple copies of the 20 amino acid repeat have a different function than those with a single copy. The maintenance of both classes of proteins could benefit a species either by serving different functions or as a means of increasing the level of proteins serving the same function.

The Em protein is the only member of this set of proteins which has been isolated and its physicochemical properties directly studied (McCubbin *et al.* 1985). Circular dichroism measurements indicated that the Em protein was 70% random coil, 13% α-helix and 17% ß-structures. It was estimated to have a high Stokes radius (2.82 nm) and a low specific volume (0.683 ml/g). These measurements are consistent with a random coil structure as predicted by my computer analysis. McCubbin *et al.* (1985) estimated the hydration potential of Em to be high, in the range of 0.5-1.0 g water/g protein. The authors suggested that Em may serve to protect cellular structures and components during desiccation by its ability to bind water and thus provide a matrix of bound water.

The 3' noncoding region of Emb5, Emb564 and their homologs also exhibit several common features. These include similar spacing (67-98 bp) between the

Figure 22. Alignment of 3' noncoding regions of Emb5 and Homologs. The 3' noncoding sequences of the maize Emb5 (Williams and Tsang, 1992), Emb564 (Williams and Tsang, 1991), wheat Em (Litts *et al.* 1987), barley B19.1, B19.3, B19.4 (Espelund *et al.* 1992), rice Emp1 (Litts *et al* 1992), radish P8B6 (Raynal *et al.* 89), and cotton LeaA4 (Galau *et al.* 1992) are aligned. TAG trinucleotides and putative polyadenylation signals are shown in bold type. Other sequence similarities are shown underlined.

```
          ***
Emb5      TAGATCTGATGCGTGCCCCCGCTTAGCATGTAGTACGTAGCTG-CAG-G
Emb564    TAGTCCTATCGCGTGCGTGCG-TACGCA-GCGTT-CGTAGCTGTCTGC
Em        TAGATGAT----------CTGTTACCCTAGC-AAGCAA
B19.1     TAGATGATGAATCGACCAGTGC----------GTACCGTAGCCTAGCAA
B19.3     TAAAGAATTGTGTAAGATGTCCAGTGCCTAGCCGA-GTAGCTAGTCACTAC
B19.4     TAAATGATGTATGCCAA-G-AT--GTCCCGTGCCA-GTAGCTAGCTAC
EMP1      TAGACTACACACACTTTTGCATCCGTGAA-TGCCA-GTGTGTCGTAGTCGTC
P8B6      TAGTCTTAAGATTTCGATGGATGTGTAGTAATAT-CGTCTTAAATCTTTTC
D19       TAATAATGTATGTTTGTTTTAGGTTTTAGGAAGAACTGAACCAGTTTATATG

Emb5                              AATGTAGT-GT-----CGTCGTAG-TAG-TAGCC
Emb564                              GTAGT-GTACCTTGGCCGTAGCTAG-T-C
Em                                    G-AGGA-----A-TC-T-CCT---TAG
B19.1                                 GCAAGACG---ACTC-T-GCT---TAG
B19.3                               TTACAAGTTAACAACTCGTAG-TAG-TAG
B19.4                                                 CGGTAG-TAG
EMP1                          TCAGTTGTAGTCATCGAGTCAGTCTTAGCTAGCTAG
P8B6                            CATATAGTTGCACGTTTTGGTTTCCTGTCAAT
D19                         TAACAAAACGCTGGACATGTTTAAGCTTTAG-CTT

Emb564              CTACTCTCTACTTTGGAGTAGCTGA-ATG-AT--AATAAT
Em                GTCGGCTGTTGGTTTGCCCTAGTGTCTACGTACCGAATAAT
B19.1             GTCGGCCGTTGGTTTACCCTAGTGTCCATGCACCGAATAAT
B19.3                                CGAGCAAGCTGACTGCTCAATAAA
B19.4                                CGAGCAAGCTGACTGCTGAATAAA
EMP1                                       CTCTCTTATCAATAAT
P8B6            CTGTCATAAGTTGTTGTT
D19             CTGTAATCTAAGCGGTGT

Emb564    GTAGCTAGCAGCATG----------CATGTAGGCGATCATACATACGTACG
Em        G----TAGTTCA-GG-C-G-----CACGTAGGCGATCGTACGTAGTATGA
B19.1     GTAG-TAGTTCA-GG-C-G-----CACGTAGGCGATCGTACGTAGTATGG
B19.3     TAATGTAGGTCAAGGTCAGGTCTTCACGTAGGCGTGAGCACGCATGTAG
B19.4     TGACGTAGGTCAAGATCAGGTCTTGACATAGGCGTGAGCACGCATGTAGGCGA
EMP1       AATGTAGGTCTTGACGGATGCACGCATTTAGGCGCCCGTATGATTTGC
P8B6           TAGTTCATAAGATGATGATCACCATGTGTGTATATACGTTTTCATCTCCT
D19          TATAGCCCTGTTGACGGTTTGTTTTAGTGTTATTAGGACTAAACCAGTTTAC

Emb564 TACACAGGTACGTGTGTGTGGATCATGTATGCTTCCAGAAGGGTATCCTAG
Em      TCATGTGTGCTTCGTAGC-TGGTAGTCTGTAGCTACTCTACGGTGAGTAGC
B19.1   TCATGTGTGCTTCGTAGAATCGTAGCTGGTAGTCAGTTGGTACTCTACGGTGAGTCG
B19.4   TCACATGTACTGTGATCATGTGTG
```

translation stop codon and the first putative polyadenylation signal and the recurrence of motifs containing the trinucleotide TAG (Fig. 22). Four TAG trinucleotides are also found in the imperfect palidrome CTAGCTAGCTACCTAGCTAG which occurs 13 nucleotides upstream of the translational start site of Emb564. It has been suggested that secondary structures such as hairpin loops in the vicinity of the translation start site might interact with the translational machinery and affect ribosome function (Bergmann and Lodish 1979, Kozak 1990). Interestingly, none of the 4 potential stop codons found in this sequence are in phase with the open reading frame.

The close similarity between these genes in maize, rice, wheat, barley, cotton, radish and carrot suggests they have evolved from a common ancestral gene. The stability of these sequences therefore is presumably maintained by a strong selection pressure, the nature of which is unknown.

4.2 Expression of Emb5 and Emb564 Differs from that of Rab-17 and Oleosin KD18.

Ribonucleic acid blot analysis of the five genes examined in this study revealed four different temporal patterns of transcript accumulation during development.

4.2.1 Regulation of Emb5 and Emb564 Transcripts

The Emb5 and Emb564 transcripts exhibited nearly identical patterns of accumulation during development (Fig. 6), shared the same tissue-specificity (Fig. 5), and exhibited similar regulation by ABA (Fig. 8, Fig. 10, Fig. 11, Fig. 12). The steady-state level of the Emb5 and Emb564 transcripts appeared to parallel the reported profile of ABA concentration during maize embryogenesis (Neill *et al.* 1987). These transcripts were also precociously stimulated in excised embryos by ABA. In addition, they were not

expressed in ABA-insensitive embryos and were expressed at low levels in embryos of the ABA-deficient mutant. These results suggest that ABA is directly involved in the regulation of Emb5 and Emb564 expression during embryogenesis. However, this simple interpretation is complicated by the finding that during late embryogenesis these transcripts were maintained at high levels in the presence or absence of exogenous ABA. Moreover, in the ABA-deficient *vp5* mutant, treatment with ABA enhanced the accumulation of Emb5 and Emb564 transcripts only in embryos isolated in the early stages of embryogenesis. Taken together, these results would imply that the effect of ABA on Emb5 and Emb564 is confined to the early to mid stages of embryogenesis. Similar results have been reported for other ABA responsive genes. For example, ABA increased the steady state mRNA levels of cruciferin and napin in *Brassica napus* during early embryogenesis (24 DAP) but not during mid embryogenesis (Delisle and Crouch 1989). In soybean, ABA maintained both 7S transcripts and 11S transcripts in mid-maturation embryos but not in late-maturation embryos (Eisenberg and Mascarenhas 1985). Similar to Emb5 and Emb564, transcripts of the the barley B19.1 gene accumulated precociously in mid stage embryos but transcript levels were not increased in mature embryos in response to exogenous ABA (Espelund *et al.* 1992). It is likely the effects of ABA during early and mid embryogenesis are at least partly due to stimulation of transcription. DeLisle and Crouch (1989) have shown that exogenous ABA increased the transcription rates of crucifern and napin in early stage (24 DAP) embryos of *Brassica napus* compared with rates observed in the freshly dissected embryos. These genes remained responsive to ABA during mid and late embryogenesis. Whether this indicates

a fundamental difference between this species and *Zea mays* is uncertain. Whereas in the present study maize embryos were incubated for 16 hours, Delisle and Crouch (1989) incubated their excised embryos for 5 days. The authors do not mention whether germination had begun in any of these embryos. It seems likely that the extended period of culture, particularly in the absence of ABA, might cause these embryos to switch to germinative development with the consequent major alteration of pattern of gene expression. Considerations such as these emphasize the caution which must be exercised when comparing data generated in different laboratories.

4.2.2 Regulation of Emb604 (Oleosin KD18)

Oleosins are small (15-26 kD), hydrophobic proteins unique to the phospholipid membrane of seed oil bodies (for a review, see Huang 1992). They are abundant seed proteins, comprising 2% to 8% of the protein in mature seed. Oleosins have been characterized from a wide range of plant species and appear to have arisen from a common ancestral gene. Oleosins all contain 3 structural domains: an N-terminal amphipathic domain, a central hydrophobic domain, and an amphipathic α-helical domain near the carboxy-terminus. It has been proposed that the central hydrophobic domain projects into the triacylglycerol core of the oleosome with the amphipathic α helix located at the surface of the oleosome, interacting with both the surface phospholipid and the cytoplasm (Qu and Huang, 1990). Oleosin has been proposed to serve a structural role, stabilizing the oleosome by its ability to sterically hinder degradation by phospholipases and, by providing the surface with a net negative charge, by preventing coalescence of oil bodies (Tzen and Huang, 1992). In addition, a lipase has been found to be tightly

bound specifically to the membrane of the maize oleosome (Lin and Huang, 1984). Accordingly, oleosins have been proposed as the determinant for lipase binding during germination (Vance and Huang, 1987). Oleosins disappear as the supply of total lipids declines.

Two isoforms of oleosin have been described in maize; having molecular weights of 16 kD and 18 kD. These genes, called Oleosin KD16 and Oleosin KD18, are coordinately expressed during seed maturation, are expressed only in the embryo and the aleurone layer and have been shown to be stimulated by ABA (Qu et al, 1990; Vance and Huang, 1988). The sequence (Qu and Huang 1990) and expression (Qu *et al.* 1990) of the 18 kD maize oleosin have been reported. I base my identification of Emb604 on the following similarities. The sequence of part of Emb604 was found to be identical to nucleotides 840 to 991 of the Oleosin KD18 gene except that Emb604 had an insertion of 8 nt at base 908 and an insertion of 5 nt at base 986 (Fig. 17A). This is in the 3' non-coding region of the transcript. Furthermore, the Emb604 transcript was estimated to be 1.2 kb. The Oleosin KD18 transcript has been reported as 1.1 kb, consistent with the Oleosin KD18 sequence which indicated a transcript of 1059 nt not including the poly(A)-tail (Qu and Huang 1990). Southern blot analyses of the genomic organization of Emb604 (Fig. 18) and Oleosin KD18 (Qu and Huang 1990) both revealed *Eco*RI fragments of 2.6 kb and 1.2 kb. I believe these similarities are sufficient to assign the identity of Emb604 as Oleosin KD18.

The maize Oleosin KD18 mRNA has been reported to be expressed at high level during embryogenesis but is present at a low level in mature embryos (Qu *et al.* 1990).

In addition, the Oleosin KD18 transcript was found to increase when mature kernels were imbibed with ABA. Interestingly, the effect of ABA on the KD18 transcript was observed only in embryos which failed to germinate and not in embryos which germinated successfully. These previous studies did not examine the effects of exogenous ABA on immature embryos. The present study provides a more detailed analysis of the developmental accumulation of the Oleosin KD18 gene and its regulation by ABA. My analysis indicates that this transcript accumulates in 0.5-mg embryos (Fig. 6) when the concentration of ABA is low. Whereas in the absence of ABA excised embryos showed a decrease of Emb604 transcript (Fig. 8), treatment with ABA at nanomolar concentrations maintained a high level of this transcript (Fig. 10). These results suggest that the accumulation of Emb604 transcript may require only a very low concentration of ABA. The observation that Emb604 is accumulated to high levels in the ABA-deficient *vp5* mutant (Fig. 12) is consistent with this idea. However, the Emb604 transcript is detected, albeit at low levels, in the ABA-insensitive *vp1* embryos (Fig. 12). The latter result implies that the synthesis of Emb604 transcript is not dependent on the availability of ABA but the maintenance of a high level of the transcript may require both ABA and VP1. One explanation for these different results is that during early and mid stages of embryogenesis ABA is not involved in the transcription of Emb604 but in the stabilization of mature Emb604 transcripts. Alternatively, this gene may be regulated by multiple ABA response systems.

4.2.3 Regulation of Emb807 (Rab-17)

An ABA-responsive maize gene, RAB-17, has been sequenced (Close *et al.* 1989,

Vilardell *et al.* 1990) and was found to encode a 17 kD protein sharing significant similarity with the dehydrins (Close *et al.* 1989). Dehydrins are small (14-23 kD) glycine-rich, hydrophilic proteins which accumulate in the dehydrated seedlings of barley, maize (Close *et al.* 1989) and rice (Mundy and Chua 1988, Kusano *et al.* 1992). They also accumulate during mid and late embryogenesis in maize (Pla *et al.* 1989, Vilardell *et al.* 1990), rice (Mundy and Chua 1988) and cotton (Baker *et al.* 1988, Hughes and Galau 1991). These studies also showed that dehydrins accumulate in response to exogenous ABA.

Sequence analysis of part of my Emb807 indicated 94% identity with nucleotides 780-990 in the 3' non-coding region of the RAB-17 gene (Vilardell *et al.* 1990). The differences consist of 8 substitutions, and 2 single-nucleotide insertions (Fig. 17B). Further evidence that the Emb807 contains the RAB-17 sequence is the similarity of transcript size, 1.1 kb, and similarity of the expression and regulation of RNA detected by these probes. Emb807 and Rab-17 have very similar genomic organization. The fragments recognized by Emb807 in the *Bam*HI, *Eco*RI and *Hin*dIII digests are similar in size to the restriction fragments detected with a Rab-17-specific probe (Vilardell *et al.* 1990).

The developmental accumulation of RAB-17 transcript was investigated (Pla *et al.* 1989). Transcripts were undetectable in embryos at 20 DAP, accumulated slightly in embryos at 40 DAP, and were abundant in embryos at 60 DAP. The RAB-17 transcript also accumulated precociously in immature embryos incubated with 1 µM ABA (Goday *et al.* 1988). Although undetectable after 48 h of germination in well-watered seedlings

(Goday *et al.* 1988), RAB-17 transcript also accumulated in the roots (Close *et al.* 1989) and leaves of dehydrated 7 d seedlings (Pla *et al.* 1989) and accumulated in 4 d seedlings incubated with 100 µM ABA (Vilardell *et al.* 1990). All these findings have been substantiated by my data. Moreover, the inclusion of more intermediate time points in my study permits a more accurate description of Emb807 accumulation. Transcripts are first detected at 23 DAP and appear to attain maximum steady-state levels in embryos at 56 DAP (Fig. 6). In addition, I showed that in wild-type (Fig. 8) and ABA-deficient embryos (Fig. 12) ABA can enhance the levels of Emb807 transcript throughout mid embryogenesis. The large increase in Emb807 transcript level during late embryogenesis, when ABA levels are low, suggests that *in vivo* a second regulator, such as osmotic potential, may be involved in the regulation of Emb807, similar to that suggested for the regulation of *Lea* genes during late embryogenesis (Finkelstein *et al.* 1987).

4.2.4 pZm13 (Maize Chloroplast 23S rRNA)

The evidence indicates that pZm13 represents sequence from the 3' end of the maize chloroplast 23S rRNA gene. Analysis of the pZm13 sequence revealed 97% identity with nucleotides 1953-2360 of this gene (Edwards and Kössel 1981). Within this sequence a stretch of 273 nucleotides exhibits 100% identity with the chloroplast 23S rRNA gene. The large accumulation of RNA complementary to pZm13 in vegetative leaf tissue is consistent with its identity as a plastid gene. In contrast to the other three genes, pZm13 RNA was found to accumulate throughout both embryonic and post-embryonic development. The steady-state level of Emb13, however, is modulated during development, declining during the maturation phase. The reduced accumulation of Emb13

transcript in *vp5* embryos compared with wild-type (Fig. 12) may be a result of disrupted chloroplast function in this mutant. Robertson *et al.* (1978) have shown that the deficiency in carotenoids in maize viviparous mutants results in disrupted plastid development. The mature, 2.9 kb RNA of 23S rRNA was barely detectable on my Northern blots. The 1.1 kb and 1.3 kb species which hybridized with pZm13 appear to be normal products of endoribonuclease processing of the primary 23S rRNA transcript (Wollgiehn and Parthier 1980). Abscisic acid does not appear to be involved in the regulation of pZm13.

4.2.5 Concentration Effects

There is a clear relationship between the accumulation of Emb5, Emb564 and Emb807 transcripts and the concentration of ABA. Very little of these transcripts accumulated in 2-mg embryos incubated with less than 1 µM ABA (Fig. 10). Emb604 transcript, on the other hand, accumulated in embryos incubated with as little as 1 nM ABA. As seen in Fig. 8, however, the levels of the Emb604 transcript became almost undetectable when 3-mg embryos were incubated without ABA. There are two possible explanations for these results. Perhaps a very low concentration of ABA is required for the accumulation of Emb604 transcripts. The relatively high steady-state level of this transcript in 0.5 mg embryos (Fig. 6) at a time when ABA levels are low is consistent with this possibility. Accumulation of the Emb604 transcript in 16-mg ABA-deficient *vp5* embryos which contain a reduced level of ABA (Neill *et al.* 1986) is also consistent with this hypothesis (Fig. 12). These results suggest that ABA may operate through multiple response mechanisms, some having a lower concentration threshhold than others. However, the low level of accumulation of Emb604 transcript in 9-mg ABA-insensitive

vp1 embryos suggests that an ABA-independent mechanism may also be involved.

Sensitivity to ABA may depend on the species and/or tissue examined. In rice protoplasts (Marcotte *et al.* 1988) and soybean embryos (Eisenberg and Mascarenhas 1985) the optimal concentration is 10 µM ABA whereas in barley aleurone 100 µM ABA has been found to be optimal (Hong *et al.* 1988). The accumulation of Emb807 transcript in seedlings treated with 100 µM ABA (Fig. 11), a concentration which, in 2-mg embryos, results in the degradation of all five transcripts (data not shown) suggests vegetative and embryonic tissues may have different sensitivities to ABA. Alternatively, these results may be attributed to different rates of uptake and/or metabolism of exogenous ABA.

4.2.6 Multiple Mechanisms are Involved in Abscisic Acid Regulation of Embryonic Transcript Accumulation

Together with the published data (see Tables 2-5), the results presented here suggest that the patterns of expression of maize embryonic, ABA-inducible genes fall into three groups. Discussed below is the basis for this conclusion.

The first class (Class I) of ABA-inducible, embryonic genes includes Glb1, oleosin KD16 and oleosin KD18. The profiles of expression during embryogenesis for these three genes are very similar. At the earliest times examined, 14-18 DAP, these transcripts have already accumulated to moderate levels (Table 3, Fig. 6). Previously it was shown that the levels of Glb1 transcript in the ABA-deficient mutants are only slightly below that of the wild type while in the ABA-insensitive mutant *vp1* there is a detectable but low level of expression (Kriz *et al* 1990). Virtually identical results were obtained for the accumulation of oleosin KD18 transcript in *vp1* and the ABA-deficient mutant *vp5* (Fig.

12). Taken together these results suggest that the elements controlling the expression of Glb1 and oleosin KD18 are probably the same. Oleosin KD16 is placed in this class primarily because of the similarity in its profile of expression to the other two genes (Vance and Huang 1988, Qu *et al.* 1990).

The early appearance during embryogenesis (Fig. 6) and the high level of accumulation in the *vp5* mutant (Fig. 12) of the oleosin KD18 transcript can be explained in part by the sensitivity of its encoding gene to ABA. While incubation of wild-type embryos in the absence of ABA resulted in a rapid loss of the oleosin KD18 transcript (Fig. 8), a moderate level of accumulation for this transcript could be maintained by incubation with ABA at a concentration as low as 1 nM (Fig. 10). However, hypersensitivity to ABA alone does not explain why the oleosin KD18 (Fig. 12) and Glb1 (Kriz *et al* 1990) transcripts also accumulate in the ABA-insensitive mutant *vp1*. One possible explanation is that the basal level of transcription for the Class I genes is not influenced by ABA but the rate of transcription and/or the stability of the mature transcripts are enhanced by ABA. Alternatively, these genes may also be regulated by a *Vp1*-independent, ABA-responsive mechanism.

The Emb564 and the Emb5 belong to the second group or Class II ABA-inducible genes. The patterns of expression for these two transcripts during embryogenesis, in viviparous mutants, and in response to ABA are almost identical (Williams and Tsang 1991, 1992) (Figs.6, 8 and 12). The Class II genes differ from the Class I genes in several respects. During embryogenesis the Class II transcripts make their appearance, 21-23 DAP, later than the Class I transcripts (Fig.6, Table 3). To stimulate the accumulation of

the Class II transcripts μM concentrations of ABA are required whereas nM concentrations of ABA can maintain a moderate level of Class I transcripts (Fig. 10). Another difference between these two classes of genes is that the Class II transcripts cannot be detected in *vp1* embryos (Fig. 12).

Besides having a similar profile of expression during embryogenesis the accumulation of pMAH9, MLG3,and Rab17 can be stimulated by ABA or desiccation in seedlings (Gómez *et al.* 1988, Pla *et al.* 1989, Triplett and Quatrano 1982, Cammue *et al.* 1989, Mansfield and Raikhel 1990, Vilardell *et al.* 1990, Fig. 11). The ability of the pMAH9, MLG3 and Rab17 genes to be stimulated by ABA and desiccation in seedlings distinguishes them from the Class II genes. Hence we refer to these genes as Class III. While the Class II and Class III transcripts appear about the same time during embryogenesis and have similar sensitivity to ABA (Fig. 10), they reach the peak levels at different times. The peak level of the Rab17 transcript occurs at about 50-60 DAP whereas the Class II transcripts peak between 20-30 DAP (Goday *et al.* 1988, Pla *et al.* 1989, Fig. 6). The high level of expression of Rab17 during late embryogenesis may be due to the influence of desiccation during this stage.

It is uncertain whether species other than maize express ABA-responsive, embryogenic genes corresponding to the maize Class I and Class II genes. Due to a lack of information regarding the time of appearance, ABA concentration-dependence, and inducibility in vegetative tissue, the majority of ABA-responsive, embryonic genes remain unclassifiable.

In contrast, genes from several other species exhibit the characteristics of the

maize Class III ABA-inducible genes. These include the wheat Em gene (Berge *et al.* 1989, Litts *et al.* 1992), wheat germ agglutinin (Triplett and Quatrano 1982, Cammue *et al.* 1989, Mansfield and Raikhel 1990), the rice Rab16A, Rab16B and Rab16C genes (Mundy and Chua 1988, Yamaguchi-Shinozaki *et al.* 1989), *Brassica napus* pLea76 (Harada *et al.* 1989), pea ABR17 (Barrat and Clark 1991), sunflower HA ds10 and HA ds11 (Almoguera and Jordano, 1992) and tomato pLEA25 (Cohen *et al.* 1991). These genes are all expressed during embryogenesis, are precociously accumulated in the presence of ABA, and are induced in vegetative tissues by environmental stresses such as water stress, high salt, and high osmoticum.

Interestingly, the wheat Em gene, the only homolog of the maize Class I Emb5 gene which I can classify, accumulates in stressed vegetative tissue, placing it in the Class III category. It is uncertain whether this is indicative of differences in the developmental programs or strategies of dealing with stress between wheat and maize. Em is a small gene family of approximately 10 members (Litts *et al.* 1987). Therefore, it is possible that some members of this family may exhibit embryo-specific expression while others exhibit vegetative-specific expression.

The existence of 3 classes of ABA-responsive embryo genes in maize suggests that several mechanisms are involved in mediating the effects of ABA on gene activity during embryogenesis. The pattern of regulation seen in Class II genes could most simply be explained by a single common ABA-dependent mechanism affecting transcription. Data presented in this thesis suggest the elements of this system are in place prior to the increase in endogenous ABA required to stimulate transcript accumulation. The expression

pattern of Class I genes is less simple and may depend on several embryo-specific mechanisms. An unique feature of Class I genes may be their transcription at a low basal level in the absence of a *Vp1*-dependent ABA signal transduction pathway (Fig. 12, Kriz *et al.* 1990). Thus, early expression of Class I genes may be regulated by a mechanism responsive to nM concentrations of ABA and independent of the *Vp1* gene product. The hypothesis that Class I genes are regulated by both *Vp1*-dependent and *Vp1*-independent pathways is consistent with the bimodal pattern of stimulation observed with increasing ABA concentration (Fig. 10). The stimulation of Class III genes in seedling tissue in response to exogenous ABA and/or various environmental stresses suggests that these genes are regulated by mechanisms which are present in vegetative as well as embryonic tissue. Further research will be required to define the characteristics of these various mechanisms.

4.3 Accumulation of Polypeptide.

Gene expression at the protein level is not necessarily a reflection of transcript accumulation. For this reason it is important to characterize the temporal and spatial accumulation of the final polypeptide product which governs the resulting biological activity or function.

There are several reasons to believe the proteins recognized by the serum generated against the ß-galactosidase-EMB564 fusion protein are the translation products of the Emb564 and/or Emb5 genes. First, the detected proteins exhibit mobilities of proteins slightly smaller than the 14.4 kD marker. This is consistent with the sizes of EMB5 (11.9 kD) and Emb564 (9.7 kD) predicted from their deduced amino acid

sequences. A second reason to believe these proteins are encoded by Emb5 and Emb564 is their developmental profile. These proteins are detectable in developing embryos at the same stage as the Emb5 and Emb564 transcripts appear, continue to accumulate during late embryogenesis and, like the transcripts, disappear during germination (Fig. 19). Third, both the proteins (Fig. 21) and the transcripts (Fig. 8) were maintained in embryos treated with ABA but not in embryos treated without ABA. Fourth, Western blot analysis of proteins extracted from the seeds of several other plant species revealed proteins similar in size to those predicted from the sequences of known Emb564 homologs (Fig. 20). In particular, the antiserum detected proteins of similar size in maize and wheat. The wheat Em protein, a homolog of EMB5 and EMB564, was isolated and estimated by sedimentation equilibrium to have a molecular weight of 11.2 kD (McCubbin *et al.* 1985), similar to the predicted size of EMB5. Thus, it seems reasonable to assign the maize proteins recognized by the antiserum as translation products of Emb5 and/or Emb564. Considering the high degree of amino acid identity predicted from these sequences it is expected that some of the polyclonal antibodies to EMB564 would react with EMB5. Although the possibility cannot be ruled out, it seems unlikely that neither EMB564 nor EMB5 accumulate to detectable levels and that the antibodies reported here recognize other proteins sharing epitopes with EMB564 and exhibit the approximate sizes, developmental expression, and ABA induction predicted for EMB564 and EMB5.

The rapid (16 h) disappearance of both the transcript (Fig.8) and its putative translation product in the absence of ABA (Fig.21) suggests that during early-to-mid embryogenesis the level of gene product is determined primarily by the steady-state level

of its transcript. A more direct effect of ABA on the stability of this protein, however, cannot be ruled out.

4.4 Role of EMB5 and EMB564.

The accumulation of EMB5 and/or EMB564 during mid embryogenesis, their abundance during late embryogenesis and their disappearance during germination suggest these proteins serve a function unique either to the maturing seed or the very young seedling. Thus, these proteins could serve functions such as osmoprotection during the desiccation and cryoprotection during the storage phase of seed development. Their abundance in the mature embryo and rapid hydrolysis during germination are also consistent with a role as storage proteins.

The cross-reaction of anti-EMB564 serum with small proteins in all plant species examined also suggests this protein and its homologs serve an essential function. It may therefore be possible to use this serum to quantify the levels of EMB564 or its homologs in a wide range of varieties or strains exhibiting phenotypic variations. The correlation of EMB564 level with such traits as seed dormancy, tolerance to desiccation, cold or other stresses may provide clues to its function. Determination of the function(s) of these proteins may be approached by genetic and/or molecular techniques such as the creation or isolation of Emb5- and Emb564-defective mutants, antisense disruptions, or transgenic overproduction constructs. The restoration of resistance in stress-sensitive mutants in simpler systems such as *Arabidopsis* or yeast by introducing the Emb5 or Emb564 genes would demonstrate their involvement in stress resistance.

Appendix 1A-C. Comparison of the developmental stages of commonly-studied angiosperm species. In order to compare events in the development of different species, the various staging schemes encountered in the literature have been compared with the revised classification used throughout this thesis. This simplified scheme divides embryogenesis into three stages: Early, Mid and Late. Where possible, representative values of the age and mass of the embryos are included.

1 Abbe and Stein 1954
2 Kowles and Phillips 1988, Marks *et al.* 1985
3 Rogers and Quatrano 1983
4 Goldberg *et al.* 1989, Eisenberg and Mascarenhas 1985
5 Finkelstein *et al.* 1987, Comai and Harada 1990
6 Hocher *et al.* 1991
7 Xu *et al*, 1990
8 Dure 1985, Hughes and Galau 1991
9 Neill *et al.* 1987

Appendix 1A. Early embryogenesis. Comparison of the developmental stages of commonly-studied angiosperm species.

SPECIES	DEVELOP-MENTAL STAGE NAME	EMBR. AGE (DAP)	EMBRYO MASS (mg FW)	MAJOR EVENTS	REF
maize	Proembryo	0-10		Embryo grows by cell division	1
	Transition	10-13		Protruberance on side of embryo at site of future axis	1
	Coleopt-ilar	13-14	? -0.5	First appearance of coleoptilar tissue as ridge around axis	1
		10-15		Zeins begin to accumulate	2
wheat	Stage I	0-7		Undifferentiated	3
	Stage II	7-14		Rapid cell divisions	
	Stage III	14-21		Completion of tissue differentiation	
soybean	Globular	0-10	0-25	Cell division	4
	Heart	10-20	25-50	Differentiation of axis and cotyledons	
	Cotyledon	20-30	50-150		
B. napus	Stage I	0-25	0-1.8	Embryo grows by cell division Differentiation of axis and cotyledons	5
tomato	Stage 1	0-10	0-2.0	Globular stage	6
	Stage 2	10-12	2.0-2.8	Heart-shaped stage	
	Stage 3	12-15	2.8-3.0	Young torpedo stage	
alfalfa	Stage I	0-10		Cell division	7
	Stage II	10-12		Globular	
	Stage III	12-14		Heart stage	
cotton	Pre--cotyledon	-	0-20	Up to heart stage	8
	Cotyledon	-27	20-40	Rapid cell proliferation	

FW, fresh weight; DAP, days after pollination

Appendix 1A-C. Comparison of the developmental stages of commonly-studied angiosperm species. In order to compare events in the development of different species, the various staging schemes encountered in the literature have been compared with the revised classification used throughout this thesis. This simplified scheme divides embryogenesis into three stages: Early, Mid and Late. Where possible, representative values of the age and mass of the embryos are included.

1 Abbe and Stein 1954
2 Kowles and Phillips 1988, Marks *et al.* 1985
3 Rogers and Quatrano 1983
4 Goldberg *et al.* 1989, Eisenberg and Mascarenhas 1985
5 Finkelstein *et al.* 1987, Comai and Harada 1990
6 Hocher *et al.* 1991
7 Xu *et al*, 1990
8 Dure 1985, Hughes and Galau 1991
9 Neill *et al.* 1987

Appendix 1B. Mid embryogenesis. Comparison of the developmental stages of commonly-studied angiosperm species.

SPECIES	DEVELOP-MENTAL STAGE NAME	EMBR. AGE (DAP)	EMBRYO MASS (mg FW)	MAJOR EVENTS	REF
maize	Stage I	14-18	0.5-4	First leaf primordium formed	1
	Stage II	18-22	4-12	2nd leaf primordium appears	
	Stage III	22-28	12-24	3rd leaf primordium appears	
wheat	Stage IV	21-31		storage protein accumulation	3
soybean	Early Maturation	30-90	150-300	Cell expansion Storage proteins and lipids accumulate	4
B. napus	Stage II	25-45	1.8-5.8	Cell division ceases Storage reserves accumulate	5
tomato	Stage 4 Stage 5	15-20 20-30	3.0-5.0 5.0-6.5	Growth phase	6
alfalfa	Stage IV Stage V Stage VI Stage VII	15-17 18-20 21-25 26-30		Elongation stage Elongation stage Filling and maturation stages	7
cotton	Maturation Post-Abscission	27-45 45-50	40-80 80-100	Finiculus atrophies Post-abscission	8

FW, fresh weight; DAP, days after pollination

Appendix 1A-C. Comparison of the developmental stages of commonly-studied angiosperm species. In order to compare events in the development of different species, the various staging schemes encountered in the literature have been compared with the revised classification used throughout this thesis. This simplified scheme divides embryogenesis into three stages: Early, Mid and Late. Where possible, representative values of the age and mass of the embryos are included.

1 Abbe and Stein 1954
2 Kowles and Phillips 1988, Marks *et al.* 1985
3 Rogers and Quatrano 1983
4 Goldberg *et al.* 1989, Eisenberg and Mascarenhas 1985
5 Finkelstein *et al.* 1987, Comai and Harada 1990
6 Hocher *et al.* 1991
7 Xu *et al*, 1990
8 Dure 1985, Hughes and Galau 1991
9 Neill *et al.* 1987

Appendix 1C. Late embryogenesis. Comparison of the developmental stages of commonly-studied angiosperm species.

SPECIES	DEVELOP-MENTAL STAGE NAME	EMBR. AGE (DAP)	EMBRYO MASS (mg FW)	MAJOR EVENTS	REF
maize	Stage IV	28-37	24-34	4th leaf primordium appears	1
		30-35		embryo and endosperm begin to dehydrate	9
	Stage V	37-50	34-43	5th leaf primordium appears	1
	Stage VI	>50	43-50	6th leaf primordium appears	
wheat	Stage V	31-50		desiccation	3
soybean	Late Maturation	90-12	300-400 (100 mature)	Water loss; RNA and protein synthesis decrease	4
B. napus	Stage III	45-60	5.8-3.6	Water content declines to 4% Metabolic activity declines Chlorophyll lost Embryo becomes quiescent	5
tomato	Stage 6	30-38	6.5-8.0	Dehydration of seed begins	6
	Stage 7	38-50	8.0-10	Embryo mature, fruit begins to ripen	
	Stage 8	>50	10-6.5	Fruit red	
alfalfa	Stage VIII	31-36		Drying stage, chlorophyll lost	7
	Stage IX	>36		Mature seed	
cotton	Pre-Desic-cation	50-52	100-120	Pre-desiccation	8
	Desicc.	>52	120-80	Desiccation	

FW, fresh weight; DAP, days after pollination

Appendix 2A-C. The concentration of embryonic ABA during development for several species of flowering plants. Estimations of ABA content of the developing embryo was taken from the cited literature. To facilitate comparison between species I have estimated the molar concentration of ABA in the embryos. For these estimations I have used either the authors' estimation of water content (r) or have assigned values based on the developmental stage of the embryo (a). Calculations of ABA concentration were performed using the formulae:

$$[ABA] = (\text{amt ABA per unit FW}) * 100/p$$

$$[ABA] = (\text{amt ABA per unit DW}) * (100-p)/p$$

where p = water content of the fresh material (in %). Molar concentration was calculated using the formula weight of ABA = 264. For the sake of these calculations I have assumed that ABA is equally distributed in the cell's aqueous component and that all cells contain similar concentrations of ABA. The papers cited were:

1 Carnes and Wright 1988
2 Neill *et al.* 1987
3 Morris *et al.* 1988
4 Walker-Simmons 1987
5 Finkelstein *et al.* 1985, 1987
6 Chang and Walling 1991
7 Ackerson 1984a, 1984b
8 Goffner *et al.* 1990
9 Xu *et al.* 1990
10 Hocher *et al.* 1991
11 Cohen *et al.* 1991
12 Piaggesi *et al.* 1991

Appendix 2A. The concentration of embryonic ABA during development for several species of flowering plants.

SPECIES	TISSUE or ORGAN	TIME	STAGE	ABA content of embryo	[ABA] (μM)*	REF	Water (%)	
							a	m
maize (cv XL12)	Kernel	3 DAP	Early	24 ng/g FW	0.10	1		90
		12 DAP	Early	19 ng/g FW	0.08			90
		15 DAP	Early	14 ng/g FW	0.06			90
maize (cv W22?)	Embryo	3 DAP	Early				90	
		15 DAP	Early				90	
		20 DAP	Mid	150 ng/g FW	0.69	2	80	
		25 DAP	Mid	140 ng/g FW	0.74		70	
		35 DAP	Late	50 ng/g FW	0.31		60	
		50 DAP	Late	25 ng/g FW	0.19		50	
wheat (cv Timmo)	Embryo	14 DPA	Early			3	92	
		21 DPA	E/M	0.67 pmol/mg FW	0.74		90	
		28 DPA	Mid	1.45 pmol/mg FW	2.0		72	
		35 DPA	Late				68	
		42 DPA	Late				66	
		49 DPA	Late				64	
		56 DPA	Late				60	
wheat (cv Brevor)	Embryo	22 DPA	Mid	0.58 ng/mg DW	0.5	4		80
		32 DPA	M/L	0.65 ng/mg DW	1.0			70
		40 DPA	Late	0.95 ng/mg DW	1.7			67
		48 DPA	Late	0.20 ng/mg DW	0.4			65
		62 DPA	Late	0.18 ng/mg DW	0.4			60
barley (cv Golden Promise)	Embryo	14 DPA	Early			3	95	
		21 DPA	Mid	0.29 pmol/mg FW	0.41		68	
		28 DPA	Mid	0.39 pmol/mg FW	0.56		70	
		35 DPA	Late				65	
		42 DPA	Late				60	
		49 DPA	Late				60	
		56 DPA	Late				50	

r, values taken from paper cited; a, values assumed when calculating ABA concentrations; *, estimated; FW, fresh weight; DW, dry weight.

Appendix 2A-C. The concentration of embryonic ABA during development for several species of flowering plants. Estimations of ABA content of the developing embryo was taken from the cited literature. To facilitate comparison between species I have estimated the molar concentration of ABA in the embryos. For these estimations I have used either the authors' estimation of water content (r) or have assigned values based on the developmental stage of the embryo (a). Calculations of ABA concentration were performed using the formulae:

$$[ABA] = (\text{amt ABA per unit FW}) * 100/p$$

$$[ABA] = (\text{amt ABA per unit DW}) * (100-p)/p$$

where p = water content of the fresh material (in %). Molar concentration was calculated using the formula weight of ABA = 264. For the sake of these calculations I have assumed that ABA is equally distributed in the cell's aqueous component and that all cells contain similar concentrations of ABA. The papers cited were:

1 Carnes and Wright 1988
2 Neill *et al.* 1987
3 Morris *et al.* 1988
4 Walker-Simmons 1987
5 Finkelstein *et al.* 1985, 1987
6 Chang and Walling 1991
7 Ackerson 1984a, 1984b
8 Goffner *et al.* 1990
9 Xu *et al.* 1990
10 Hocher *et al.* 1991
11 Cohen *et al.* 1991
12 Piaggesi *et al.* 1991

Appendix 2B. The concentration of embryonic ABA during development for several species of flowering plants.

SPECIES	TISSUE or ORGAN	TIME	STAGE	ABA content of embryo	[ABA] (μM)*	REF	Water (%)	
							r	a
B. napus (cv Tower)	Embryo	25 DPA	E/M	2 ng/mg FW	8	5	95	
		35 DPA	Mid	3.3 ng/mg FW	20		62	
		40 DPA	Late	1.5 ng/mg FW	10		56	
		45 DPA	Late	1 ng/mg FW	8		46	
		60 DPA	Late	0.2	3		30	
soybean (cv Dare)	Cotyledon	25 DAF	Early	10 ng/mg FW	44	6		85
		35 DAF	Mid	18 ng/mg FW	89			75
		55 DAF	Mid	20 ng/mg FW	120			60
		70 DAF	Mid	16 ng/mg FW	150			40
		85 DAF	M/L	8 ng/mg FW	99			30
soybean (cv Wye)	Embryo	5 DAF	Early	1 μg/g FW	4	7		95
		15 DAF	Early	7 μg/g FW	29			90
		22 DAF	Early	10 μg/g FW	44			85
		30 DAF	Mid	6 μg/g FW	28			80
		40 DAF	Mid	3 μg/g FW	16			70
		50 DAF	Mid	2 μg/g FW	12			60
		65 DAF	Mid	1 μg/g FW	8			45
sunflower (cv Mirasol)	Seed	8 DAF	Early	0.6 ng/mg DW	0.2	8	90	
		11 DAF	E/M	1.0 ng/mg DW	0.9		80	
		14 DAF	Mid	2.8 ng/mg DW	4.4		70	
		16 DAF	Mid	2.0 ng/mg DW	4.9		60	
		17 DAF	Mid	0.8 ng/mg DW	3.0		50	
		21 DAF	Mid	0.5 ng/mg DW	1.9		50	
		23 DAF	M/L	0.3 ng/mg DW	1.4		45	
		25 DAF	Late	0.5 ng/mg DW	2.8		40	
		28 DAF	Late	0.4 ng/mg DW	2.8		35	
		35 DAF	Late	0.3 ng/mg DW	10		10	
		42 DAF	Late	0.3 ng/mg DW	10		10	

r, values taken from paper cited; a, values assumed when calculating ABA concentrations; *, estimated; FW, fresh weight; DW, dry weight.

Appendix 2A-C. The concentration of embryonic ABA during development for several species of flowering plants. Estimations of ABA content of the developing embryo was taken from the cited literature. To facilitate comparison between species I have estimated the molar concentration of ABA in the embryos. For these estimations I have used either the authors' estimation of water content (r) or have assigned values based on the developmental stage of the embryo (a). Calculations of ABA concentration were performed using the formulae:

$$[ABA] = (\text{amt ABA per unit FW}) * 100/p$$

$$[ABA] = (\text{amt ABA per unit DW}) * (100-p)/p$$

where p = water content of the fresh material (in %). Molar concentration was calculated using the formula weight of ABA = 264. For the sake of these calculations I have assumed that ABA is equally distributed in the cell's aqueous component and that all cells contain similar concentrations of ABA. The papers cited were:

1 Carnes and Wright 1988
2 Neill *et al.* 1987
3 Morris *et al.* 1988
4 Walker-Simmons 1987
5 Finkelstein *et al.* 1985, 1987
6 Chang and Walling 1991
7 Ackerson 1984a, 1984b
8 Goffner *et al.* 1990
9 Xu *et al.* 1990
10 Hocher *et al.* 1991
11 Cohen *et al.* 1991
12 Piaggesi *et al.* 1991

Appendix 2C. The concentration of embryonic ABA during development for several species of flowering plants.

SPECIES	TISSUE or ORGAN	TIME	STAGE	ABA content of embryo	[ABA] (μM)*	REF	Water (%)	
							r	a
alfalfa (cv Excalibur	Embryo	III	Early	40 pmol/mg DW	4	9		90
		IV	Mid	130 pmol/mg DW	33			80
		V	Mid	100 pmol/mg DW	43			70
		VI	Mid	90 pmol/mg DW	60			60
		VII	Mid	25 pmol/mg DW	25			50
		VIII	Late	5 pmol/mg DW	8			40
		IX	Late	2 pmol/mg DW	5			30
tomato (cv Vilmorin B686)	Seed	stage 1	Early	10 nmol/g DW	1.1	10	90	
		stage 2	Early	10 nmol/g DW	1.6		86	
		stage 3	Early	12 nmol/g DW	2.1		85	
		stage 4	Mid	33 nmol/g DW	3.7		90	
		stage 5	Mid	55 nmol/g DW	8.2		87	
		stage 6	Late	53 nmol/g DW	13		80	
		stage 7	Late	40 nmol/g DW	19		68	
		stage 8	Late	2 nmol/g DW	1		61	
tomato (cv Vilmorin B686)	Embryo	stage 6	Late	100 nmol/g DW	25	10	80	
		stage 7	Late	35 nmol/g DW	16		68	
		stage 8	Late	4 nmol/g DW	3		61	
tomato (cv Ailsa Craig)	Seed	MG1		2.2 μg/g FW	9.0	11	90	
		MG2		5.5 μg/g FW	24		85	
		MG3		3.6 μg/g FW	17		80	
		MG4		2.4 μg/g FW	13		70	
		B		4.2 μg/g FW	26		60	
		T		2.2 μg/g FW	16		50	
		P		1.0 μg/g FW	9.2		40	
		R		0.5 μg/g FW	4.6		40	
peach (cv Spring-crest)	embryo	84 DAP		20 ng/g FW	0.08	12		90
		87 DAP		15 ng/g FW	0.07			80
		96 DAP		30 ng/g FW	0.16			70
		105 DAP		50 ng/g FW	0.32			60
		112 DAP		150 ng/g FW	1.14			50

r, values taken from paper cited; a, values assumed when calculating ABA concentrations; *, estimated; FW, fresh weight; DW, dry weight.

Appendix 3. The inhibitory effect of ABA on precocious germination of isolated embryos.

E, Early
M, Mid
L, Late
E/M, transition between Early and Mid embryogenesis
E-M, from Early into Late embryogenesis

1 Neill *et al.* 1987
2 Rivin and Grudt 1991
3 Triplett and Quatrano 1982
4 Mansfield and Raikhel 1990
5 Walker-Simmons 1987
6 Xu *et al.* 1990
7 Bartels *et al.* 1988
8 Black *et al.* 1974
9 Bewley and Fountain 1972
10 Finkelstein *et al.* 1985, 1987
11 Eisenberg and Mascarenhas 1985
12 Galli *et al.* 1979

Appendix 3. The inhibitory effect of ABA on precocious germination of isolated embryos.

SPECIES	EMBRYO AGE	STA-GE	[ABA] (μM)	GERMINAT-ION (%)	PERIOD (days)	REF
maize	15-25 DAP	M	10	0	6	2
	26 DAP	M	10	0	4	1
	27-35 DAP	L	0	0	10	2
	40 DAP	L	10	10	6	2
	42 DAP	L	10	40	4	1
	42 DAP	L	50	0	4	1
	45 DAP	L	10	20	6	2
	50 DAP	L	10	40	6	2
	55 DAP	L	10	100	6	2
wheat	15 DAP	M	100	0	4	4
	15-30 DAP	E-M	1	0	5	3
	25-35 DAP	M-L	5	0	12	5
	25-35 DAP	M-L	50	0	12	5
	48 DAP	L	5	25	12	5
	48 DAP	L	50	10	12	5
	60 DAP	L	5	75	12	5
	60 DAP	L	50	15	12	5
alfalfa	Stage III	E	0	85	5	6
	Stage IV	M	0	80	5	6
	Stage V	M	0	60	5	6
	Stage VI	M	0	20	5	6
	Stage VI	M	0	100	10	6
	Stage VII	M	0	40	5	6
	Stage VIII	L	0	100	5	6
	Stage IX	L	0	100	5	6
	St.III-VII	E-M	10	0	5	6
barley	12 DAP	E	>1	0	4	7
lettuce	mature	L	3	0	2	8
	mature	L	30	0	2	9
Brassica napus	0-40 DPA	all	1	0	3	10
	all stages	all	10	0	3	10
soybean	50-100 mg	E	1	0	10	11
	150-200 mg	M	1	15	10	11
	275-325 mg	M/L	1	40	10	11
	450-500 mg	L	1	40	10	11
	all stages	all	10	0	10	11
Haplopappus gracilis	seeds	L	10	0	5	12

Appendix 4. Construction of pWR590::EMB564 Fusion Plasmid.

1) A single *Dra* II endonuclease site was identified at the junction between the Emb56 cDNA and the oligo-dG tail: (This site is 35 nucleotides from the start codon, at nucleotide 83 of Emb564.)

2) Because of the presence of *Dra* II sites in the Blue Script vector an *Eco*R I-*Bam* HI fragment containing the entire Emb56 cDNA was isolated from agarose prior to digestion with Dra II.

3) The sticky ends were filled in with Klenow and *Eco*R I linkers added.

4) Following digestion with *Eco*R I and *Pst* I the fragment was isolated by electrophoretic size fractionation through polyacrylamide gel.

5) The isolated *Eco*R I-linkered fragment was then ligated with kinased, *Eco*R I, *Pst* I-cut Bluescript Plus.

6) The nature of the *Eco*R I junction was verified by sequencing. Then the *Eco*R I- *Pst* I insert was removed from Bluescript and ligated with kinased, *Eco*R I, *Pst* I-digested pWR590-1.

7) This construct results in the removal of an undetermined number of amino acids (at least 15) from the end of the truncated ß-galactosidase protein and the addition of the entire coding sequence of Emb564 preceded by 13 amino acids encoded by the (previously) 5' untranslated sequence.

1)

Emb56 GGGGGGGGGGGGGGGGGGGGGGACCTAGCTAGCTACCTAGCTAGTTCAG
CCCCCCCCCCCCCCCCCCCCCCCCTGGATCGATCGATGGATCGATCAAGTC

↓ *Eco*R I/ *Bam* HI
↓ isolate fragment
↓ *Dra* II

2)

GGGGGGGGGGGGGGGGGGGGGG GACCTAGCTAGCTACCTAGCTAGTTCAG
CCCCCCCCCCCCCCCCCCCCCCCTG GATCGATCGATGGATCGATCAAGTC

3) ↓ Klenow + dNTPs

GACCTAGCTAGCTACCTAGCTAGTTCAG
ctgGATCGATCGATGGATCGATCAAGTC

+ *Eco*R I linkers ↓ T4 DNA ligase

cccgaattcgggcccgaattcgggGACCTAGCTAGCTACCTAGCTAGTTCAG
gggcttaagcccgggcttaagccccctgGATCGATCGATGGATCGATCAAGTC

4) ↓ *Eco*R I/ *Pst* I
↓ isolate fragment

aattcgggcccg aattcgggGACCTAGCTAGCTACCTAGCTAGTTCAG
gcccgggcttaa gcccctgGATCGATCGATGGATCGATCAAGTC

5) + kinased, *Eco*R I, *Pst* I-cut BS+

BS 710
CCCCGGGCTGCAGG aattcgggGACCTAGCTAGCTACCTAGCTAGTTCAG
GGGGCCCGACGTCCTTAA gcccctgGATCGATCGATGGATCGATCAAGTC

↓ T4 DNA ligase

BS
CCCCGGGCTGCAGGaattcgggGACCTAGCTAGCTACCTAGCTAGTTCAG
GGGGCCCGACGTCCTTAAgcccctgGATCGATCGATGGATCGATCAAGTC

6) ↓ *Eco*R I/ *Pst* I

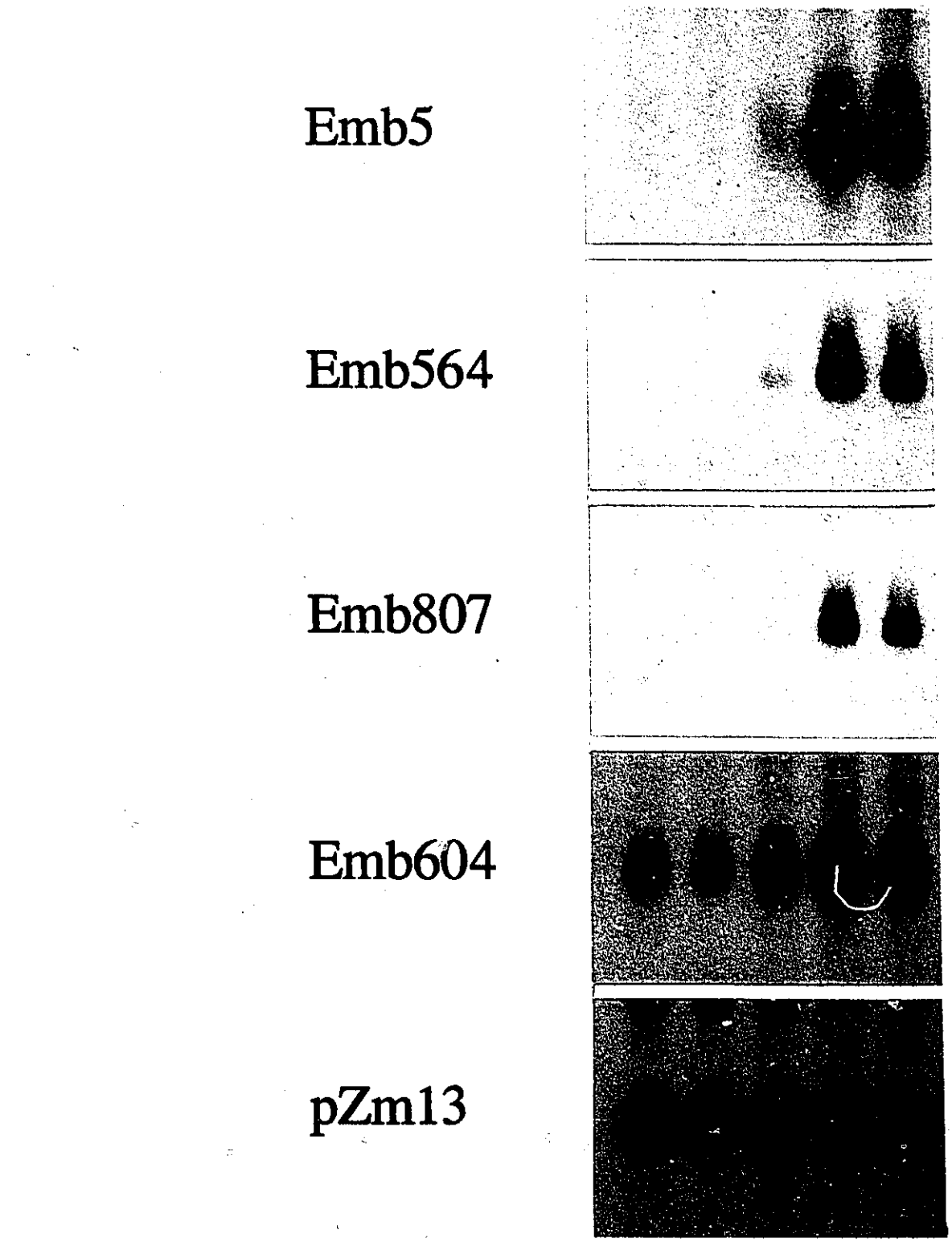
*Eco*R I, *Pst* I-pWR590-1
Gly-Asn-CCG-GGC-GAG-CTC-G AATTCGGGGACCTAGCTAGCTACCTAGCTAGTTCAG
GGC-CCG-CTC-GAG-CTT-AA GCCCCTGGATCGATCGATGGATCGATCAAGTC

7) + *Eco*R I, *Pst* I-pWR590-1 ↓ T4 DNA ligase

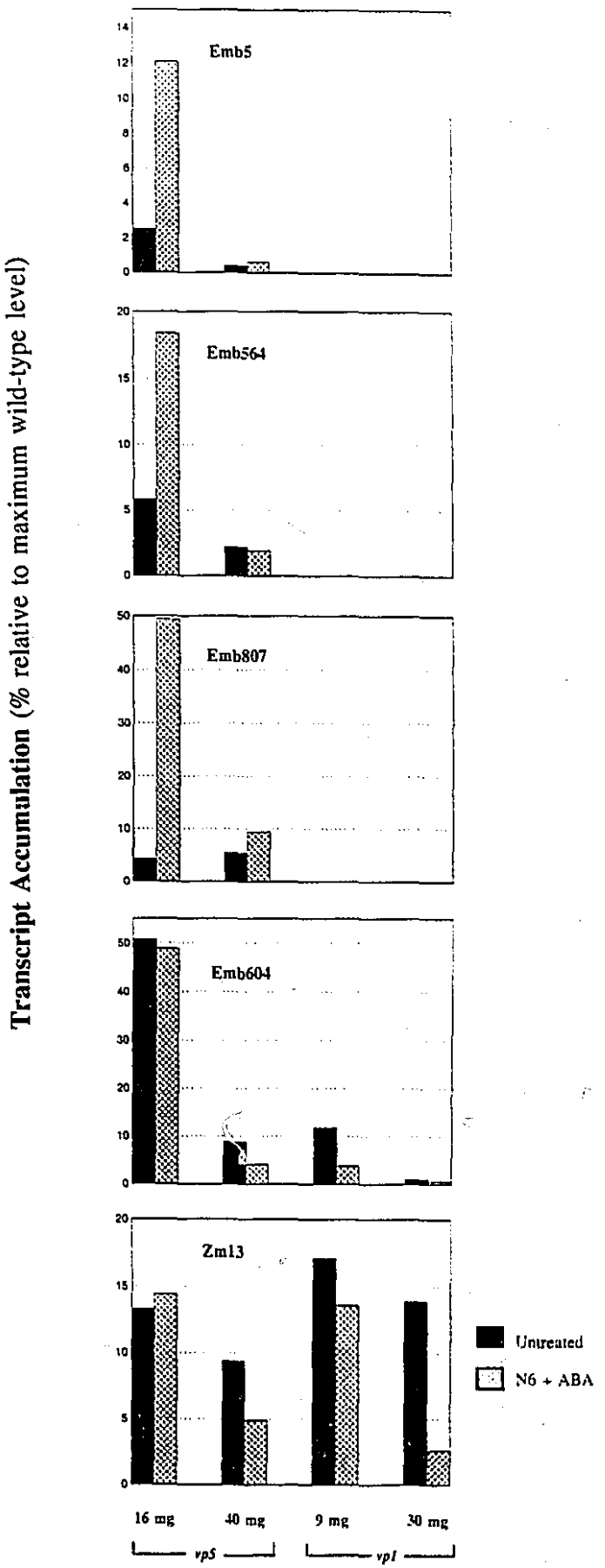
←——pWR590-δ-ß-Gal————————┤ 5′ untranslated region Emb564
Gly Asn Pro Gly Glu Leu Glu Phe Gly Asp Leu Ala Ser Tyr Leu Ala
Gly Asn ccg ggc gag ctc gAA TTC GGG GAC CTA GCT AGC TAC CTA GCT

├————————EMB564 Open Reading Frame————————→
Ser Ser Gly Asp Asp MET Ala Ser Gly Gln Glu Ser Arg Lys Glu....
AGT TCA GGC GAC GAT ATG GCG TCT GGT CAG GAG AGC AGG AAG GAG....

Appendix 5. Concentration Dependence of ABA-Regulated Gene Expression. Two-mg embryos were incubated on N6 medium supplemented with 1 nM (-9), 10 nM (-8), 100 nM (-7), 1μM (-6), or 10μM (-5) ABA for 16 h. Total RNA was isolated and analyzed by RNA blot hybridization with probes complementary to Emb5, Emb564, Emb604, Emb807, and pZm13. Autoradiograms were quantified by densitometry (see Fig. 10).

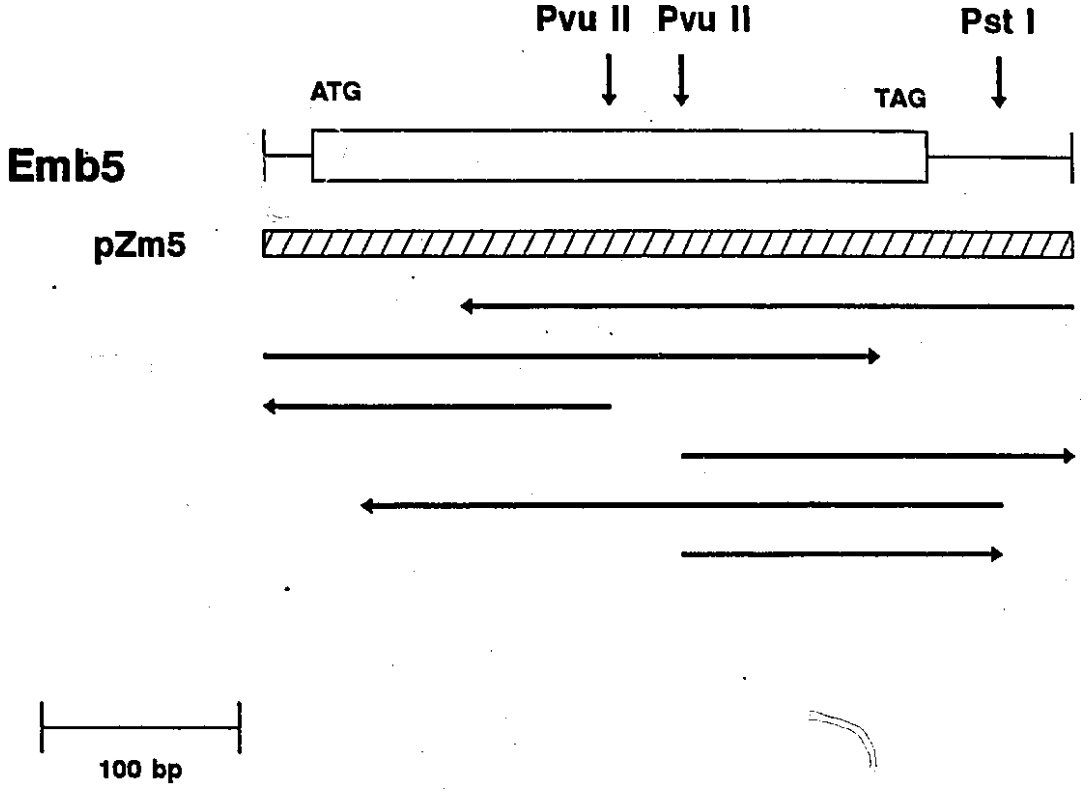

[ABA] (M)
-9 -8 -7 -6 -5
Emb5
Emb564
Emb807
Emb604
pZm13

Appendix 6. Densitometric Estimation of Relative Accumulation of Transcript in Viviparous Embryos Incubated with ABA. The relative intensities of signals from autoradiographs shown in Fig. 12 were estimated by densitometry and graphically displayed. One set of embryos was frozen immediately and kept as untreated controls (C). The second set of embryos was incubated on N6 medium supplemented with 1 µM ABA for 16 h (A). Maximum accumulation in wild-type embryos was taken as 100%.


Transcript Accumulation (% relative to maximum wild-type level)
Emb5
Emb564
Emb807
Emb604
Zm13
Untreated
N6 + ABA
16 mg
40 mg
9 mg
30 mg
vp5
vp1

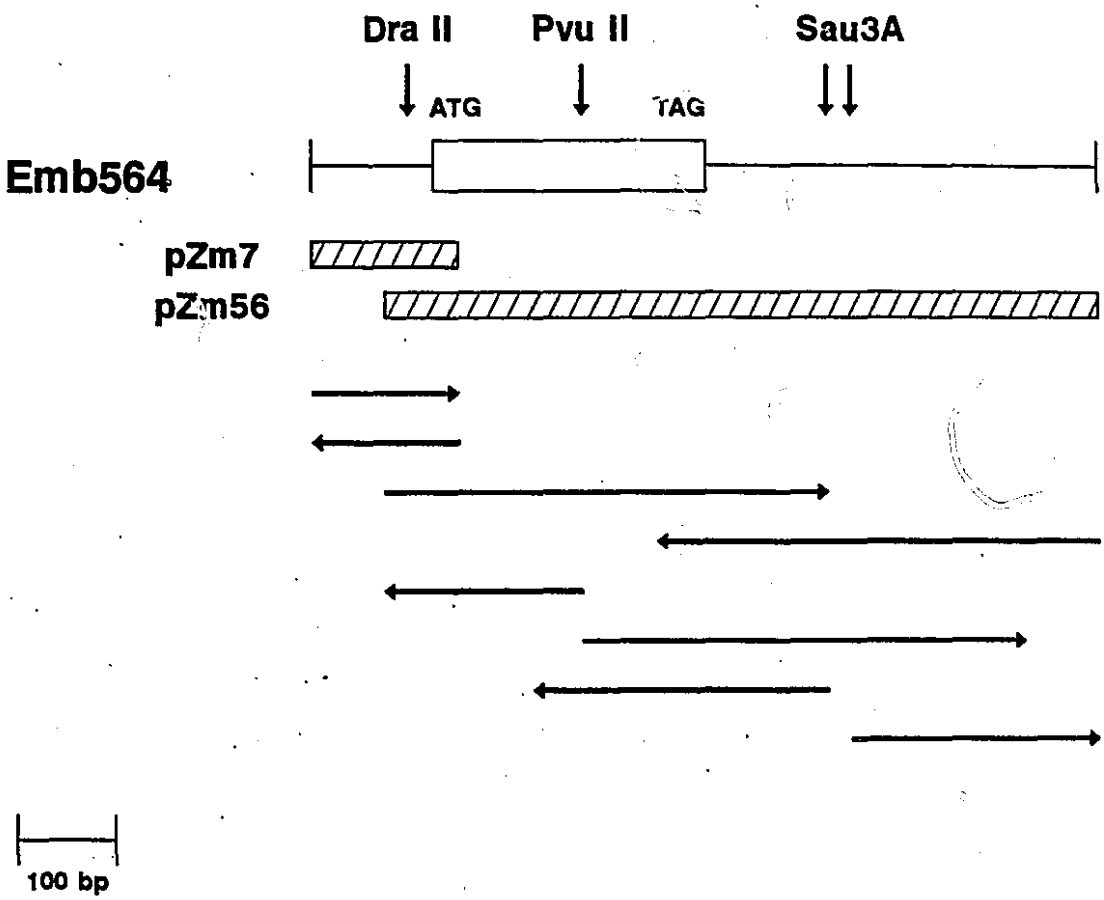
Appendix 7. Sequencing Strategy for cDNA Clone Emb5.

The clone pZm5, represented by the shaded rectangle, consists of a cDNA inserted into the *Pst* I site of pBR322. This clone was sequenced using the double-stranded method. Restriction fragments (*Pst* I, *Pvu* II-*Pst* I, and Pvu II-Bam HI) from this clone were subcloned into Bluescript vectors and sequenced using the single-stranded technique. The open reading frame is represented by the open rectangle; untranslated sequences are indicated by a line. The strategy used to determine the nucleotide sequence is indicated by arrows.


Pvu II
Pvu II
Pst I
ATG
TAG
Emb5
pZm5
100 bp

Appendix 8. Sequencing Strategy for cDNA clone Emb564.

The original clones, pZm7 and pZm56, are represented by shaded rectangles. These were sequenced using the double-stranded technique. Restriction fragments (*Pst* I, *Pvu* II-*Pst* I, and *Sau* 3A-*Pst* I) from these clones were subcloned into Bluescript vectors and sequenced using the single-stranded technique. The open reading frame of Emb564 is represented by the open rectangle; untranslated sequences are indicated by a line. The strategy used to determine the nucleotide sequence is indicated by arrows.


Dra II
Pvu II
Sau3A
ATG
TAG
Emb564
pZm7
pZm56
100 bp

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