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**THE ROLE OF ABSCISIC ACID AND ABSCISIC
ACID-ANALOGS IN INDUCING DESICCATION
TOLERANCE IN MICROSPORE-DERIVED
EMBRYOS OF BRASSICA NAPUS**

by

XIAOBING PENG, B.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Biology
Carleton University
Ottawa Canada

September 12, 1994

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**THE ROLE OF ABSCISIC ACID AND ABSCISIC ACID-ANALOGS
IN INDUCING DESICCATION TOLERANCE IN MICROSPORE-
DERIVED EMBRYOS OF BRASSICA NAPUS**

submitted by **XIAOBING PENG, B.Sc.**

in partial fulfilment of the requirements for the degree of Master of Science

A handwritten signature in black ink, appearing to read "Daniel C. W. Brown", is written over a horizontal dashed line.

Thesis Supervisor

A handwritten signature in black ink, appearing to read "J. H. S.", is written over a horizontal dashed line.

Chairman, Dept. of Biology

ABSTRACT

S-ABA, R-ABA and a group of analogs were used in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus*. Results indicated that, in inducing desiccation tolerance in microspore-derived embryos, the ABA structure requirement for biological activity is more flexible than in other systems. It was found that S-ABA at 10-100 μ M was the most effective of the compounds tested in inducing desiccation tolerance, resulted in the best vigour after rehydration, and that with concentrations applied below 0.1 μ M, there was no observable effect. R-ABA was not as effective as S-ABA at higher concentrations (10-100 μ M), but was more persistent than S-ABA at lower concentrations 0.1-1 μ M. Three S-configuration analogs, P-89 (Dihydro ABA), P-82 (Dihydroacetylenic ABA) and P-63 (Dihydroacetylenic alcohol ABA) were equally effective in inducing desiccation tolerance, but the embryos needed an exposure to a 5 times higher concentration to reach the same survival level of survival as with S-ABA. P-185, an enantiomer of P-82, was found to be as effective as P-82 at 50 μ M. This is first time that P-185, a R-configuration analog, was shown to have biological activity although its activity dropped very fast when the concentration was lowered to 2 μ M. Another two R-configuration enantiomers, P-59 and P-51, were found to be inactive. Results also indicated that an ABA metabolite, phaseic acid, and $\pm$ T-T ABA did not induce desiccation tolerance. Experiments demonstrated that:

1. The applied ABA was taken up and metabolised during subsequent incubation in culture.

2. During the treatments with R-ABA and ABA-analogs, the endogenous ABA increased a little suggesting that R-ABA and ABA-analogs were not converted to S-ABA, even when the concentration was high enough to be toxic to the embryos.

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LIST OF ABBREVIATIONS

ABA	Absciscic Acid
ABH	Aminobenzoyl Hydrazone
BB	Borate Buffer
BSA	Bovine Serum Albumin
C-T ABA	$\pm$ <i>cis-trans</i> ABA
DW	Dry Weight
EtOH	Ethanol
HPLC	High Performance Liquid Chromatography
MAb	Monoclonal Antibody
M ABA	ABA With a Mixture of Isomers
MW	Molecular Weight
P-89	(S)-2', 3'-Dihydro ABA or PBI-89
P-59	(R)-2', 3'-Dihydro ABA or PBI-59
P-82	(S)-2', 3'-Dihydroacetylenic ABA or PBI-82
P-185	(R)-2', 3'-Dihydroacetylenic ABA or PBI-185
P-63	(S)-2', 3'-Dihydroacetylenic alcohol ABA or PBI-63
P-51	(R)-2', 3'-Dihydroacetylenic alcohol ABA or PBI-51
P-240	Phaseic acid or PBI-240
PBI	Plant Biotechnology Institute
PBS	Phosphate Buffer Saline

p-NPP	para-Nitro-Phenyl Phosphate
R-ABA	Unnatural ABA, PBI-145 or P-145
RH	Relative Humidity
S-ABA	Natural ABA, PBI-58 or P-58
SD	Standard Deviation
TBS	Tris-buffer Saline
T-T ABA	$\pm$<i>Trans-trans</i> ABA , PBI-49 or P-49

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INTRODUCTION

A. AGRONOMIC POTENTIAL OF CANOLA - *BRASSICA NAPUS*

Rapeseed/canola is one of the most valuable economic crops in the world. It ranks in third place among the oilseed crops after soybean and palm in production of vegetable oil (FAO, 1992). The major producers of rapeseed/canola are: China, India, Canada, Pakistan, Australia, England, Poland and France. Canada is the world's leading exporter of rapeseed/canola.

Rapeseed/canola defines a group of plant species derived from oil yielding members of the *Brassica* family. In this family, *Brassica nigra* (n=8), *Brassica oleracea* (n=9) and *Brassica campestris* (n=10) are the three basic species. *Brassica napus* (n=19), which is derived genetically from a natural hybridization of *Brassica campestris* and *Brassica oleracea*, is one of the most important and widely grown species with summer varieties grown in North America as well as in Europe and in parts of India (Shahidi, 1990). *Brassica napus* varieties can also be grown at lower temperatures in moist climates so it is becoming a favoured species in the cooler areas where soybean and sunflower may not survive.

Although rapeseed oil has been used for cooking and as lamp oil in China and India for thousands of years, its use as an edible oil in Northern America is a recent development.

Due to a high content of erucic acid, which is considered toxic to human beings (Shahidi, 1990), and a high content of glucosinolate, which is an inhibitor of animal growth (Shahidi, 1990), rapeseed oil and its meal have been mainly used in lamps and industries and as a fertilizer.

Canola refers to varieties of *Brassica*, containing less than 2% erucic acid and low or no glucosinolates in their seeds. It was first grown in Canada in 1968 (NRC, 1992). The name "canola" was initially registered as a trademark in 1978. As a quality edible seed oil, canola oil was recognized by the US Food and Drug Administration in 1985 and granted GRAS (generally recognized as safe) status. Containing 40% oil and yielding a meal with 38-43% protein, canola expansion in agricultural land use was very fast. In 1988, 63% of all deodorized vegetable oil production in Canada was canola oil (Canola Council of Canada, 1990). In 1991, over 3.2 million hectares of canola (19.8 million hectares of rapeseed/canola in the world) were planted in Canada (FAO, 1992). The canola industry is one of the major industries in Canadian agriculture and canola is now the second most valuable crop behind barley in Canada (NRC, 1992). According to CBC news in July 1994, the total value of canola in Canada this year will be over 10 billion dollars.

At this time, canola breeding efforts are focused on eliminating all forms of the glucosinolate and lowering the erucic acid in the seed, increasing the seed-oil yield, improving the oil quality, earlier maturity, aiding the "traditional" breeding with

biotechnology, and the development of integrated methods for the control of insect pests and disease. Advances in plant biotechnology, especially in molecular biology, are providing the necessary tools for the above objectives.

B. TISSUE CULTURE OF *BRASSICA NAPUS* AND ARTIFICIAL SEED

Since the first plant was produced from microspore culture in *Datura innoxia* in 1964 (Guha and Maheshwari, 1964), great progress has been made in using the *in vitro* culture of gametophytic cells to produce haploid and homozygous diploid plants for various purposes (reviewed by Heberle-bors, 1985; Keller, Arnison and Cardy, 1987), mainly in plant breeding and genetics.

In aid of plant breeding, the microspore-derived embryo system may be used to select lines producing low glucosinolates (Lichter, et al., 1988). The haploid nature of these embryos allows for the rapid selection of traits associated with recessive genes. Comparisons of agronomic traits in microspore-derived and conventional populations of spring *Brassica napus* (Charne and Beversdorf, 1991) showed similar results in the main economic traits of flowering, maturity, lodging, seed yield and content of oil. This favourable comparison predicts a bright future for the application of microspore-derived embryos in plant breeding.

The use of tissue culture-derived embryos as artificial seed or synthetic seed was first mentioned in 1978 (Murashige, 1978) and is rapidly becoming feasible as the advances in tissue culture technology define conditions for the induction and development of embryos. During the last two decades, four artificial seed delivery systems, fluid drilling of the embryos (Drew, 1974), encapsulation of embryos in an alginate gel (Redenbaugh,

1984), desiccation of the embryos at high humidity (Gray, 1986; Gray and Purohit, 1991) and desiccation of embryos encapsulated in a water soluble resin (Kitto and Janick, 1982; 1985_{a,b}), have been proposed and practiced. The aim of the artificial seed study is to set up a well-developed species-independent productive system to produce a high quality and a large quantity of artificial seed, a well-developed reliable storage system to maintain the high conversion abilities of the artificial seeds after rehydration and a well-developed regeneration system to induce the normal development of the artificial seeds without secondary embryogenesis. Nevertheless, the application of artificial seeds will also depend on the cost of utilization of the above systems compared with natural breeding systems.

As occurs in a natural seed, if the artificial embryo is dried to a moisture content of approximately 10%, the propagation system has an additional advantage of serving as a germplasm storage system, in which the propagule is maintained in a quiescent state for an extended period of time. Artificial embryos can also be produced continuously year-round in a central facility, shipped to local nurseries and can be stored for future planting.

Anthers, microspores, ovaries and ovules can all be used to regenerate haploids via tissue culture. Anther and microspore cultures have been used extensively to provide a rapid and a highly efficient source of genetic homozygosity (Huang, 1992; Keller et al., 1987).

Anther culture using *Brassica napus* was first reported in 1975 by both Keller et al. (1975) and Thomas and Wenzel (1975) with low frequency embryo production. Since

then, this culture technique has been improved significantly with reliable embryo production. Direct embryo formation from isolated microspores was another milestone for *Brassica napus* haploid production technology (Lichter, 1982). Siebel and Pauls (1989) compared anther and microspore culture in *Brassica napus*. They found that, in *Brassica napus*, microspore culture is ten times more efficient than anther culture in terms of embryo production and is the most efficient *in vitro* culture system developed to date. Not only does microspore embryogenesis provide a valuable system for developmental as well as genetic study, but also the use of microspore culture as a recipient system for gene transfer has been considered (Neuhaus et al., 1986; Swanson and Erickson, 1989).

During the last decade, a number of factors influencing embryogenesis have been evaluated and the conditions optimized. The microspore culture procedure has been considerably improved and has become a standard method for plant breeding in canola (Lichter, 1985).

Donor plant genotype has been suggested to be the most important determining factor influencing embryogenesis in anther and microspore culture (Chuong et al., 1985; Dunwell et al., 1985; Huang and Keller, 1989; Takahata, et al., 1991) Chuong and coworkers found that embryogenesis in microspore culture appeared to be genotypically dependant, and, for different genotypes, the range of embryo yield was quite different. The amount of the cotyledon-stage embryos varied from 6-70% of the total numbers of embryos produced. Genetic control for some components of embryo yield was confirmed

by the evidence that hybrids of *Brassica napus* with a common parent responded similarly under certain treatments (Dunwell et al., 1985). *Brassica napus* cv. Topas, a cultivar from a single cloned cell, used in these experiments, is not a commercially available species but highly responsive in microspore culture. This line was crossed with Westar, a widely used species in Canada, and the resultant plants were very good in several main economic traits (Charme and Beversdorf, 1991).

It is important to accurately identify and select buds containing potentially embryogenic microspores (Kott et al., 1987; Pechan and Keller, 1988; Swanson et al., 1987). The results from the above-cited studies showed that fifty percent of buds between 2.5-3.5 mm in length contain potentially embryogenic microspores, increasing to nearly 100 percent between 4-4.5 mm in length and decreasing sharply thereafter. Cytological studies (Fan et al., 1988) indicated that buds 3-4.5 mm in length, containing microspores mainly at the late uni- and early binucleate stages of development, are the most likely to undergo embryogenesis. General morphological traits could thus be used to determine those buds that were likely to contain potentially embryogenic microspores, but not to predict the frequency of microspore embryogenesis of individual buds (Pechan et al., 1989).

Other conditions of microspore culture, including pH, autotoxicity, medium and temperature have been studied and optimized (Chuong et al., 1988; Chuong and Beversdorf, 1985; Gland, 1988; Huang and Keller, 1989; Keller and Long, unpublished; Kott et al., 1988).

The system of *in vitro* production and maturation of microspore-derived embryos has the advantage of having a precisely defined window of embryo induction (the first 8 hours after isolation of the specially staged microspore, Pechan et al., 1991), the elimination of an external growth regulator influence to trigger embryogenesis, the induction and sustainment of the embryogenic process by a single stress treatment of a defined duration, up to 50% of microspores in a given culture undergoing embryogenesis (Pechan and Keller, 1988) and high quality and quantity synchronous development of embryos (Brown et al., 1993; Swanson et al. 1987). A typical and efficient system for isolated microspore embryogenesis has been outlined by Huang and Keller (1989). The system used in this study is generally based on this published protocol with modifications as outlined by Brown et al. (1993).

C. BIOLOGY AND CHEMISTRY OF ABA AND ABA-ANALOGS

Abscissic acid was first identified and characterized chemically in 1963 by Frederick T. Addicott and his co-workers in California, who were studying compounds responsible for abscission of cotton fruits (Salisbury and Ross 1985). ABA is a 15-carbon sesquiterpenoid synthesized in chloroplasts and other plastids. Its structure is shown in Figure 1.

The ABA molecule contains, in position C1', an asymmetric carbon atom and possesses, therefore, optical activity. Only the S-ABA (or +ABA) enantiomorph occurs naturally in plants (Dorffling and Tietz, 1983). Commercially available synthetic ABA is a racemic 1:1 mixture of S(+)-ABA and R(-)-ABA. Most of the ABA present in plants is the S-2-*cis*, 4-*trans* isomer. Only small amounts of S-2-*trans*, 4-*trans* isomer occur naturally and this isomer is virtually inactive in all the experiments (Milborrow, 1978).

In contrast to its sensitivity to light, especially UV, ABA is relatively heat stable. The melting point of natural ABA is at 161° C, whereas racemic ABA has a melting point of 191° C. Usually, S-ABA is easily converted to the 1:1 mixture of +2-*cis*-4-*trans* and +2-*trans*-4-*trans* isomers by light (Plancher, 1979). Recently, Dunstan et al. (1992) tested the metabolism of $\pm$ ABA in somatic embryo suspension cultures of white spruce and found that, when the cultures were incubated in the light, the levels of $\pm$ 2-*trans*-4-*trans* ABA did not increase significantly. In addition to its optical activity, ABA strongly absorbs UV radiation. The absorption of UV by S-ABA dissolved in ethanol shows

Figure 1. Structure of (S)-ABA and the ABA Analogs.

1. S-ABA Natural ABA, shown carbon atom position
2. S-ABA Natural ABA or PBI-58
3. R-ABA Unnatural ABA or PBI-145
4. S-*trans-trans* ABA
5. R-*trans-trans* ABA
6. PBI-240 Phaseic acid
7. PBI-89 (S)-2', 3'-Dihydro ABA
8. PBI-59 (R)-2', 3'-Dihydro ABA
9. PBI-82 (S)-2', 3'-Dihydroacetylenic ABA
10. PBI-185 (R)-2', 3'-Dihydroacetylenic ABA
11. PBI-63 (S)-2', 3'-Dihydroacetylenic alcohol ABA
12. PBI-51 (R)-2', 3'-Dihydroacetylenic alcohol ABA

Note: a. C-T ABA is a mixture of S-ABA and R-ABA;

b. M ABA is a mixture of isomers of ABA ($\pm$ 2-*cis*-4-*trans* and $\pm$ 2-*trans*-4-*trans* ABA);

c. 1, 3, 5 and 7 are S-ABA configuration analogs and 2, 4, 6 and 8 are R-ABA configuration analogs.

PBI-89

PBI-59

PBI-82

PBI-185

PBI-63

PBI-51

maximum at 260 nm.

S-ABA can be dissolved in many solvents such as methanol, ethanol, ethylacetate, ether and chloroform. Containing a carboxyl group, S-ABA is a weak acid (pK 4.8); therefore, the solubility in aqueous solvents depends on the pH. At low pH (2-3), the compound is slightly insoluble. In its acid form, S-ABA is easily solubilized in organic solvents. At high pH (8-9), the situation is entirely opposite and the salt of S-ABA shows high solubility in water and low solubility in less polar solvents.

Two pathways for S-ABA biosynthesis in plants have been proposed (Zeevaart et al., 1983):

- (1) the direct pathway, in which a C15 precursor, presumably farnesyl pyrophosphate, is cyclized and then converted to S-ABA,
- (2) the indirect pathway, in which S-ABA is synthesized by degradation of xanthophyll (C40); cleavage of a terminal ring with six C's of the backbone would yield a C15 compound, which would be further metabolized to S-ABA.

It is possible that both these pathways operate in plants, depending on various environmental and internal physiological factors. In both pathways, mevalonic acid (MVA) would be the initial precursor.

Inactivation of ABA can also occur in two ways (Zeevaart et al., 1983). One is by

attachment of glucose to the carboxyl group to form an ABA-glucose ester. Another inactivation process is oxidation with oxygen to form phaseic acid and further dihydrophaseic acid, a compound in which the ketone of phaseic acid is reduced to a hydroxyl group. The site of the oxidative degradation of ABA occurs principally in the cytosol (Gillard and Walton, 1976; Hartung et al., 1980).

The major defined biological functions of ABA in plants are the initiation of stomata closure when water stress occurs or when elevated CO₂ levels occur in guard cells; induction and maintenance of bud dormancy; promotion of seed dormancy and prevention of seed germination; and acceleration of leaf abscission. Like other plant growth substances, ABA does not always suppress the growth activity of plant tissue. Evidence reveals that ABA can promote a few physiological processes; ABA can stimulate storage protein accumulation in embryos (Sussex et al., 1980; Finkelstein et al., 1985) and proline accumulation in barley leaves (Stewart, 1980); increase desiccation resistance (Long, 1979) and desiccation tolerance of somatic embryos (Kermode and Bewley, 1987); and induce callus formation in *Citrus sinensis* bud culture (Altman and Goren, 1971). ABA not only suppresses the synthesis of a group of proteins that are related to seed germination but also induces another new set of proteins (Dure et al., 1980). Observations have shown that, in germinating barley seeds, ABA can inhibit germination at high concentrations (10-100 μ M) but can enhance the germination process at lower concentrations (0.01-0.001 μ M) (Ho, 1983).

ABA seems to have three major sites of action, depending on the tissue involved (Ho, 1983). Available evidence indicates that ABA is able to affect membrane-located processes, the expression of RNA synthesis (transcription), and the expression of protein synthesis (translation). The mechanisms of these effects is still unknown.

The structure/activity relationship of the ABA molecule was studied as soon as ABA and ABA-analogs were chemically synthesized. Up to now, more than 100 ABA-analogs have been used in various bioassays. The activity of the structure was compared with S-ABA and outlined by Walton (1983). *Trans-trans* ABA is virtually inactive in all experiments in the dark. An acetylenic linkage at the C-4 position, in the side-chain, had little effect as long as there was a 2-*cis* double bond (Orton and Mansfield, 1974). Changes in the free carboxylic group with nitrile or alcohol have both low (Oritani and Yamashita, 1970) and high (Taylor and Burden, 1972) activity depending on the species. It is assumed that this group is required for binding. Esterification of the carboxyl group of S-ABA has been reported to both increase activity (Milborrow, 1966; Taylor and Burden, 1972) and decrease activity (Milborrow, 1966). It is likely that increased activity of the esters may involve enhanced uptake and reduced metabolism. It is difficult to test and determine the absolute requirement of the ring system. Some results indicate that changes in the ring system resulted in less activity than ABA and some analogs can be converted to ABA (Milborrow, 1972; Tamura and Nagao, 1970). Another important point that should be mentioned is that the C1' is the only asymmetric carbon atom in the ABA molecule. The effect of the pair of optically pure enantiomers in plant development has been compared.

In several experimental systems, the synthetic R-enantiomer is as active as the natural one except in the closure of barley leaf stomata (Cummins and Sondheimer, 1973).

During last decade, the precise role of ABA in plants has been studied at different levels, especially at the molecular level. ABA is thought to play a key role in two processes; namely the maturation of seeds and the mediation of responses to drought stress (Bray, 1992; Parry and Horgan, 1991). ABA levels will increase and ABA-responsive genes can be induced to express in the above-mentioned conditions, although different sets of ABA-responsive genes exhibited different patterns of expression (Skriver and Mundy 1990). Under some conditions, the ABA-responsive genes can also be induced to express in unstressed tissue by the application of exogenous ABA or ABA-analogs.

In recent years, many ABA-analogs and optical isomers of ABA have been used to study the structural requirements of the ABA recognition response in barley (Van der Meulen et al, 1993), *Brassica napus* (Wilen, et al., 1993), bromegrass (Abrams et al., 1989; Lamb et al., 1993), sunflower (Barthe and Le Page-Dgivy, 1991), tomato (Linthorpe et al., 1990) and wheat embryos (Qureshi et al., 1989; Walker-Simmons et al., 1991 and 1992). Different species or different organs of the same species have different structure requirements for ABA or ABA-analogs. The recognition of structure activity of ABA-analogs and their effects on expression of a set of ABA-response genes in the above species have been studied with both empirical methods and molecular biological methods. This will be discussed in the next section.

D. ANALYSIS OF ABA IN PLANT TISSUE

Since the discovery of ABA, methods for its purification and quantification have been performed and developed both in bioassay and in physical and chemical assays. A comparison of several common methods of quantification of ABA is presented in Table I. Details were reviewed by Dorffling and Tietz (1983) and Zeevaart and Creelman (1988).

To further understand the mechanism of action and the physiological role of ABA, and the effects of ABA in molecular biology, it is important to develop a rapid, convenient and sensitive method to detect endogenous ABA levels in particular plant organs, tissues, and even individual cells. Immunoassays provide rapid, sensitive and specific tools to identify and quantify the low physiological concentrations of the plant growth substance ABA (recently reviewed by Walker-Simmons and Abrams, 1992). These methods, ELISA (enzyme-linked immunosorbent assay) (Leroux et al., 1985; Norman et al., 1988; Ross et al., 1987), RIA (radioimmunoassay) (MacKay et al., 1990; Quarrie et al., 1988) and FIA (fluoroimmunoassay) (Banowetz, 1989) are based on the specific antigen-antibody reaction. Since the ABA molecule is not big enough to be used as an antigen, it is usually coupled as a hapten to protein carriers (BSA, bovine serum albumin, or HSA, human serum albumin). After immunization with one of the conjugates, rabbits produce antibodies to ABA. The specificity of the antibodies toward ABA depends on conjugation of ABA to the carrier protein, because functional groups distant to the binding site are

most important for specific antibody formation (Dorffling and Tietz, 1983). ABA-analog cross-reaction with antibodies also depend on the antibody toward ABA bound by C1 or C4'. If the binding protein coupling ABA is through C1, the produced antibody can be used for total ABA determination and if the coupling site is through C4', the produced antibody can be used for free ABA determination. The immunoassay is the most specific and sensitive method available at present.

ELISA is less expensive than RIA and eliminates radioactive tracers. To avoid the disadvantages listed in Table I, a new indirect ELISA technique was developed recently (Norman et al., 1988; Walker-Simmons, 1987). The major advantages of indirect ELISA are the requirement of considerably less commercially available monoclonal antibody than direct ELISA, and the reduction of the possibility of interaction between enzyme and potential enzyme inhibitors in the plant tissue. Indirect ELISA has been used in crude extracts of wheat grain and the identity and quantity of ABA were validated by HPLC-UV and GC-MS (Walker-simmons, 1987). This technique was used to measure the endogenous ABA levels in microspore-derived embryos of *Brassica napus*. Special advantages for this experimental system are that relatively small amounts of sample, 10 mg, are needed, and several samples could be analyzed together on one ELISA plate.

TABLE I. COMPARISON OF COMMON METHODS FOR THE DETECTION AND MEASUREMENT OF ABA^a

Method	Advantages	Disadvantages
GC-FID	Inexpensive and easier	Low selectivity and sensitivity; requires derivation
GC-ECD	High sensitivity and selectivity; small tissue sample needed	Requires derivation
GC-MS	High selectivity and/or sensitivity	Expensive equipment
HPLC	Rapid; easy; multiple uses	Low selective and sensitivity
ELISA	Rapid; more sensitive than EIA; specific for ABA	Samples need purification by TLC or HPLC; expensive to develop
RIA	Rapid; more precise than ELISA; specific for ABA	Recovery with internal radioactivity standards not possible; samples need purification with TLC or HPLC; expensive and time-consuming to develop

a: Modified from Creelman and Zeevaart (1987)

E. ABA, ABA-ANALOGS AND PLANT EMBRYOGENESIS AND MATURATION

ABA one of the well recognized plant growth substances, which is defined as a compound that, in low concentration, moves from a site of production to a site of action and functions to regulate a physiological process (Van Overbeek et al., 1954), ABA has been implicated in the control of many events during embryogenesis and seed formation; namely, embryo morphogenesis (Quatrano, 1987), storage protein synthesis (Crouch and Sussex, 1981; Finkelstein et al., 1985), desiccation tolerance (Kermode and Bewley, 1987) and the onset and maintenance of dormancy (Koornneef, 1986). Several ABA-responsive genes have been isolated (Baker et al., 1988; Bartels, et al., 1991; Gomez et al., 1988; Marcotte et al., 1988; Mundy and Chua 1988; Vilardell et al., 1990; Williamson and Quatrano, 1988), but their functions are unknown.

The effects of ABA on protein and RNA synthesis were first suggested by the works of Ho and Varner (1976) and Jacobsen and coworkers (1979). They detected new peptides when the barley aleurone layer was treated with ABA alone. These peptides turned over rapidly and the quantities formed were reduced by inhibitors of transcription and translation. Crouch et al., (1985) and Finkelstein et al., (1985) demonstrated that ABA could both inhibit precocious germination of rapeseed (*Brassica napus*) embryos in culture, and maintain the expression of the embryo-specific storage protein genes.

Late embryogenesis abundant (*lea*) proteins, which become very abundant in the last

stages of seed formation, have been described with various species, including barley (Hong et al., 1988), *Brassica napus* (Harada et al., 1989), carrot (Seffens et al., 1990), cotton (Dure et al., 1981) and wheat (Curry et al., 1991). Experiments with environmental induction and exogenously-applied ABA show that their expression is indeed responsive to the growth substance (Dure et al., 1981; Harada et al., 1989; Hughes and Galau, 1991; Skriver and Mundy, 1990). Wheat *Em* mRNA and protein, which is a major protein in mature wheat embryos, accumulated during late embryogenesis (Williamson et al., 1988) is also a ABA-regulated gene and associated with ABA content. Marcotte et al. (1989) demonstrated that a reporter gene (β -glucuronidase (GUS)) linked to an ABA-regulated wheat-*Em*-gene promoter were correctly expressed in transgenic tobacco plants induced by ABA. Other ABA-regulated genes were also isolated from different species, eg. *rab* in rice (Mundy and Chua, 1988), LE16, LE20 and LE25 in tomato (Cohen and Bray, 1990) and PI-II in potato (Pena-Cortes et al., 1989). ABA regulation of gene expression was recently reviewed by Bray (1992).

ABA is essential for the induction of seed dormancy. Results from ABA-deficient and ABA-insensitive mutants of *Arabidopsis thaliana* (Koornneef et al., 1989; Meurs et al., 1992; Ooms et al., 1993) showed that seeds of ABA-deficient as well as ABA-insensitive mutants failed to desiccate at the normal time, remained green until ripe and lost viability upon drying. Meanwhile, these double-mutant seeds could not accumulate the major storage proteins.

Dormant seed embryos of wheat make a good system for examining ABA responsive mechanisms during seed germination because these embryos are highly responsive to ABA. The application of the different ABA concentrations that block germination of dormant seed embryos resulted in the expression of a set of ABA-response genes in the growth-arrested tissue (Morris et al., 1991). If ABA is removed from the media, the immature wheat embryos will undergo precocious germination (Quatrano et al., 1983) and the precociously germinated seeds can be inhibited by exogenously applied high concentrations of ABA (10-100 μ M).

The successful induction of desiccation tolerance is the key step for the use of *in vitro* embryogenesis in an artificial seed system. The general effect of ABA in enhancing desiccation tolerance is well documented (Bartels et al., 1988; Brown et al., 1990, 1993; King, 1982; Ross, 1984; Senaratna et al., 1989, 1991; Skriver and Mundy 1990; Takahata et al., 1990 and 1992; Walbot, 1978). ABA has been shown to play a key role in enhancing desiccation tolerance during the maturation of seeds and in response to drought stress. But the precise role of ABA in inducing desiccation tolerance is still not clear, especially at the molecular level. Application of ABA prior to water removal increases survival of desiccated somatic embryos of alfalfa (Senaratna, 1989), celery (Park et al., 1988), microspore-derived embryos of *Brassica oleracea*. L (Takahata et al., 1992) and *Brassica napus* (Brown et al., 1993).

Desiccation tolerance is a complex property. The phenomenon has been frequently

described at the morphological, physiological and biochemical levels, but is still far away from being understood at the molecular level (reviewed by Bewley and Krochko, 1982; Bartels et al., 1988). Developing embryos, whether somatic, zygotic or microspore-derived, can be used as experimental systems to study desiccation tolerance. Under constant environmental conditions, the onset of desiccation tolerance, together with that of correlated metabolic events, can be precisely monitored. The degree of precision with an *in vitro* system is far greater than in an *in vivo* system. Moreover, the *in vitro* cultivation of the microspore-derived embryos, somatic embryos and isolated embryos offers the possibility of testing the influence of exogenously supplied ABA and ABA-analogs *ex planta*. In this system, you can separate the induction time from the time of water stress temporally. This is not possible in an *in vivo* system.

Some attempts have been made to desiccate microspore-derived and other somatic embryos without encapsulation, but success has been limited to a few species: alfalfa (Senaratna et al., 1990), canola (Brown et al., 1990, 1993), carrot (Iida et al., 1992), cotton (Ackerson et al., 1982) and grape (Gray, 1987).

Desiccation tolerance was successfully induced in alfalfa (*Medicago sativa*. L) somatic embryos by exogenous application of ABA via incorporation in the medium (Senaratna et al., 1989, 1990). Embryos can be dried to 10-15% moisture and stored for several weeks in the dry state. In the process, the timing of this ABA treatment is critical because the somatic embryos are responsive to the induction treatment for only a few specific

days in their development. Generally, alfalfa somatic embryos were most responsive to ABA at the torpedo to cotyledonary stage of development. In carrot (Iida et al., 1992), 15-day-old torpedo stage somatic embryos that were treated with 10 μ M exogenous ABA for 8 days shew the highest viability after desiccation without encapsulation, which is considered to maintain the viability of the embryos.

As described above, *lea* proteins are expressed during seed development and, in other tissues, in response to water stress or ABA (Skiver and Mundy, 1990). Analysis of amino acid composition of these proteins, Dure et al. (1989), suggested that these proteins were unlikely to be either enzymes or elements of structure in the cells, but they were likely to be the proteins located in cytoplasm and thus have been proposed to play an important role in increasing desiccation tolerance. To study the relationship between the accumulation of *lea* proteins and ABA induction, and the accumulation of *lea* proteins and desiccation tolerance of the developing seed, experiments were carried out in several plants; namely alfalfa (Xu and Bewley, 1991), canola (Johnson-Flanagan et al., 1992), pea (Robertson and Chandler, 1992) and rice (Still et al., 1994). Johnson-Flanagan et al., (1992) showed that applied-exogenous ABA can induce desiccation tolerance but *lea* transcripts increasing only slightly, and water stress induced a high level of *lea* transcript only without inducing desiccation tolerance. *lea* transcripts could also be increased by low temperature. They concluded that the *lea* protein studied was correlated with desiccation itself, and the increases of desiccation tolerance did not reflect the increased levels of *lea* mRNAs' transcripts. Still et al., (1994) indicated similar results in rice.

To investigate ABA perception in embryogenic tissue, ABA and a small number of ABA-analogs with structures very similar to the natural growth substance and recognized by monoclonal antibodies raised against (S) ABA (Walker-Simmons et al., 1991), were used to probe the stereochemical requirement of the ABA recognition response for germination inhibition and for regulation of ABA-responsive genes in wheat embryos. In wheat embryo germination (Walker-Simmons et al., 1992), optically pure ABA-analogs, which were modified in various functional groups (similar to the ABA-analogs used in our experiments, structures shown in Figure 1), were identified as having differential effects on ABA-responsive gene expression. All the analogs that were effective germination inhibitors induced two ABA-responsive genes, *dhn* (*rab*) and wheat *lea*, by 24 hs, but had varying effects on the *Em* gene induction. R-ABA, S- and R-2',3'-dihydro ABA and R-2',3'-dihydroacetylenic ABA had little effect on *Em* gene expression. ABA and ABA-analogs induced expression of a reporter gene (GUS) linked with a wheat *Em* gene promoter, electroporated into protoplasts derived from an embryogenic white spruce culture (Dong et al., 1994). Both S-ABA and ABA-analogs with the same C-1' configuration as S-ABA were effective but not their enantiomers. The wheat *Em* gene showed different expressions in the above-mentioned two plant systems.

Only a limited number of studies have reported comparisons of the biological activity of (S)- and (R)-ABA. The two forms have often been found to have equivalent biological activity (Walton 1983), although the (R)-enantiomer has been found to be less effective than (S)-ABA in inhibiting root growth of barley seedlings and in inducing stomatal

closure (Cummins and Sondheimer 1973; Sondheimer, 1971). (R)-ABA is found to be more persistent than (S)-ABA in culture filtrates of bromegrass cell cultures (Abrams et al., 1989). Gusta et al. (1992) found that S-ABA was more effective than R-ABA in inhibiting both root and shoot emergence of cress seed; thereby, suggesting that R-ABA has a different effect in monocots and dicots.

Churchill et al. (1992) compared the activity of 32 racemic ABA-analogs, in which systematic alterations were made in four functional groups of the ABA molecule, in induction of freezing tolerance in cultured bromegrass cells. They got similar results to Walker-Simmons et al. (1991) in monoclonal antibody recognition of ABA-analogs. Results indicated that the most active compounds were ABA and ABA-analogs with a saturated ring or/and a triple bond at C-4 and C-5 of the sidechain; although, all of the alternated structures showed a reduced activity of compounds to ABA.

The application of pure ABA-analogs provides a new tool to probe the ABA recognition response. It would be very valuable to identify the role of ABA and ABA-analogs in inducing desiccation tolerance both theoretically and practically. It would also contribute to the understanding of gene expression and regulation during embryo desiccation.

With several advantages listed in part B, microspore-derived embryos of *Brassica napus* offer a suitable system for above problematic studies. The approach that was successful in wheat and bromegrass can be applied to *Brassica napus*. Meanwhile, the application

of ABA and ABA-analogs in inducing the desiccation tolerance in microspore-derived embryos of *Brassica napus* will be more helpful to recognize the structure-activity of the ABA and ABA-analogs. Natural occurring ABA is S-(+)-ABA or S-ABA. Only a few experiments have been performed by using the purified natural form of ABA. Most experiments in which ABA has been added to plants were done using racemic ABA which is a 1:1 mixture of S-(+)-ABA and R-(-)-ABA. Some experiments have been done using ABA mixed isomers which is a mixture of four compounds: S-(+)-ABA, R-(-)-ABA, S-*trans-trans*-ABA and R-(-)-*trans-trans*-ABA. Most researchers do not report the quality of the ABA they use. Unless otherwise stated, one should assume that exogenously supplied ABA means either racemic or mixed isomers. The reason for emphasizing this matter is that, in some assays, R-(-)-ABA is active and in others it is not. It therefore becomes difficult to compare levels of activity induced by ABA forms in different systems.

In this thesis, the activity of S-(+)-ABA, (R)-(-)-ABA and racemic T-T ABA were studied separately. Six ABA-analogs and the ABA metabolite, phaseic acid, (structures shown in Figure 1) were also used to induce desiccation tolerance in microspore-derived embryos. By treating the microspore-derived embryos with ABA or ABA-analogs and assessing the success of ABA or ABA-analogs in inducing the desiccation tolerance, it was speculated that the functional group of ABA could be recognized. This association was also important for the identification of the desiccation tolerance specific messages in future work.

In order to further recognize and determine the effects of exogenously applied ABA or ABA-analogs on endogenous ABA pools, as well as, to determine if the ABA-analogs were converted to ABA in the embryo, the endogenous ABA levels of the embryos in different developmental stages and conditions were also tested.

MATERIALS AND METHODS

A. PLANT GROWTH

Donor plants, *Brassica napus*. L. (cv. Topas), were grown from seeds (produced at the Plant Research Centre, Agriculture Canada, Ottawa), which were the progeny of a selfed, single microspore-derived plant that was selected for its response *in vitro*. Seeds were germinated in soil (sandy loam: peat moss: perlite; 4:4:1; v/v/v) in 23cm fibre pots in a growth chamber at 20/15° C (day/night) with 16-hour photoperiod from 7am to 11pm and $300\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity, watered daily as required and supplied with 20:20:20 (K/N/P) once weekly. Three weeks later (at this time plants were 5-7 cm high), 26 grams of solid Nutricote (14/14/14, K/N/P, Plant Products Co. Ltd., Brampton, ONT.) were added to each pot.

Five weeks after germination, the growth chamber temperature was lowered to 10/5° C (day/night) with a 16-hour photoperiod from 7am to 11pm, 70% relative humidity and $300\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. At this stage, the plants also received Hoagland's (a mixture solution of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, KH_2PO_4 , KNO_3 and $\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$, Plant Products Co. Ltd., Brampton, ONT.) solution weekly and were watered automatically using a timed nutrient trickle system.

B. MATERIALS AND MEDIA

1). CHEMICALS

$\pm$ *cis-trans* ABA (Sigma A2784), mixed ABA (Sigma A7383), *trans-trans* ABA (Sigma A0560), para-nitro-phenyl phosphate and anti-mouse alkaline phosphatase conjugate antibody (Sigma A-1902) were purchased from Sigma Chemical Company, St. Louis, Missouri.

Monoclonal antibody was purchased from Idetec Inc., Sunnyvale, California.

Natural ABA, unnatural ABA, 2',3'-dihydro (S-) ABA, 2',3'-dihydro (R-) ABA, 2',3'-dihydroacetylenic (S-) ABA, 2',3'-dihydroacetylenic (R-) ABA, 2',3'-dihydroacetylenic alcohol (S-) ABA, 2',3'-dihydroacetylenic alcohol (R-) ABA, and phaseic acid were kindly provided by Dr. Suzanne R. Abrams, Plant Biotechnology Institute, National Research Council, Saskatoon.

ABA-C₄-ABH was kindly provided by Drs. Patricia Ross and Suzanne R. Abrams, and CB positive control conjugate was kindly provided by Cheryl Bock, Plant Biotechnology Institute, National Research Council, Saskatoon.

All other chemicals were obtained from Sigma, Fisher or Aldrich.

2). EQUIPMENT

Dynatech immulon 2 plates and Nalgene disposable sterilization filter units for ELISA were purchased from Fisher Scientific, Ottawa, Ontario.

Centricon 3 microconcentrators for ELISA were purchased from Amicon Canada Ltd., Oakville, Ontario.

3). MEDIA

The standard liquid media used for microspore isolation and culture were B₆-13 and NLN-13, as described in Table I of Appendix 1. A 10 times stock solution of NLN medium containing all compounds except reduced glutathione, serine, L-glutamine and sucrose was prepared and frozen. At the time of medium preparation, the remaining compounds were added to the thawed stock solution, its pH was checked and the solution filter sterilized (0.22 µm filter). For B₆-13 and B₆-0, which were used for rehydration of the desiccated embryos, media stock solution of 10 times salts, micronutrient and vitamins, except sucrose and agar (for B₆-0 medium only), were separately kept in the freezer at -20°C. For medium preparation the stock solutions were thawed, combined and the remaining compound(s) added. The pH was checked and the solution filter sterilized or autoclaved (for B₆-0 only) for 18 minutes at pressure.

C. MICROSPORE ISOLATION, CULTURE, DESICCATION AND REHYDRATION

The protocol used in embryo production were based on Huang and Keller (1989) and modified as shown in Figure 2 to include the embryo collection and the desiccation treatments. All work was carried out under sterile conditions. The protocol can be divided into a number of stages.

Microspore Isolation

Stage I: Flower buds (3.0-4.0 mm in length) obtained prior to the opening of more than one flower in each inflorescence from ten to twenty-four week-old plants were surface sterilized in a Lipshaw basket suspended for 15 minutes in 7% calcium hypochlorite and then given 3 successive five-minute rinses in sterile distilled water. Buds were transferred to B₅ medium (Gamborg et al., 1968) modified to contain 13% sucrose and macerated with a glass pestle in a glass beaker to release the microspore. The homogenate was filtered through a 44 μ m mesh nylon screen, transferred to a centrifuge tube, and centrifuged at 100x g for 3 min and the supernatant decanted. The microspores were washed with fresh F₆-13 medium and the centrifuge step was repeated 2x with the speed being progressively reduced to 75x g and 50x g.

The microspores were resuspended in filter-sterilized NLN-13 medium (Huang and Keller 1989) at a density of 20,000 - 30,000 cells per mL. A higher or lower microspore

Figure 2. Experiment Steps

(modified from Brown et al., 1993)

density reduces microspore embryo numbers (Brown et al., 1990). Aliquotes of 10.0 mL of the microspore suspension were plated onto 95x15 mm Fisherbrand plastic Petri dishes. The plates were wrapped with Parafilm and the microspores cultured as indicated.

Embryo Induction and Development

Stage II: To induce embryo formation, plates were immediately transferred to 32.5° C, in the dark, for 4 days. A high temperature pretreatment is necessary and important for the induction of embryogenesis. The above procedure (from maceration of the buds to putting cultures into the incubator) should be finished within one hour. Longer manipulation times drastically reduces microspore embryogenesis (Huang and Keller, 1989; Pechan et al., 1991).

Stage III: On day 4 of culture, dishes were transferred to 25° C, in the dark, to allow embryo development.

Stage IV: On day 14, embryos in 95x15 mm plates were divided into five 60x15 mm Falcon plastic Petri dishes and the medium replaced with 3 mL fresh NLN-13 medium.

Embryo Treatment and Samples Collection For ELISA

Stage V: On day 21, NLN-13 medium was replaced by NLN-13 medium containing various concentrations of ABA and embryos were diluted 2-fold and cultured for the required days (1 and 2 hours and 1, 3 or 7 days). To add ABA, the old medium was

carefully pipetted off and new medium, containing the appropriate level of ABA, was added. EtOH was used to dissolve ABA in preparation of the ABA stock solution. To study the effects of EtOH in embryo development and inducing desiccation tolerance, different concentrations of EtOH were applied the same as with ABA. For sample collection, embryos treated 1 day, 3 days and 7 days by different concentrations of ABA, and were separately washed through a 1000 μ m mesh screen with sterile distilled water. Only the embryos retained by the screen were collected and immediately frozen in liquid nitrogen and stored at -80°C until used.

Stage VI: On day 28, embryos (treated by ABA or ABA-analogs for 7 days) were selected for size by sieving through a 1000 μ m mesh screen. Previous experiments indicated that large-size embryos were most responsive to ABA treatment (Brown et al., 1993). Embryos retained on the 1000 μ m mesh screen (2.5-3 mm in length) were collected for desiccation.

Desiccation and Rehydration:

Stage VII: For desiccation, size-selected embryos were collected on damp filter paper, and then divided into two sets. One set was regenerated directly on B₅-O medium. The other set, in 60x15 mm plastic Petri dishes, was transferred to a series of desiccators and exposed to sequentially lowered relative humidities (97%-43% RH under 25°C, see Table II). Every 24-48 hs, the dishes were transferred to desiccators with progressively lower relative humidity levels. Relative humidity levels were controlled by including specific

saturated salt solutions at the base of each desiccator as outline in Table II (Brown et al., 1993). The saturated salt solutions were selected for their constant relative humidity rating based on Winston and Bates (1960) and Weast (1977).

Relative water lost from the plant material was monitored with relative humidity gauges and samples were transferred when the relative humidity in the chamber approached the theoretical value, representing atmosphere equilibration. After desiccation, dried embryos were stored in the dark at room temperature and a relative humidity of 43%.

Stage VIII: For rehydration, dried embryos were transferred to 0.35% agarose-solidified, growth substance-free, B₅-0 medium containing 2% sucrose. Embryos were cultured at 25° C with a 16-h photoperiod under fluorescent light. A minimum of 20 embryos were cultured per replica treatment.

TABLE II. RELATIVE HUMIDITY CONDITIONS USED DURING DESICCATION

a: Results based on Winston and Bates (1960) and Weast (1977).

b: Measured by gauge at 25 °C

Desiccator	Saturated Compound	Theoretical ^a % RH	Measured ^b % RH
1	K ₂ SO ₄	97	93 - 96
2	Na ₂ CO ₃	87	92 - 95
3	NaCl	75	85 - 88
4	NH ₄ NO ₃	63	64 - 66
5	Ca(NO ₃) ₂ •4H ₂ O	51	51 - 53
6	K ₂ CO ₃ •2H ₂ O	43	43 - 45

D. PLANT ASSESSMENT

Stage IX: For assessment of survival, embryos were viewed with a dissecting microscope 14 days after rehydration and scored for root growth and shoot greening and extension. Only the embryos that showed both shoot growth, which developed a true leaf, and root growth, which emerged at least 5 mm, but showed no obvious swelling of the hypocotyl and no visible evidence of secondary embryo formation, were scored as survivors. Past experiments indicated that 100% embryos from this stage can normally be recovered as whole plants in the greenhouse.

To compare the vigour of the emblings of microspore-derived embryos and seeds (shown in Table III), emblings were measured 14 days after rehydration from treated and desiccated embryos and 14 days after development from untreated embryos and natural seeds (developed in B₅-0 medium directly).

E. ABA AND ABA-ANALOG SOLUTIONS

A 1000 μ M C-T ABA stock solution was made by dissolved C-T ABA in small amount of 95% EtOH, and then made up with culture medium to a final volume. The final concentration of EtOH in the stock solution was 10% (v/v). The same method was used to prepare 1000 μ M S-ABA, R-ABA and ABA-analog stock solutions, but the amount of each ABA-analog dissolved in solutions was modified with regard to the different molecular weight of the compound. The effect of 1-10% EtOH in the induction of desiccation tolerance in microspore-derived embryos was also tested.

Six ABA-analogs with three concentrations, 2, 10 and 50 μ M, were used to induce desiccation tolerance in microspore-derived embryos of *Brassica napus* and their biological-activities were investigated. Compared with S-ABA and R-ABA, P-89 and P-59 are a pair of enantiomers in which the functional group was changed in the C2', C3' double bond of the ring with a saturated ring. P-82 and P-185 are another pair of enantiomers in which its functional groups were changed in the C2', C3' double bond of the ring as P-89, P-59 and C4, C5 *trans*-double bond of the side chain with the triple bond. P-63 and P-51 are the third pair of enantiomers in which the functional groups were changed in the C2', C3' double bond of the ring. The C4, C5 *trans*-double-bond of the side chain as P-82 and P-185, and C1 acid was further replaced by a alcohol group. Among the six ABA-analogs, P-89, P-82 and P-63 are the S-ABA configuration, and P-59, P-185 and P-51 are the R-ABA configuration (shown in Figure 1).

The stock solutions were frozen at -20° C in the dark for no longer than one month if not used immediately. The purification of the stored compounds was monitored by HPLC during each experiment. This was done in Dr. S. Abrams's laboratory. This was to confirm that the chemicals used in the experiments were of the same purification (quality). Only the compounds in which purification was above 99% were used in the experiments.

F. ABA EXTRACTION FROM MICROSPORE-DERIVED EMBRYOS

The ABA extraction procedure followed that of Walker-Simmons (1987) and was modified to suit the microspore-derived embryo system. Microspore-derived embryos frozen in -80°C were lyophilized for 36 to 48 hours before extraction, and were powdered in liquid nitrogen. For extractions, 10 mg of powdered sample was weighed into a 1.5 mL Eppendorf tube, 1000 μL extraction buffer (shown in Appendix II) was added, and stirred overnight in dark (150 rpm, 25°C). To monitor the efficiency of each step of the purification process and the recovery of ABA during extraction, a series of authentic ABA standards were added to the original sample dilutions as the internal standards, and then the amounts of ABA measured by ELISA were checked and compared.

After incubation, samples were transferred to a microfuge, and centrifuged 15-20 min at 12,000 rpm with a Benchtop centrifuge. The supernatant was transferred to a fresh Eppendorf tube, and evaporated in a speed-Vac for 3 hours. The evaporated samples were resuspended with 100 μl methanol, and vortexed well. 900 μl TBS was added and the samples were vortexed well again. The resultant sample was filtered through a 0.2 μm nylon filter, and the filtrate was transferred to the upper reservoir of a Centricon 3 microconcentrator. It was spun at 7500x g, until only a thin film of liquid remained in the upper reservoir (2.5 hours). The endogenous S-ABA levels of the samples in the filtrate were determined by ELISA.

G. ABA ANALYSIS

i) Preparation of the ABA-C₄-ABH-BSA conjugate

ABA-4'-p-aminobenzoyl hydrazone (ABA-C₄-ABH) was prepared based on Quarrie and Galfre (1985). ABH was used as a bridge to connect ABA with BSA. ABA-C₄-ABH was kindly provided by Drs. Rose and Abrams, PBI, Saskatoon.

ABA-C₄-ABH crystal was resuspended in 200 μ L MeOH and 0.1 M 800 μ L HCL.

a. The following solutions were prepared and cooled to 0° C on ice.

1. BSA: 160 mg/8 mL 0.1 M BB was dialysed overnight against 0.25 M BB, pH 9.2.
2. NaNO₂: 25 mg per mL water (prepare fresh for steps b. and h.)
3. 500 μ l of ABA-C₄-ABH-HCl (1/2 of recipe).

(The following b through d was done on ice.)

b. 70 μ L NaNO₂ was added dropwise to 500 μ l ABA-C₄-ABH-HCL.

c. The sample was allowed to diazotize for 20 min.

d. The diazotized solution of ABA-C₄-ABH-HCl was added dropwise to 1.5 mL BSA in BB.

e. The sample was incubated overnight at 2° C in the dark.

f. The sample was then dialysed against 0.1 M BB pH 9.0 for 3 days, 4° C, in the dark (0.32 mL/cm #2 dialysis tubing in approximately 3.5 litres of buffer was used. The buffer was changed at 25 hour intervals).

(The following g through j was done on ice.)

- g. The remaining 500 μ L ABA-C₄-ABH-HCL was taken and added to ABA-C₄-ABH-BSA.
- h. 70 μ l NaNO₂ was added to the ABA-C₄-ABH-BSA solution (added dropwise).
- i. The solution was allowed to diazotize for 20 min.
- j. The diazotized solution was added to 1.5 mL BSA in 0.1 M BB (added dropwise).
- k. The solution was incubated overnight at 2° C in the dark.
- l. The solution was dialysed for 2 days against 5 litres PBS (1.7 mL/cm #1 dialysis tubing was used. The buffer was changed after 24 hours, using 2.5-3.0 litres of buffer each time.)
- m. The final product was frozen in 100 μ l aliquots in 0.5 mL Eppendorf tubes. The conjugate was stored at -20° C in the dark.

ii). Optimization

a. MAb titre

This is based on a ratio; K. Walker-Simmons (1987) suggested a ratio range of 5/60 to 25/60. Our final range was 12/60, 13/60, 14/60, 15/60, 16/60 and 17/60.

The numerator of the ratio is units (mL) of prepared monoclonal antibody to ABA. The denominator is total units (mL) of MAb diluted in TBS buffer (eg. 15 mL MAb + 45 mL TBS buffer).

MAb, 15-I-C5, used in this experiment is specific for S-ABA and does not cross-react

with the analogs used for immunoassays except P-89, which has a 75% cross-reaction with 15-I-C5 (Walker-Simmons et al., 1991). Natural ABA standards were incubated with each MAb titre, and an ELISA plate was run. The optimum titre produced a curve with a high value for the y-intercept when absorbency was plotted on the y-axis against the logarithm (base 10) of the concentration on the x-axis (shown in Figure 4). The log of 0 is infinity, so the 0 (zero) standards were deleted from the semi-log plot.

b. ABA-BSA conjugate

In a similar manner, the conjugate level was optimized. Suitable concentrations were used in the range of 15, 17, 19, 20 and 23 μ l conjugate per 20 mL 0.05 M NaHCO_3 solution and CB conjugate was used as positive control.

A range of standards incubated with MAb at its optimum titre was run on a plate which had each column at a different conjugate concentration. Plot absorbency (y-axis) against log conc. (x-axis) (shown in Figure 3). We chose a conjugate concentration which gave a linear fit curve over the range of the standards.

iii) Determination of the water content in microspore-derived embryos of *Brassica napus*

Samples for ELISA were collected and weighed at day 21 (for control only) and day 28 (for both control and treated), and the weights were measured again after lyophilization.

Figure 3. Conjugate Optimization

- 1: 15uL Conjugate/20mL TBS**
- 2: 17uL Conjugate/20mL TBS**
- 3: 20uL Conjugate/20mL TBS**
- 4: 19uL Conjugate/20mL TBS**
- 5: 23uL Conjugate/20mL TBS**
- 6: CB, positive control**

Conjugate Optimazation

Figure 4. MAb Optimization

1: 17mL MAb/60mL TBS

2: 16mL MAb/60mL TBS

3: 15mL MAb/60mL TBS

4: 14mL MAb/60mL TBS

5: 12mL MAb/60mL TBS

6: 13mL MAb/60mL TBS

MAb Optimization

MAB-1

The effects of ABA in the development of microspore-derived embryos were compared by measuring the changes of the dry weights and the percentage of the water in the embryos (shown in Table VI).

iv). Indirect ABA ELISA

The effects of ABA and ABA-analogs on the endogenous ABA pool can be measured by the indirect Enzyme-Linked Immunesorbent Assay method (Walker-Simmons, 1987). The indirect ELISA assay procedure, as currently established, is presented and has been optimised according to microspore-derived embryo requirements. All samples and solutions were kept in the dark during incubation.

a. Coating of wells with ABA-C₄-BSA conjugate.

A 17 μ L aliquot of ABA-C₄-BSA conjugate was added to 20 mL of 0.05 M NaHCO₃, pH 9.6. A 200 μ L aliquot of the diluted conjugate was added to each well of the microtitration plate, excluding the upper and lower rows. Plates were incubated at 4°C overnight with constant agitation at 100x rpm.

b. Preparation of ABA standards.

2.5 mg S-ABA was weighed and dissolved in 0.5 mL MeOH, then diluted with TBS buffer (shown in Table II of Appendix 1) to a precise volume of 50 mL. A 2 mL aliquot of the stock was filtered. Further dilutions were made of this filtered ABA stock solution

with TBS buffer to concentrations of 400 through 10 pg S-ABA per 100 μ L. These standards were made up ahead of time and fresh ABA standard dilutions were prepared once biweekly.

c. Preparation of Mab stock solution.

2 mg Monoclonal antibody (Idetek Inc.) was dissolved in 135 mL TBS buffer, containing 0.2% (W/V) BSA. Aliquots (3.0 mL) of this stock solution were stored at -25°C.

For each ELISA plate, MAb was further diluted with TBS buffer in the proportion 1 mL of MAb stock solution to 3 mL TBS buffer. Each new batch of Idetek MAb was calibrated.

d. Incubation of ABA samples with MAb.

A 350 μ l aliquot of the ABA standards or samples was pipetted into individual glass test tube. Then, 350 μ l of MAb solution was added and mixed in each tube. The solutions were incubated overnight at 4°C with constant agitation. This was enough material for three replicate well assays.

e. Addition of ABA samples incubated with MAb.

Plate wells coated with conjugate were washed three times with TBS washing buffer (shown in Table II of Appendix 1). The final washing solution was left in the plates for 10 min and then discarded. Then, 200 μ l aliquot of the samples incubated with MAb was

added to two replicate wells (three replicate wells for standards). Plates were incubated for 2 hours in the dark. This incubation and all the following steps were performed at room temperature.

f. Addition of the second antibody.

Wells were washed three times with washing TBS buffer. Rabbit antimouse alkaline phosphatase solution (200 μ l) was added to each well. This solution was a 1:1000 mL dilution into TBS buffer of a Sigma conjugate (Sigma A-1902). Plates were incubated for 2 hours in the dark.

g. Measurement of alkaline phosphatase.

Wells were washed three times with washing TBS buffer (as above). 200 μ L p-Nitrophenyl phosphate substrate (1 mg/mL in 0.05 M NaHCO₃ pH 9.6) was added to each well. Plates were incubated for around 1 h until the absorbance in the wells at 405 nm of control samples containing no ABA, was approximately 1.0. The incubation was stopped by adding 50 μ l 5 N KOH, and the sample absorbency measured at 405 nm with a Bio-Rad Enzyme Immunoassay Reader. The absorbency of the samples was inversely proportional to the amount of ABA in the original sample incubated with MAbs (Shown standard curve in Figure 5). Bio-Tek KinetiCalc EIA application software version 2.12 (BIO-TEKINSTRUMENTS, INC, 1992) was used at the above calculations.

Triplicate ABA standards were assayed for each plate. A linear regression analysis was

Figure 5. Standard curve for Indirect ELISA Quantitation

Standard Curve for Indirect ELISA Quantitation

computed using the ABA standards, and the amount of ABA in the plant extract samples calculated based on the coefficient of the ABA standard curve for each plate. All sample results were the average of three separate experiments, and each experiment sample was duplicated with three serial dilutions within the linear range of the ABA standard curve.

RESULTS

A. INITIAL RESULT TO ESTABLISH AN ABA CONCENTRATION RESPONSE CURVE USING TWO TYPES OF ABA ISOMERS, C-T ABA AND M-ABA, IN INDUCING DESICCATION TOLERANCE IN MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

Desiccated microspore-derived embryos (shown in Figure 9) could survive after being stored for several weeks at room temperature, 43% relative humidity in the dark. But for this survival, an ABA treatment was necessary before the embryos were desiccated. Observations confirm that the shoot meristems appear to be more sensitive to desiccation damage than root meristems (Figures 7,8 and 9). ABA treatment at concentrations of 10 μ M gave the highest viability of the desiccated embryos and, at this optimum concentration, C-T ABA gave a better result than M ABA (shown in Figure 8). C-T or M ABA, at a concentration of 1000 μ M, appears to be toxic as all of the embryos turned dark and died; while at 100 μ M, only a few of the embryos turned dark and those which did not turn dark survived. When the ABA concentration was lowered to 0.1 μ M, there was no a obvious effect on embryo survival after desiccation (shown in Figures 7, 8 and 9). This agrees with the results obtained previously (Brown et al., 1993). ABA treatment stimulated normal development of the embryos without formation of secondary embryos. A range of concentrations for S-ABA and R-ABA from 0.1-100 μ M were then used.

Figure 6. Embryo Development After Treatment with C-T and M ABA

Microspore-derived embryos were treated with C-T and M ABA for 7 days and germinated and regenerated directly on B₅-0 solid medium as described in "Materials and Methods". Only those embryos that showed both shoots, which developed a true leaf, and roots, which emerged at least 5 mm, were scored as survivors after 14 days development.

Values represent the means of at least three separate experiments, there were three duplicates for each experiment. Vertical bars show $\pm$ SD.

EMBRYO SURVIVAL 14 DAYS DEVELOPMENT

Figure 7. Embryo Root Survival After Treatment with C-T and M ABA

Microspore-derived embryos were treated with C-T and M ABA for 7 days and then were desiccated and rehydrated as described in "Materials and Methods". Only those embryos that showed roots, which emerged at least 5 mm, were scored as root survivors after 14 days rehydration.

Values represent the means of at least three separate experiments, there were three duplicates for each experiment. Vertical bars show $\pm$ SD.

EMBRYO SURVIVAL

14 DAYS AFTER REHYDRATION (ROOT)

Figure 8. Embryo Survival After Treatment with C-T and M ABA

Microspore-derived embryos were treated with C-T and M ABA for 7 days and then were desiccated and rehydrated as described in "Materials and Methods". Only those embryos that showed both shoots, which developed a true leaf, and roots, which emerged at least 5 mm, were scored as survivors after 14 days rehydration.

Values represent the means of at least three separate experiments, there were three duplicates for each experiment. Vertical bars show $\pm$ SD.

EMBRYO SURVIVAL 14 DAYS AFTER REHYDRATION

Figure 9. *In vitro* Microspore-derived Embryo and Embling Morphology

- A.**
1. 21-day-old microspore-derived embryos of *Brassica napus*;
 2. 28-day-old microspore-derived embryos of *Brassica napus*;
 3. 28-day-old microspore-derived embryos of *Brassica napus* before desiccation;
 4. Desiccated microspore-derived embryos of *Brassica napus*.
- B.** Treated and desiccated microspore-derived embryos, 14 days after rehydration:
1. Untreated and desiccated control embryos;
 2. Treated with 0.1 μ M C-T ABA for 7 days;
 3. Treated with 1 μ M C-T ABA for 7 days;
 4. Treated with 10 μ M C-T ABA for 7 days;
 5. Treated with 100 μ M C-T ABA for 7 days;
 6. Treated with 1000 μ M C-T ABA for 7 days;
- C.** Treated and undesiccated microspore-derived embryos, 14 days after development:
1. Untreated and undesiccated embryos;
 2. Embryos treated with 0.001% EtOH for 7 days;
 3. Embryos treated with 0.01% EtOH for 7 days;
 4. Embryos treated with 0.1% EtOH for 7 days;
 5. Embryos treated with 1% EtOH for 7 days;
 6. Embryos treated with 10% EtOH for 7 days.

Arrows show secondary embryos and callus.

B. EFFECT OF EtOH IN EMBRYO DEVELOPMENT, REGENERATION AND INDUCING DESICCATION TOLERANCE OF MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

EtOH was used to dissolve the ABA and ABA-analogs. As a solvent, EtOH was 1% (v/v) in a 100 μ M ABA solution. The effects of EtOH on the development, regeneration and induction of desiccation tolerance in microspore-derived embryos of *Brassica napus* were also studied (Figure 10). The highest EtOH concentration (10% v/v) appeared to inhibit embryo growth and development but stimulated the embryo conversion on the regeneration B₅-O medium without occurrence of secondary embryos (shown in Figure 9).

The effect of EtOH in induction of desiccation tolerance of microspore-derived embryos was also tested. EtOH itself was not effective in inducing embryo desiccation tolerance in the above range (results not shown). In the beginning of the experiment, some of the untreated control embryos and EtOH treated embryos survived after desiccation, perhaps because the embryos were too large to be desiccated to less than 20% moisture using the RH levels and timing used during the desiccation protocol.

Figure 10. Effect of EtOH in Development of Microspore-derived Embryos of *Brassica napus*

Microspore-derived embryos were treated with EtOH for seven days then regenerated directly on B5-0 solid medium as described in "Materials and Methods". Only those embryos that showed both shoots, which developed a true leaf, and roots, which emerged at least 5 mm, were scored as survivors after 14 days development.

Values represent the means of at least three separate experiments, there were three duplicates for each experiment. Vertical bars show $\pm$ SD.

EFFECTS OF EtOH 14 DAYS DEVELOPMENT

C. EFFECT OF CONCENTRATION OF OPTICALLY PURE S-ABA OR R-ABA IN INDUCING DESICCATION TOLERANCE IN MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

Based on the results from part A, four concentrations, 0.1, 1, 10 and 100 μ M, of (S)-ABA and (R)-ABA were used to treat the microspore-derived embryos and draw accurate responsive curves of the embryo survival after desiccation. The results are shown in Figures 11 and 12.

Both S-ABA and R-ABA were effective in inducing desiccation tolerance in microspore-derived embryos in the range of concentrations from 0.1-100 μ M. In contrast with C-T and M ABA, in the concentration range between 10-100 μ M, both S- and R-ABA did not turn the embryos dark and resulted in the highest embryo survival. However, survival of S-ABA-treated embryos is higher than R-ABA at these concentrations (shown in Figure 11). In lower concentrations (0.1-1 μ M), R-ABA was more effective (77% and 59%) than S-ABA (56% and 26%). To determine the effective range of concentrations of ABA and the threshold of the concentrations which can be used to induce the survival of embryos after desiccation, the S- and R-ABA concentrations were lowered to 0.01-0.0001 μ M. In these concentrations, survival rates of the desiccated embryos dropped fast. All of the embryos treated with S-ABA died after desiccation except a small percentage of the 0.01 μ M of R-ABA-treated embryos which survived (shown in Figure 11).

Figure 11. Embryo Survival After Treatment with S-ABA and R-ABA

Microspore-derived embryos were treated with S-ABA and R-ABA for 7 days, desiccated and rehydrated as described in "Materials and Methods". Only those embryos that showed both shoots, which developed a true leaf, and roots, which emerged at least 5 mm, were scored as survivors after 14 days rehydration.

Values represent the means of at least three separate experiments, there were three duplicates for each experiment. Vertical bars show $\pm$ SD.

EMBRYO SURVIVAL

14 DAYS AFTER REHYDRATION

Figure 12. Emblings Recovery From Desiccated Microspore-derived Embryos of *Brassica napus*

A. Treated and desiccated microspore-derived embryos, 14 days after rehydration:

1. Untreated and desiccated control embryos;
2. Embryos treated with 0.1 μ M S-ABA for 7 days;
3. Embryos treated with 1 μ M S-ABA for 7 days;
4. Embryos treated with 10 μ M S-ABA for 7 days;
5. Embryos treated with 100 μ M S-ABA for 7 days.

B. Treated and desiccated microspore-derived embryos, 14 days after rehydration:

1. Untreated and desiccated control embryos;
2. Embryos treated with 0.1 μ M R-ABA for 7 days;
3. Embryos treated with 1 μ M R-ABA for 7 days;
4. Embryos treated with 10 μ M R-ABA for 7 days;
5. Embryos treated with 100 μ M R-ABA for 7 days.

C. Treated and desiccated microspore-derived embryos, 14 days after rehydration:

1. Untreated and desiccated control embryos;
2. Embryos treated with 2 μ M P-89 for 7 days;
3. Embryos treated with 10 μ M P-89 for 7 days;
4. Embryos treated with 50 μ M P-89 for 7 days.

D. Treated and desiccated microspore-derived embryos, 14 days after rehydration:

1. Untreated and desiccated control embryos;
2. Embryos treated with 2 μ M P-82 for 7 days;
3. Embryos treated with 10 μ M P-82 for 7 days;
4. Embryos treated with 50 μ M P-82 for 7 days.

E. Treated and desiccated microspore-derived embryos, 14 days after rehydration:

1. Untreated and desiccated control embryos;
2. Embryos treated with 2 μ M P-185 for 7 days;
3. Embryos treated with 10 μ M P-185 for 7 days;
4. Embryos treated with 50 μ M P-185 for 7 days.

D. EFFECT OF OPTICALLY PURE ABA-ANALOGS IN INDUCING DESICCATION TOLERANCE IN MICROSPORE-DERIVED EMBRYOS OF *BRASSICA NAPUS*

Compared with S-ABA, alternation of the C2', C3' double bond in S-ABA (P-89) does not reduce the biological activity very much in inducing the desiccation tolerance in microspore-derived embryos. However, in the R-ABA configuration (P-59), the activity of the compound is reduced more than 60% at the concentration of 1 μ M (Figure 13). When the altered structures were changed again to replace the C4, C5 *trans* double-bond with a triple bond (P-82 and P-185) and the C1 acid was replaced with a C1 alcohol (P-63 and P-51), the results presented in Figures 14 and 15 showed that these two pairs of enantiomers, P-82 and P-185, and P-63 and P-51, had similar results in inducing desiccation tolerance as P-89 and P-59 which were only altered in C2', C3' double bond.

The three (S)-ABA-analogs showed higher, stable and similar effects in inducing desiccation tolerance as S-ABA in the three concentrations. However, the best results in inducing desiccation tolerance with these (S)-ABA-analogs required a concentration five times higher than that needed for S-ABA. P-185, the enantiomer of P-82, had as high as and, a similar effect as P-82 at the concentration of 50 μ M, but the effect dropped rapidly when the concentration was reduced to 2 μ M (shown in Figure 14). Another two (R)-ABA-analogs, P-59 and P-51 had less than half of the promotive effect of S-ABA at the concentration of 10 μ M. These three (R)-ABA-analogs were very ineffective in inducing

desiccation tolerance in microspore-derived embryos at concentrations below 2 μ M
(Shown in Figures 13 and 15).

Figure 13. Embryo Survival After Treatment with P-89 and P-59

Microspore-derived embryos were treated with P-89 and P-59 for 7 days, desiccated, and rehydrated as described in "Materials and Methods". Only those embryos that showed both shoots, which developed a true leaf, and roots, which emerged at least 5 mm, were scored as survivors after 14 days rehydration.

Values represent the means of at least three separate experiments, there were three duplicates for each experiment. Vertical bars show $\pm$ SD.

EMBRYO SURVIVAL 14 DAYS AFTER REHYDRATION

Figure 14. Embryo Survival After Treatment with P-82 and P-185

Microspore-derived embryos were treated with P-82 and P-185 for 7 days, desiccated, and rehydrated as described in "Materials and Methods". Only those embryos that showed both shoots, which developed a true leaf, and roots, which emerged at least 5 mm, were scored as survivors after 14 days rehydration.

Values represent the means of at least three separate experiments, there were three duplicates for each experiment. Vertical bars show $\pm$ SD.

EMBRYO SURVIVAL 14 DAYS AFTER REHYDRATION

2 of/de 2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Figure 15. Embryo Survival After Treatment with P-63 and P-51

Microspore-derived embryos were treated with P-63 and P-51 for 7 days, desiccated, and rehydrated as described in "Materials and Methods". Only those embryos that showed both shoots, which developed a true leaf, and roots, which emerged at least 5 mm, were scored survivors after 14 days rehydration.

Values represent the means of at least three separate experiments, there were three duplicates for each experiment. Vertical bars show $\pm$ SD.

EMBRYO SURVIVAL 14 DAYS AFTER REHYDRATION

E. COMPARISON OF VIGOUR OF EMBLINGS OF DESICCATED MICROSPORE-DERIVED EMBRYOS AND SEEDLINGS OF SEEDS OF *BRASSICA NAPUS* CV. TOPAS.

Effects of ABA and ABA-analogs in inducing desiccation tolerance in microspore-derived embryos were tested (shown in Figure 11-15). Although embryo survival rates indicated that S-, R-ABA and S-configuration ABA-analogs were very effective in inducing desiccation tolerance, results also indicated that the vigour of the emblings of various treated embryos were very different (shown in Table III and Figure 12). Compared with the seedlings of seeds, emblings from treated and desiccated microspore-derived embryos were obviously smaller during the same developmental period. Among the desiccated microspore-derived embryos, S-ABA treated embryos showed the best results with respect to the vigour of the emblings at high concentrations from 10-100 μ M, and R-ABA treated embryos showed better results than S-ABA treated embryos at lower concentrations from 0.1-1 μ M. Both the embryo survival rate and the vigour of the emblings from the desiccated embryos treated at concentration 0.1 μ M of S-ABA dropped very fast (Table III and Figure 11). The vigour of the emblings from (S)-ABA-analogs treated embryos was lower than that of the emblings from S-ABA treated embryos. Regeneration observations confirmed these results (data not shown). Most of the emblings from untreated 28-day-old embryos developed abnormally with many secondary embryos and callus formation (shown in Figure 9).

**TABLE III. VIGOUR OF EMBLINGS OF DESICCATED MICROSPORE-
DERIVED EMBRYOS AND SEEDLINGS OF SEEDS OF BRASSICA NAPUS CV.
TOPAS MEASURED 14 DAYS AFTER REHYDRATION**

a: Microspore-derived embryos were treated and desiccated as described in "Materials and Methods", shoots were measured 14 days after rehydration.

b: 28-day-old untreated microspore-derived embryos were regenerated directly on B₅-0 medium and measured 14 days after rehydration. Arrows (Figure 9 and 16) show the secondary embryos and callus.

c. Seeds were germinated on B₅-0 medium and measured 14 days after rehydration.

Values represent the means of at least 15 emblings or seedling $\pm$ standard deviation

Treatment	Concentration (μ M)	Shoot Length ^a (cm)	Treatment	Concentration (μ M)	Shoot Length ^a (cm)
P-58	0.1	0.78 $\pm$ 0.25	P-145	0.1	1.33 $\pm$ 0.28
	1	1.20 $\pm$ 0.20		1	1.95 $\pm$ 0.49
	10	2.03 $\pm$ 0.30		10	1.63 $\pm$ 0.36
	100	2.82 $\pm$ 0.26		100	2.35 $\pm$ 0.29
P-89	2	0.98 $\pm$ 0.18	P-59	2	0.28 $\pm$ 0.12
	10	1.25 $\pm$ 0.71		10	0.67 $\pm$ 0.34
	50	1.05 $\pm$ 0.36		50	0.95 $\pm$ 0.33
P-82	2	1.02 $\pm$ 0.29	P-185	2	0.60 $\pm$ 0.30
	10	1.12 $\pm$ 0.19		10	0.68 $\pm$ 0.41
	50	1.05 $\pm$ 0.21		50	1.30 $\pm$ 0.33
D-28 ^b	0	1.97 $\pm$ 0.39	Seeds ^c	0	8.53 $\pm$ 0.64

F. EFFECT OF ABA METABOLIC PRODUCTS, PHASEIC ACID, AND T-T ABA IN INDUCING DESICCATION TOLERANCE IN MICROSPORE-DERIVED EMBRYOS OF *BRASSICA NAPUS*

In the preceding experiments, the use of S-ABA, R-ABA, C-T ABA, M-ABA and six other ABA-analogs, PBI-89, 59, 82, 185, 63 and 51, to induce the desiccation tolerance in microspore-derived embryos of *Brassica napus* was tested. The response was found to be dependent on ABA or ABA-analog concentration and structure. At a concentration of 10 μ M, all of the compounds were effective.

In order to further understand and examine the induction requirement of desiccation tolerance and test the effects of ABA metabolic products in inducing desiccation tolerance in microspore-derived embryos, two compounds, P-240 and T-T ABA were selected as potential negative controls. Phaseic acid, P-240, which is the primary metabolic product of S-ABA, was found to be active (Ho, 1979; Nolan and Ho, 1988) or inactive (Gusta, 1990) in different species. +*Trans-Trans* ABA, which is an isomer of S-ABA, can be easily converted by S-ABA to 1:1 mixture of S-ABA:+T-T ABA under light conditions (Dorffling and Tietz, 1983). In the results of part A, it was found that the commercially available M ABA, was not as effective as C-T ABA, possibly because it contained a high level $\pm$ T-T ABA. It was suspected that $\pm$ T-T ABA was not active in inducing desiccation tolerance.

Three concentrations, 1, 10 100 μ M were used in these experiments. In the previous results, these three concentrations were among those tested and were considered to be the optimum concentrations to induce desiccation tolerance.

Embryos treated with three concentrations of P-240 and $\pm$ T-T ABA for one week, without desiccation, were regenerated directly in B5-0 medium. After 7 days development, the embryos treated with high concentrations of P-240 and +T-T ABA (100 μ M was shown to be toxic to embryos) were obviously smaller and paler than embryos treated with concentrations 1 and 10 μ M of P-240 and $\pm$ T-T ABA and the control samples, which were not treated (shown in figure 17). Most of the desiccated embryos treated with a high concentration of P-240 and $\pm$ T-T ABA (100 μ M) had turned dark, especially P-240 treated embryos, after rehydration (data not shown). Only about 5% of desiccated embryos treated with the high concentration of (100 μ M) P-240 survived (shown in Table IV). P-240 and $\pm$ T-T ABA in the above range of concentrations had no effect in inducing desiccation tolerance in microspore-derived embryos (shown in Table IV).

Figure 16. Effect of C-T ABA, Phaseic Acid and $\pm$ T-T ABA In Microspore-derived Embryos of *Brassica napus*

A. Treated and undesiccated microspore-derived embryos, 14 days after development:

1. Untreated and undesiccated embryos;
2. Embryos treated with 0.1 μ M C-T ABA for 7 days;
3. Embryos treated with 1 μ M C-T ABA for 7 days;
4. Embryos treated with 10 μ M C-T ABA for 7 days;
5. Embryos treated with 100 μ M C-T ABA for 7 days;
6. Embryos treated with 1000 μ M C-T ABA for 7 days.

Arrows show secondary embryos and callus.

B. Treated microspore-derived embryos:

1. Untreated 28-day old embryos;
2. Embryos treated with 0.1 μ M C-T ABA for 7 days;
3. Embryos treated with 1 μ M C-T ABA for 7 days;
4. Embryos treated with 10 μ M C-T ABA for 7 days;
5. Embryos treated with 100 μ M C-T ABA for 7 days;
6. Embryos treated with 1000 μ M C-T ABA for 7 days.

C. Treated and undesiccated microspore-derived embryos, 7 days after development:

1. Untreated and undesiccated embryos;
2. Embryos treated with 1 μM $\pm$ T-T ABA for 7 days;
3. Embryos treated with 10 μM $\pm$ T-T ABA for 7 days;
4. Embryos treated with 100 μM $\pm$ T-T ABA for 7 days.

D. Treated and undesiccated microspore-derived embryos, 7 days after development:

1. Untreated and undesiccated embryos;
2. Embryos treated with 1 μM phaseic acid for 7 days;
3. Embryos treated with 10 μM phaseic acid for 7 days;
4. Embryos treated with 100 μM phaseic acid for 7 days.

TABLE IV. EFFECTS OF $\pm$ T-T ABA OR PHASEIC ACID IN INDUCING DESICCATION TOLERANCE IN MICROSPORE-DERIVED EMBRYOS OF *BRASSICA NAPUS*

Embryos were treated with $\pm$ T-T ABA or phaseic acid (P-240) for 7 days and desiccated to 43% RH in a series of desiccators. The results were scored 14 days after rehydration in B5-0 medium.

a: Not recorded; all of the control embryos died after desiccation.

The results presented are the sum of three separate experiments and in each experiment at least 25 embryos were used.

	Concentration (uM)	Embryo Total	Embryo Survival
$\pm$ T-T ABA	2	91	0
	10	86	0
	50	90	0
Phaseic Acid	2	93	0
	10	90	0
	50	78	4
Control	0	N ^a	0

G. EFFECT OF EXPOSURE TIME TO OPTIMUM CONCENTRATION OF S-ABA OR R-ABA IN INDUCING DESICCATION TOLERANCE OF *BRASSICA NAPUS*

When 21-day-old microspore-derived embryos were treated with concentrations of 10 μ M S-ABA and R-ABA for 7 days, over 85% of the embryos survived desiccation. In order to clarify the relationship between the exposure time course and desiccation tolerance, embryos were treated by S-ABA and R-ABA with the optimum concentration (10 μ M) for 1, 2 and 24 hours to induce desiccation tolerance.

Results shown in Table V indicated that, during 24 hours of treatment, embryos began to acquire tolerance to desiccation although the embryo survival rates were not high. At this stage, S-ABA and R-ABA had similar effects. With shorter times, 1 or 2 hours, embryos could not be induced to acquire desiccation tolerance.

TABLE V. EFFECT OF EXPOSURE TIME TO OPTIMUM CONCENTRATION S-ABA OR R-ABA IN INDUCING DESICCATION TOLERANCE IN MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

21-day-old embryos were cultured, treated and desiccated as outlined in "Materials and Methods".

Exposure Length	S-ABA (Survival ^a %)	R-ABA (Survival %)
1 hour	0	0
2 hours	0	0
24 hours	15.6±0.3	17.2±1.1

a: Only the embryos that showed both a shoot, which developed a true leaf, and a root, which emerged at least 5 mm, were scored as survivors.

Values represent the means of three separate experiments ± standard deviation.

H. EFFECT OF ABA IN THE DEVELOPMENT AND CONVERSION OF MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS CV. TOPAS

ABA is considered to be effective in promoting plant development and seed maturation. In this experiment, 21-day-old microspore-derived embryos were treated at concentrations from 0.0001 to 100 μ M of C-T ABA for 7 days and then regenerated on B₅-0 medium. 28-day-old untreated microspore-derived embryos were used as controls. Results indicated that ABA treatment inhibited embryo development, but could stimulate the microspore-derived embryo conversion and regeneration. On B₅-0 medium, the embryos treated with the concentrations from 1-100 μ M of C-T ABA converted and regenerated normally, and the embryos treated at concentrations below 1 μ M and the untreated embryos had been found to form many secondary embryos and callus (shown in Figure 9). ABA appears to be a toxic to the embryos at the highest concentration (1000 μ M). At this concentration, all of the embryos turned dark and died after 7 days treatment. ABA also inhibited embryo development; the size of the embryos treated with ABA was smaller than the same stage of untreated embryos (Figure 16). In this experiment, the dry weight of the embryos was also measured at 1, 3 and 7 days of the treatment. Results indicated that the ABA treatment did not increase the dry weight of the embryos as compared to the same stage of untreated embryos (shown in Table VI).

TABLE VI. WATER CONTENT OF NORMAL DEVELOPING AND ABA TREATED MICROSPORE-DERIVED EMBRYO OF BRASSICA NAPUS

Embryo Age (day)	D.W/Embryo ^a (mg)	Moisture (%)
22	0.242±0.008	75.83±0.8
28	0.288±0.021	71.11±3.41
21+1/ABA (10 µM)	0.242±0.013	75.85±1.34
21+3/ABA (10 µM)	0.244±0.005	75.61±0.5
21+7/ABA (10 µM)	0.277±0.0351	72.32±3.51

a: Microspore-derived embryos were treated and collected as described in "Materials and Methods" and the collected samples were immediately frozen in liquid nitrogen. Samples were measured after lyophilization for 36 to 48 hours.

Values represent the means of six separate experiments ± standard deviation.

I. OPTIMIZATION OF MONOCLONAL ANTIBODY AND CONJUGATE

1. Optimization of the Conjugate

Before use, the conjugate concentration was first optimized. In constant MAb, the results for various concentrations of conjugate are presented in Figure 3.

Two concentrations, 17 μ L/20mL and 23 μ L/20mL, and a control sample (CB, positive control, from PBI, Saskatoon) showed the best R Squared results, 0.9645, 0.9699 and 0.9608. R Squared, or variance, is a statistic that shows the strength of the relationship between the independent and dependent variables (Quattro Pro, 1993). It ranges from 0 to 1, where 1 shows a perfect relationship. Another related factor is the Constant, which is the y-axis intercept of the regression. A concentration of 17 μ L/20mL showed the highest Constant result of 1.534. This concentration was selected for the experiments.

2. Optimization of the Monoclonal Antibody

Using a similar method as above, the optimum concentrations of MAb were selected under constant and optimum concentration of conjugate. For both, the first and the second batch of MAb, the concentrations of 15 mL/60 mL showed the best R-Squared results (0.9824 and 0.9501), the highest Constant results (1.6427 and 1.1239) and were used in the experiments (shown in Figure 4).

J. EFFECT OF EXOGENOUS APPLIED ABA OR ABA-ANALOGS ON THE ENDOGENOUS ABA POOL IN MICROSPORE-DERIVED EMBRYOS OF *BRASSICA NAPUS*

In the previous experiments on desiccation tolerance and results from other research papers, it was found that the embryos treated with effective compounds for three to seven days showed the best survival results after desiccation (Brown et al., 1993). In order to further understand the relationship between exogenous applied ABA and ABA-analogs and the endogenous ABA level, ABA and ABA-analogs and desiccation tolerance, and if the applied ABA-analogs were converted to S-ABA in embryo, embryos treated with ABA or ABA-analogs at various concentrations and times. The times selected were:

- a) day one, which is the earliest stage for treated embryos to acquire desiccation tolerance,
- b) day three, which is the middle stage for treated embryos to obtain desiccation tolerance, and
- c) day seven, at which treated embryos showed the best survival results after desiccation.

Embryos were collected and tested for endogenous ABA content by indirect ELISA as outlined in the "Materials and Methods". Known quantities of authentic ABA were added to appropriate extract dilutions and the added ABA was measured by ELISA. Recoveries of 87.5% (SD, 4%) ABA were obtained for the ABA added to the extracts.

i) Control Samples

Two untreated samples, day-21 (c21), which was the first day that the embryos were treated with exogenous ABA, and day-28 (c28), which is the last day when the treated embryos were collected, were used as the controls of endogenous ABA level in microspore-derived embryos. The endogenous ABA level at c21 was found to be around 0.3 μ M and at c28 was around 0.4 μ M. These concentrations were considered to be the basic and natural levels for normal developing microspore-derived embryos. The ABA level in c21 samples was 25-30% lower than in c28 samples (shown in Table VII). At day-21, most of the embryos were in torpedo and early cotyledon stages and at day-28 most of the embryos were in the cotyledon stage (shown in Figure 9). Embryos in the cotyledon stage had higher levels of ABA than the embryos in earlier stages of development.

ii) Samples Treated With Natural (S) ABA

Embryos were treated by S-ABA at seven different concentrations from 0.0001-100 μ M and collected after 1 day (d1), 3 days (d3) and 7 days (d7). The trends show that, at high treatment concentrations from 0.1-100 μ M, the endogenous ABA levels at d1 were higher than d3s' and d3s' were higher than d7s'. At lower concentrations from 0.0001-0.01 μ M, the endogenous ABA levels at d1 were lower than d3s' and d3s' were lower than d7s'. In the lower concentrations, the endogenous ABA levels were similar to endogenous ABA levels from the same stage of untreated developing microspore-derived embryos.

The endogenous ABA levels from 0.0001-0.01 μ M ABA treated samples appeared not to be changed from the same stage c21 or c28 samples. But ABA levels of the samples treated by higher concentrations of ABA from 0.1-100 μ M were increased from 1-100 fold depending on the concentrations of applied ABA (shown in Table VII).

From the above results, the following trends were drawn:

- 1) The initial uptake of the exogenous applied ABA by the embryos was very fast, however the taken up ABA was gradually metabolised by the embryos;
- 2) The embryos can regulate their own ABA levels; during the embryo development, they gradually accumulate a certain amount of endogenous ABA. The ABA levels of untreated developing embryos are around 0.3 μ M at day 21 and 0.4 μ M at day 28;
- 3) The normal ABA levels at day 21 and day 28 of the embryo development appear to be too low to induce the desiccation tolerance. Thus, the control embryos at day 21 and day 28 did not survive after desiccation;
- 4) The concentrations of exogenous applied ABA below the endogenous ABA level of the embryos appear not to affect the endogenous ABA level.

Previous experimental observations also confirmed the above results. With concentrations 0.1-1 μ M of applied S-ABA, embryos started to gain desiccation tolerance, but the endogenous ABA pool increased only 1 fold presumably because most of the applied ABA was metabolised by the embryos. When the concentrations of the applied exogenous ABA were up to 10-100 μ M, over 95% of treated embryos could survive the desiccation

TABLE VII. EFFECT OF S-ABA ON ENDOGENOUS ABA CONTENT IN MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

Embryo samples were treated, collected and analyzed as outlined in "Materials and Methods".

Values represent the means of at least three separate experiments $\pm$ standard deviation.

There were duplicates with three dilutions in each experiment.

a: Not Measured.

Treatment	Concentration (μ M)	S-ABAContent ng/g (DW) (1 day)	S-ABAContent ng/g (DW) (3 day)	S-ABAContent ng/g (DW) (7 day)
S-ABA	100	21000 $\pm$ 5500	11000 $\pm$ 2100	2300 $\pm$ 800
	10	1500 $\pm$ 400	1460.0 $\pm$ 520	1300 $\pm$ 500
	1	690 $\pm$ 88	510 $\pm$ 68	480 $\pm$ 37
	0.1	330 $\pm$ 106	370 $\pm$ 54	280 $\pm$ 58
	0.01	300 $\pm$ 3	270 $\pm$ 72	400 $\pm$ 137
	0.001	200 $\pm$ 63	200 $\pm$ 27	300 $\pm$ 44
	0.0001	370 $\pm$ 171	300 $\pm$ 133	300 $\pm$ 78
Control	medium	250 $\pm$ 74	NM ^a	330 $\pm$ 97

treatment and the endogenous ABA levels increased 5 to 100 fold.

iii) Samples Treated With Unnatural (R) ABA

The same concentrations from 0.0001-100 μ M of R-ABA as S-ABA were used to treat the microspore-derived embryos. Indirect ELISA assay indicated (shown in Table VIII) that endogenous ABA levels did not increase at low concentrations from 0.0001-1 μ M of the applied R-ABA. When the concentrations of applied R-ABA were increased to 10-100 μ M, the endogenous ABA levels began to increase; but, only 0.5 - 2 fold. The results also indicated that with high applied concentrations of R-ABA, from 1-100 μ M, endogenous ABA levels of the embryos treated for 7 days were higher than in the embryos treated for 1 day.

iv) Three concentrations (2, 10 and 50 μ M) of ABA-analogs were used to induce desiccation tolerance in microspore-derived embryos and samples were analyzed by ELISA.

a. Samples treated with P-89 and P-59

Embryos treated with the above concentrations of P-89 could induce desiccation tolerance to a slightly lower degree than S-ABA at the same concentrations. This was the only compound used which showed a cross-reaction with the S-ABA-derived monoclonal antibody 15-I-C5 (Walker-Simmons et al., 1991). The indirect ELISA indicated that, with applied P-89 at the above three concentrations, the endogenous ABA levels of the samples

TABLE VIII. EFFECT OF R-ABA ON ENDOGENOUS ABA CONTENT IN MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

Embryo samples were treated, collected and analyzed as outlined "Materials and Methods".

Values represent the means of at least three separate experiments $\pm$ standard deviation.

There were duplicate samples with three dilutions in each experiment.

Treatment	Concentration (μ M)	S-ABA Content ng/g (DW) (1 day)	S-ABA Content ng/g (DW) (3 day)	S-ABA Content ng/g (DW) (7 day)
R-ABA	100	440 $\pm$ 10	350 $\pm$ 29	670 $\pm$ 190
	10	340 $\pm$ 56	500 $\pm$ 120	590 $\pm$ 184
	1	290 $\pm$ 134	330 $\pm$ 142	320 $\pm$ 80
	0.1	250 $\pm$ 46	270 $\pm$ 50	200 $\pm$ 44
	0.01	220 $\pm$ 81	190 $\pm$ 22	250 $\pm$ 56
	0.001	220 $\pm$ 53	220 $\pm$ 46	220 $\pm$ 80
	0.0001	290 $\pm$ 67	270 $\pm$ 80	240 $\pm$ 107

were much higher than at the same level of applied P-89 (data not shown) because the measured ABA levels were the total levels of S-ABA and the taken up P-89.

P-59, the enantiomer of P-89, was not as effective as P-89 in inducing desiccation tolerance at a concentration of 10 μ M. The indirect ELISA showed that with applied 10 μ M of P-59, the endogenous ABA level increased 2 fold (shown in Table IX).

b. Samples treated with P-82 and P-185

PBI-82 was as effective as S-ABA in inducing desiccation tolerance, but P-185 was not as effective as S-ABA at lower concentrations (2-10 μ M). The indirect ELISA showed (shown in Table X) that applied P-82 did not result in an increase of endogenous ABA. The endogenous ABA level showed a slight decrease when P-82 was applied at 2-10 μ M. The results with applied P-185 indicated that endogenous ABA levels changed in a similar manner to those as with applied P-82. At this point, both P-82 and P-185 appear not to be converted to S-ABA in microspore-derived embryos of *Brassica napus*.

c. Samples treated with P-63 and P-51

P-63 and P-51 had similar results to P-89 and P-59 in inducing desiccation tolerance but showed different effects on the endogenous ABA levels (Shown in Table XI). With applied P-63, at concentrations of 10-50 μ M, the endogenous ABA increased 2 times. With applied P-51, at concentrations from 10-50 μ M, endogenous ABA levels increased only 0.5-1 times over the control. It seems that both P-63 and P-51 were not converted

TABLE IX. EFFECT OF P-59 ON ENDOGENOUS ABA CONTENT IN MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

Embryos samples were treated, collected and analyzed as outlined in the "Materials and Methods".

Values represent the means of at least three separate experiments $\pm$ standard deviation.

There were duplicates with three dilutions in each experiment.

Treatment	Concentration (μ M)	S-ABA Content ng/g (DW) (1 day)	S-ABA Content ng/g (DW) (3 day)	S-ABA Content ng/g (DW) (7 day)
P-59	2	260 $\pm$ 70	380 $\pm$ 130	150 $\pm$ 25
	10	220 $\pm$ 75	230 $\pm$ 70	220 $\pm$ 60
	50	1800 $\pm$ 1300	1700 $\pm$ 780	1300 $\pm$ 940

to S-ABA in microspore-derived embryos of *Brassica napus*.

v) Samples treated with metabolic products of ABA, phaseic acid, and $\pm$ T-T ABA

Both P-240 and $\pm$ T-T ABA had a negative effect on induction of desiccation tolerance in microspore-derived embryos of *Brassica napus*. ELISA analysis indicated that application of PA could not increase the endogenous ABA levels but decreased the levels 10-50% (shown in Table XII).

TABLE X. EFFECT OF P-82 OR P-185 ON ENDOGENOUS ABA CONTENT IN MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

Embryo samples were treated, collected and analyzed as outlined in the "Materials and Methods".

Values represent the means of at least three separate experiments $\pm$ standard deviation.

There were duplicates with three dilutions in each experiment.

Treatment	Concentration (μ M)	S-ABA Content ng/g (DW) (1 day)	S-ABA Content ng/g (DW) (3 day)	S-ABA Content ng/g (DW) (7 day)
P-82	2	110 $\pm$ 13	120 $\pm$ 5	130 $\pm$ 28
	10	170 $\pm$ 28	150 $\pm$ 14	190 $\pm$ 63
	50	220 $\pm$ 11	260 $\pm$ 64	390 $\pm$ 160
P-185	2	200 $\pm$ 47	110 $\pm$ 22	200 $\pm$ 52
	10	250 $\pm$ 105	300 $\pm$ 74	190 $\pm$ 48
	50	250 $\pm$ 81	240 $\pm$ 96	210 $\pm$ 57

TABLE XI. EFFECT OF P-63 OR P-51 ON ENDOGENOUS ABA CONTENT IN MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

Embryo samples were treated, collected and analyzed as outlined in the "Materials and Methods".

Values represent the means of at least three separate experiments $\pm$ standard deviation.

There were duplicates with three dilutions in each experiment.

Treatment	Concentration (μ M)	S-ABA Content ng/g (DW) (1 day)	S-ABA Content ng/g (DW) (3 day)	S-ABA Content ng/g (DW) (7 day)
P-63	2	190 $\pm$ 26	150 $\pm$ 32	150 $\pm$ 20
	10	440 $\pm$ 110	190 $\pm$ 64	150 $\pm$ 42
	50	600 $\pm$ 120	330 $\pm$ 102	200 $\pm$ 59
P-51	2	200 $\pm$ 28	270 $\pm$ 37	310 $\pm$ 130
	10	360 $\pm$ 69	340 $\pm$ 66	330 $\pm$ 110
	50	560 $\pm$ 105	310 $\pm$ 19	310 $\pm$ 100

TABLE XII. EFFECT OF $\pm$ T-T ABA OR PHASEIC ACID ON ENDOGENOUS ABA CONTENT IN MICROSPORE-DERIVED EMBRYOS OF *BRASSICA NAPUS*

Embryo samples were treated, collected and analyzed as outlined in the "Materials and Methods".

Values represent the means of at least three separate experiments $\pm$ standard deviation.

There were duplicates with three dilutions in each experiment.

Treatment	Concentration (μ M)	S-ABA Content ng/g (DW) (1 day)	S-ABAContent ng/g (DW) (3 day)	S-ABAContent ng/g (DW) (7 day)
$\pm$ T-TABA	100	180 $\pm$ 24	230 $\pm$ 36	444 $\pm$ 67
	10	330 $\pm$ 45	290 $\pm$ 32	318 $\pm$ 46
	1	190 $\pm$ 22	267 $\pm$ 24	345 $\pm$ 88
P-240	100	170 $\pm$ 36	200 $\pm$ 55	270 $\pm$ 64
	10	150 $\pm$ 17	210 $\pm$ 64	100 $\pm$ 3
	1	240 $\pm$ 70	270 $\pm$ 50	130 $\pm$ 4

DISCUSSION

A. EFFECT OF EXOGENOUS-APPLIED ABA ISOMERS, C-T OR M ABA, IN INDUCING DESICCATION TOLERANCE IN MICROSPORE-DERIVED EMBRYOS OF *BRASSICA NAPUS* AND EFFECT OF ABA IN DEVELOPMENT AND CONVERSION IN THE MICROSPORE-DERIVED EMBRYOS OF *BRASSICA NAPUS*

Results from the C-T and M ABA treatments clearly demonstrated that ABA plays a key role in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus* (Figures 7, 8 and 9). The optimum concentrations of C-T and M ABA in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus* were around 10 μ M. At high ABA treatment concentrations of 1000 μ M of C-T or M ABA, embryos turned dark and died. The concentrations of applied C-T and M ABA below 0.1 μ M were not effective in inducing desiccation tolerance, as the control untreated and desiccated 21-day old and 28-day old embryos did not survive desiccation.

Microspore-derived embryos produced and accumulated certain levels of endogenous ABA during culture with the *in vitro* protocol used in the study. It was speculated that before maturation and germination of the microspore-derived embryos, the older the embryos were, the higher the ABA level would be. The endogenous ABA level of 28-day-old cotyledon stage microspore-derived embryos was found to be around 0.4 μ M and

was 25% higher than the ABA level of 21-day-old late-torpedo-stage embryos. The 21-day-old embryos had an ABA level of around 0.3 μ M. The situation is different with *Brassica* seed, in which the endogenous ABA concentration showed two peaks at 35 and 38 days post anthesis during embryogenesis (Finkelstein et al., 1985), and these ABA level peaks were correlated with induction of dormancy. The usual levels of ABA in 21-day-old or 28-day-old microspore-derived embryos *in vitro* appear to be too low to induce sufficient desiccation tolerance to allow embryos to survive desiccation.

The induction of desiccation tolerance by ABA depends on the developmental stage of the embryo (Brown et al., 1993; Senaratna et al., 1991), and embryo survival after desiccation depends on the concentrations of the exogenous applied ABA during *in vitro* microspore embryogenesis. Early cotyledonary stage embryos of alfalfa were most responsive to ABA. Early-staged embryos such as globular and heart-staged embryos were not responsive to ABA treatments. At the same developmental stage, different species have difference sensitivity to ABA. For alfalfa, the optimum concentration was 10 μ M (Senaratna et al., 1990), and in barley embryo culture, 100 μ M ABA gave the best viability after desiccation (Bartels et al., 1988). In the present study, the optimum concentration of C-T and M ABA in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus* was 10 μ M (Figure 8) and the optimum concentrations of S-ABA was 100 μ M (Figure 11). These results indicated that the synthetic ABA isomers, C-T or M ABA, at concentration of 100 μ M appeared to show toxic effects on the embryos. Some of the embryos turned dark during the 7 day treatment at this

concentration with C-T or M ABA but not with S-ABA. This noteworthy observation should be kept in mind in the future ABA studies. In the previous papers, the optimum concentrations in inducing desiccation tolerance varied from 50 to 100 μ M (Brown, et al., 1993; Senaratna et al., 1991; Takahata et al., 1992). This variation is perhaps caused by the differing purities of the applied compounds. At the concentration of 10 μ M, C-T ABA is more effective than M ABA because C-T ABA is the mixture of $\pm$ cis-trans ABA whereas M ABA is the mixture of $\pm$ cis-trans ABA and $\pm$ trans-trans ABA. $\pm$ trans-trans ABA has no effect in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus* (Table 17). This observation will be discussed in part D. Senaratna et al. (1991) indicated that in 21-day-old microspore-derived embryos treated with 10 μ M of C-T ABA with a light intensity of 30-40 μ mol m⁻²sec⁻¹ for 7 days, only root meristems survived after desiccation. In the present study, more than 25% of the desiccated embryos survived when the concentrations of C-T ABA were lowered to 0.1 μ M. One possibility is that S-ABA was degraded during the 7 day incubation under the 16-h photoperiod. But the relationship between S-ABA degradation and light exposure and photoperiod is not clear. Endogenous ABA analysis indicated that when the applied ABA was at or above the basic levels of 21-day microspore-derived embryos, the survival rates of the desiccated embryos increased proportionally with the increases of the applied exogenous ABA until the apparent toxic level (Figures 8 and 9). The effective range of concentrations of applied ABA in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus* is wider than those observed in previous tests.

Uses of the microspore-derived embryos in plant breeding and genetic studies have been well documented. Microspore-derived embryos can be regenerated directly in B₆ medium and then chromosomes can be doubled by colchicine treatment of the root meristem (Huang and Keller, 1989). ABA treatment during microspore embryogenesis can promote embryo maturation. In contrast with cell cultures of white spruce (Dunstan et al., 1992), in which ABA could increase the size of both embryos and their suspensors during the culture period, ABA treatment inhibited the growth and development in microspore-derived embryos of *Brassica napus*. The size of the embryos treated with higher concentrations (10-100 μ M) of C-T ABA was smaller than the same stage of untreated embryos (Figure 16); this agrees with previous results in oilseed rape microspore-derived embryos (Senaratna et al., 1991), in somatic embryos of alfalfa (Senaratna et al., 1990) and with somatic embryos of celery (Kim and Janick, 1991). In the study of induction of desiccation tolerance in carrot somatic embryos, the ABA response gene was induced to express and some of the proteins began to accumulate in the first 24 hours of the treatment (Iida et al., 1992). By contrast, in the present study, the dry weight (Table VI) of the microspore-derived embryos did not increase very much during the 7-day treatment compared to the untreated embryos. Both EtOH and ABA treatment can stimulate microspore-derived embryo conversion into plants by reducing abnormal morphology. ABA treatment can also increase the vigour of the emblings derived from the desiccated embryos after rehydration (Figure 12). Most of the emblings from treated microspore-derived embryos converted to plantlets in growth cabinets (data not shown). Embryos untreated and treated at lower concentrations of EtOH or ABA produced secondary

embryos from swollen hypocotyls and formed callus (Figure 9), thus preventing "normal" conversion into plants. These abnormalities of secondary embryo formation and callus formation can also be overcome by cold treatment (Huang and Keller, 1989) suggesting that stress exposure is necessary to prevent the formation of secondary embryo during microspore embryogenesis and plant conversion from microspore-derived embryo. In another words, stress exposure can trigger a gene or set of genes which are involved in embryo maturation and plant conversion..

ABA has been shown to inhibit the expression of a group of mRNAs and proteins, and at the same time also stimulate the expression of a set of related mRNAs and proteins (reviewed by Lazarus, 1991; Skiver and Mundy, 1990). The best example is the ABA response of cereal aleurone layers in which ABA inhibits GA-regulated proteins and induces its own proteins (reviewed recently by Jacobsen and Chandler, 1987). Le Page-Degivry and Garello (1991) pointed out that membrane stability played a key role in survival of desiccated seeds. To achieve tolerance to desiccation, it is necessary to synthesize some new proteins in the embryo. In the study of somatic embryos of celery (Kim and Janick, 1991) and microspore-derived embryos of *Brassica napus* (Pomeroy et al., 1994), fatty acids, mainly unsaturated acids, and storage proteins were significantly increased by applied ABA. However, the increased levels of fatty acids and proteins were still lower than those of the zygotic embryos. In inducing desiccation tolerance in carrot, Iida et al. (1992) suggested that the cellular components were modified with the accumulation of certain substances during the period of ABA treatment. They also

indicated and implied that the synthesis of a group of proteins of 20-23 kD molecular weight enhanced by ABA treatment were directly associated with desiccation tolerance. These proteins have shown homology to *lea* proteins. As outlined in the introduction, *lea* proteins which were found to be accumulated during embryo maturation in several plants have been implicated in the development of desiccation tolerance. *lea* genes have been isolated based on their responsive characteristics to ABA, however the function of these *lea* proteins in the embryo is still not clear. Chilling and heat stresses can also induce desiccation tolerance (Kott and Beversdorf, 1990; Senaratna et al., 1989). The synthesis of stress proteins were considered to be mediated by endogenous ABA. Bray (1992) assumed that drought stress triggered the synthesis of ABA biosynthetic enzyme(s) and resulted in an increase of the endogenous ABA. The increased endogenous ABA then regulated the increased transcription of a number of ABA-induced genes resulting in specific mRNA accumulation and further specific protein accumulation which may be involved in drought tolerance.

B. EFFECT OF EXOGENOUS APPLIED OPTICALLY PURE S-ABA OR R-ABA IN INDUCING DESICCATION TOLERANCE AND ON THE ENDOGENOUS ABA POOL IN MICROSPORE-DERIVED EMBRYOS OF BRASSICA NAPUS

i) Effect of exogenous applied S-ABA

When exogenous S-ABA was applied to embryos in culture medium, the embryos took up and accumulated the applied S-ABA. The endogenous ABA levels increased depending on the levels of applied S-ABA. When the concentrations of the applied S-ABA were lower than 0.1 μ M, the endogenous ABA levels of the embryos did not change during 1-day incubation and the embryos did not acquire desiccation tolerance (Figure 10 and Table VII). After 3 days treatment with 0.1 μ M of S-ABA, endogenous ABA levels were higher. They increased to 13% more than the endogenous ABA level of untreated c28 embryos. Although 7 days later, the applied ABA appeared to be metabolised to the usual observed levels, some of the embryos acquired desiccation tolerance. ABA accumulation and the survival rates of desiccated embryos increased proportionally with an increase of the concentrations of applied ABA. When the concentration of the exogenous applied S-ABA was at 100 μ M, during a 1-day incubation, the endogenous ABA levels were around 30 μ M. At this concentration, the embryos did not turn dark as the embryos treated with C-T or M ABA at the same concentration and the survival rate of the desiccated embryos increased to more than 95% suggesting that synthetic ABA was somewhat toxic at concentrations above 100 μ m. This result showed a similar trend with previous papers. In the study of the relationship between the accumulation of storage

proteins in maturation of the rapeseed embryos with or without exogenous-applied ABA, Finkelstein et al., (1985) found that it was necessary to increase the concentrations of exogenous ABA to result in the accumulation of the storage proteins *in vitro*. They also indicated that the accumulation of the storage proteins in embryos was correlated with the desiccation process. In studying the changes of fatty acids and proteins after addition of ABA in microspore-derived embryos of *Brassica napus*, Pomeroy et al. (1994) found that applied ABA caused an increase in total unsaturated fatty acids on a dry weight basis and stimulated the synthesis of cruciferin- and napin-like proteins. As the embryos were promoted to mature by high exogenous ABA levels, the embryo metabolised most of the ABA taken up to maintain the growth substance balance. Whether the endogenous ABA of the matured microspore-derived embryos decreased to very low levels before germination, as occurs normally in seeds, should be investigated in future experiments. In somatic embryo suspension cultures of white spruce (Dunstan et al., 1992), exogenous applied 15 μ M S-ABA was completely metabolised during the following 7-day incubation *in vitro*. In the present study, most of the taken up ABA was metabolised by embryos during the 7 day incubation. The endogenous ABA levels of the treated embryos were much higher than the usual ABA levels of the developing embryos, such as embryos treated with 100 μ M of S-ABA (Table VII). If the endogenous ABA levels of the treated embryos increased only 5-10 times or less, such as embryos treated with 1-10 μ M of S-ABA, than the usual levels of the developing embryos, the increased ABA was metabolised more slowly. The endogenous ABA levels of the treated embryos were still higher after the 7-day incubation. One possibility to explain these observations is that the

higher concentrations of endogenous ABA can induce and promote the synthesis of ABA degradative enzyme(s).

ii) Effect of exogenous applied R-ABA

R-ABA, a enantiomer of natural ABA, has been shown to have different effects in different species, even in different tissues of the same species (reviewed by Walton, 1983). R-ABA was found to be as effective as S-ABA as a wheat germination inhibitor (Walker-Simmons et al., 1992). Nevertheless, it was less effective in inducing the wheat *Em* gene within the time that embryogenic germination was inhibited. Wilen et al. (1993) indicated that a 30-times R-ABA level was necessary to significantly stimulate the accumulation of napin transcripts in microspore-derived embryos of *Brassica napus*.

Combined with the desiccation tolerance data, the results of this study suggest:

1) R-ABA was not as effective as S-ABA in inducing desiccation tolerance in

microspore-derived embryos of *Brassica napus* at higher concentrations from 10 to 100 μ M, but was more persistent than S-ABA at lower concentrations from 0.001 to 1 μ M. One possibility to explain the above phenomenon is that R-ABA is effective in inducing desiccation tolerance in microspore-derived embryos and is metabolized more slowly than S-ABA in the embryo.

2) R-ABA is recognized by the ABA receptor(s) in the embryos. Endogenous ABA

analysis indicated that desiccation tolerance of the embryos treated with R-ABA was not induced by the increases of the endogenous S-ABA in the embryos (Table VIII).

During the treatment, the endogenous ABA levels increased a little.

3) R-ABA was not converted into S-ABA in the embryos. The 1-2 fold increases of endogenous ABA in the embryos at high concentrations from 10 to 100 μ M of R-ABA treatment were because that, as a stress, applied high concentrations of R-ABA could stimulate the embryos to produce more endogenous ABA, but was not caused by converting R-ABA to S-ABA. In bromegrass and corn cell cultures, Abrams et al. (personal communication) recently got similar results.

In studying desiccated alfalfa somatic embryos, McKersie et al. (1989) found that both the length of the shoot and fresh weight of the seedling from desiccated alfalfa somatic embryos were 100% and 45% higher respectively than in undesiccated embryos. They suggested that desiccation could trigger the synthesis of relative mRNAs or proteins with germination. In the present experiment, although 5-fold-higher concentrations of three (S)-ABA-analogs had similar effects in inducing desiccation tolerance, comparison of the vigour of the emblings of ABA and ABA-analogs treated and desiccated embryos indicated that the mean shoot length from S- and R- ABA treated embryos was 30% longer than the shoots from the emblings treated with the three (S) ABA-analogs and 60% longer than the shoots from the emblings treated with the three (R) ABA analogs (Table III). This suggests that applied S- or R-ABA can not only induce protein accumulation related to desiccation tolerance, but also enhance storage protein levels and regulate the synthesis of other proteins associated with germination. The initiation of specific proteins for germination may promote the growth and development of the emblings after

rehydration. However more evidence is needed to conform this hypothesis. The above-mentioned stimulation needs a higher concentration level of at least 1uM of S-ABA.

C. EFFECT OF EXOGENOUS-APPLIED OPTICALLY-PURE ABA-ANALOGS IN INDUCING DESICCATION TOLERANCE AND ON THE ENDOGENOUS ABA POOL IN MICROSPORE-DERIVED EMBRYOS OF *BRASSICA NAPUS*

All of the six ABA-analogs tested were shown to be effective in inducing desiccation tolerance in microspore-derived embryos at a concentration of 10 μ M but they had very different capabilities (Figures 13, 14 and 15). Although the three (S) configuration analogs had very strong biological activity, they still needed 5-times-higher concentrations to reach to the same activity as S-ABA. The three (R) configuration analogs had only 30% as strong of an activity as (S)-configuration analogs. Indirect ELISA analysis indicated that exposure to R-ABA and (S)-configuration ABA-analogs did not increase endogenous ABA contents except for P-89 which cross-reacted with the monoclonal antibody derived from S-ABA. For the three R-configuration ABA-analogs, they had very weak capability in inducing desiccation tolerance except P-185 at the higher concentrations of 50 μ M. At this concentration, it had a similar effect as its enantiomer P-82 and the effect dropped very fast when the concentration was lowered to 2-10 μ M.

i) Effect of P-89 and P-59

The biological activity of P-89 and P-59 have been studied in several species; namely black spruce (Blake et al., 1990a,b), bromegrass (Churchill et al., 1992; Lamb et al., 1993), and wheat (Walker-Simmons et al., 1992). In previous experiments, P-89 was found to be effective in antitranspiration in droughted plants, induced freezing tolerance

in bromegrass, inhibited wheat embryo germination and induced two ABA-responsive genes, wheat group 3 *lea* and *rub*. But it was found to increase transpiration in unstressed Douglas-fir and be ineffective in inducing wheat *Em* gene. P-59 was inactive in the above experiments.

In the present study, P-89 had a very strong effect in inducing desiccation tolerance in microspore-derived embryos, but not P-59 which showed an effect only in the higher concentration of 50 μ M. Combined with endogenous ABA analysis (Figure 12 and Table IX), these results suggest that:

- 1) The endogenous ABA levels measured from P-89 treated samples were the total amount of endogenous S-ABA plus P-89, as P-89 cross-reacted with the S-ABA monoclonal antibody to give a false high level of endogenous ABA.
- 2) P-89 was accumulated, and was not immediately metabolised in the embryos as was S-ABA, as the measured endogenous ABA stayed higher (more persistent) during the 7 day incubation.
- 3) Added high concentrations of P-89 (above 10 μ M) may stimulate the embryos to produce more endogenous ABA as the measured endogenous ABA levels were higher than the same level of the applied P-89 levels.
- 4) The increase of endogenous ABA in P-59 treated samples may be because a) added P-59 may cause the embryos to produce more endogenous ABA; b) P-59 may compete with S-ABA at the oxidation enzyme active site(s);
- 5) It is suggested that part of desiccation tolerance of the P-59 treated embryos was

induced by the increase of the endogenous ABA but not by P-59 itself; as at the concentration of 50 μ M, the endogenous ABA increased 5-10 times.

Because P-89 has a cross-reaction with S-ABA MAb, it is difficult to factor the endogenous ABA from P-89 effect except by using another S-ABA monoclonal antibody.

ii) Effect of P-82 and P-185

The effect of P-82 and P-185 in inducing desiccation tolerance and on the endogenous ABA pool was shown in Figure 14 and Table X. P-82 were effective in inducing desiccation tolerance and did not increase the endogenous ABA levels, and P-185 were as effective as P-82 at a concentration 50 μ M but not at concentrations below 10 μ M. The fact that endogenous ABA levels decreased nearly 50% when treated with P-82, suggested two possibilities:

- 1) P-82 promoted the synthesis of the ABA degradative enzyme; or
- 2) P-82 inhibited the synthesis of the ABA biosynthetic enzyme(s).

More details may be revealed if the ABA-responsive mRNAs and proteins were to be studied. As with R-ABA, P-185 didn't increase the endogenous ABA levels of the treated samples (Table X). These results indicated that P-185 was recognized by the ABA receptor(s) and was active in inducing desiccation tolerance. This is the first time that P-185 has been shown to have biological activity suggesting that the c1' S-configuration is not essential (or crucial) in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus* although its effect dropped fast at lower concentration of 2 μ M.

iii) Effect of P-63 and P-51

Further changes in the C1 position did not decrease the biological activity of the chemicals. Both P-63 and P-51 were as effective as another pair of enantiomers, P-89 and P-59, and P-82 and P-185, but P-63 had a much stronger effect than P-51 in inducing desiccation tolerance (shown in Figure 15). ABA analysis indicated that endogenous ABA levels increased 0.5-2 fold when the embryos were treated with higher concentrations (10-50 μ M) of P-63 and P-51 (Table XI). Obviously, P-63 was effective in inducing desiccation tolerance, but with P-51, as was postulated with P-59, part of the desiccation tolerance of the P-51 treated embryos was likely induced by the increase of the endogenous ABA but not by P-51 itself. These results agreed with previous experimental results (Wilén et al., 1993), in which P-63 was effective in promoting the expression of genes encoding storage proteins in microspore-derived embryos of *Brassica napus* and P-51 was inactive.

D. EFFECT OF EXOGENOUS-APPLIED ABA METABOLIC PRODUCTS, PHASEIC ACID AND $\pm$ T-T ABA, IN INDUCING DESICCATION TOLERANCE AND ON THE ENDOGENOUS ABA POOL IN MICROSPORE-DERIVED EMBRYOS OF *BRASSICA NAPUS*

i) Effect of phaseic acid

In cell suspension cultures of white spruce, exogenous applied S-ABA was completely metabolised and converted to an equal quantity of phaseic acid during 7 days of culture (Dunstan et al., 1992). PA, which is the major metabolite of S-ABA in some species, was found to have different effects, active or inactive, in different species. In barley aleurone layer culture, PA could inhibit α -amylase production and the accumulation of a group of GA-regulated mRNAs and proteins and induce the accumulation of a group of other mRNAs and proteins as did S-ABA (Ho, 1979; Nolan and Ho, 1988; Uknes and Ho, 1984). However, PA was inactive in inducing freezing tolerance in bromegrass cell culture (Gusta, 1990) and in inhibiting wheat embryo germination (Milborrow, 1969). In the present study, PA was found to inhibit embryo development at a high concentration of 100 μ M (Figure 16), as did ABA, but was inactive in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus*. Endogenous ABA analysis also indicated that PA appeared not to be reconverted to S-ABA in the embryo although its concentration was high enough to be toxic in culture medium. This result agrees with those observed in cell cultures of white spruce (Dunstan et al., 1992). This analysis provided further evidence that PA was active in some species. PA was also found to

inhibit the development of microspore-derived embryos of *Brassica napus* (Figure 16).

ii) Effect of $\pm$ T-T ABA

All of the experiments have indicated that $\pm$ T-T ABA has no biological activity (Reviewed by Walton, 1983) including inducing desiccation tolerance of microspore-derived embryos of *Brassica napus* (Table IV). The *cis* C2, C3 configuration of ABA isomers plays a key role for activity in recognition of these compounds in plant (Churchill et al., 1992). Change of ABA-analogs in the *cis* C2, C3 configuration will lead to elimination or reduction of their ABA-like biological activity. Once S-ABA is converted to (S)-*trans-trans* ABA, it is hard to reconvert to S-ABA in the embryo. At a high concentration of 100 μ M, $\pm$ T-T ABA was found to inhibit the development of microspore-derived embryos of *Brassica napus* (Figure 16). If this compound has other biological activities, it is not clear from this result.

CONCLUSIONS

In this study, a range of positive and negative, physiological and biochemical effects of ABA and ABA-analogs in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus* were demonstrated. ABA inhibits growth and induces desiccation tolerance in microspore-derived embryos, and these inhibitions and inductions show dose-, purification- and time-dependencies. The concentrations of exogenous-applied ABA below the usual levels of the developing microspore-derived embryos did not induce desiccation tolerance. In the processes of microspore embryogenesis and plant recovery and regeneration from microspore-derived embryo, stress appears to be necessary to maintain plant development and growth.

S-ABA and C-T ABA had similar effects in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus*. Synthetic ABA (C-T and M ABA) appeared to be toxic to the embryos at concentrations above 100 μ M but not S-ABA. The ABA metabolite, phaseic acid, was not associated with the acquisition of desiccation tolerance. Any change in the structure of S-ABA appeared to reduce its activity. R-ABA was not as effective as S-ABA, but was metabolized more slowly and showed more persistence than S-ABA at the concentrations below 1 μ M. Three (S)-configuration analogs needed five times higher concentrations to reach the same levels as S-ABA and two of the (R)-configuration analogs lost activity in inducing desiccation tolerance in microspore-derived embryos. Another (R)-configuration analog, P-185 showed its activity in inducing

desiccation tolerance in all of the experiments. This is the first report of the activity of P-185 in inducing desiccation tolerance. We assumed that this compound was recognized by the ABA receptor(s) because induction of desiccation tolerance was not correlated with an increase of endogenous S-ABA. Meanwhile, we also can not eliminate the possibility that P-59 and P-51 have very weak activity in this experiment system. The above facts can be further distinguished and confirmed by investigating the relative changes of specific mRNAs and proteins with the above conditions. The results suggest that ABA plays a key role in inducing desiccation tolerance in microspore-derived embryos of *Brassica napus* cv. Topas but the structural requirement of ABA in this experimental system is more flexible than other experimental systems such as wheat, cress or white spruce. These analogs appear not be converted to S-ABA in the microspore-derived embryos.

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Appendix I. Composition of Media Used in Microspore Culture

	B5	B5-13	NLN-13
Chemicals	Concentration mg/L	Concentration mg/L	Concentration mg/L
Salts			
NaH ₂ PO ₄ ·H ₂ O	150	150	
KNO ₃	3,000	3,000	125
(NH ₄) ₂ SO ₄	134	134	
MgSO ₄ ·7H ₂ O	500	500	125
CaCl ₂ ·2H ₂ O	150	150	
Fe(III) EDTA	42.9	42.9	
FeEDTA,Na			80
Ca(NO ₃) ₂ ·4H ₂ KH ₂			500
PO ₄			125
Micronutrients			
MnSO ₄ ·4H ₂ O	10	10	22.3
H ₃ BO ₃	3	3	6.2
ZnSO ₄ ·7H ₂ O	2	2	8.6
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	0.25
CuSO ₄ ·5H ₂ O	0.025	0.025	
CoCl ₂ ·6H ₂ O	0.025	0.025	0.025
KI	0.75	0.75	0.025
Vitamins			
Nicotinic acid	1	1	5.0
Thiamine.HCl	10	10	0.5
Pyrodoxine.HCl	1	1	0.5
Myo-Inositol	100	100	
Glycine			100
Folic acid			2.0
Biotin			0.5
Glutathione, reduced			0.05 30
L-glutamine			
L-serine			800
Sucrose	20,000	130,000	130,000
pH	5.5	6.0	6.0

Appendix II. Composition of Buffers Used in ELISA

1. Borate Buffer: 0.1 M boric acid, pH 9.0 (adjust with concentrated NaOH).

2. PBS Buffer: 10x stock solution, one liter, pH 7.3

80 g	NaCl
2 g	KCl
11.5 g	Na ₂ HPO ₄ · 7H ₂ O
2 g	KH ₂ PO ₄

3. TBS Buffer: one liter, pH 7.8

6.05 g	Tris (MW = 121.1)
0.20 g	MgCl · 6H ₂ O
8.80 g	NaCl

4. TBS Buffer with BSA: one liter, pH 7.8

6.05 g	Tris (MW = 121.1)
0.20 g	MgCl · 6H ₂ O
8.80 g	NaCl
0.02% (w/v)	BSA

5. TBS Buffer with BSA and Tween: one liter, pH 7.8

6.05 g	Tris (MW = 121.1)
0.20 g	MgCl · 6H ₂ O
8.80 g	NaCl
0.1% (w/v)	BSA
0.05% (v/v)	Tween 20

END

0 1 - 0 6 - 9 5

FIN