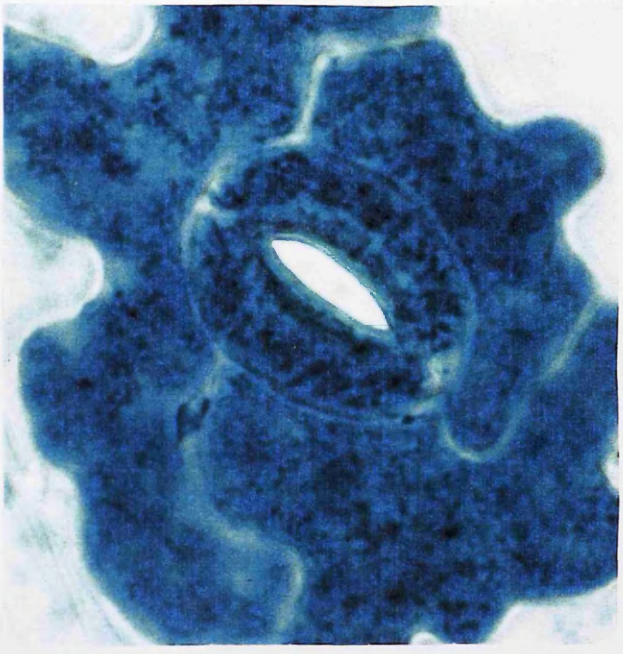
Lucy Theresa Montgomery, BSc. (Hons) (Leicester)

Investigations of ABA Signalling Pathways in Stomatal Guard Cells



A thesis submitted to the University of Lancaster

for the degree of Doctor of Philosophy,

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The photograph on the front page shows the histochemical localization of GUS activity driven by an ABA-regulated gene promoter in stomatal guard cells of *Arabidopsis thaliana* (see Chapter 4).

Contents

Abstract

Montgomery, Lucy Theresa B.Sc. (Hons.) (Leicester). **Investigations of ABA Signalling Pathways in Stomatal Guard Cells**. A thesis submitted to the University of Lancaster for the degree of Doctor of Philosophy, September 1997.

The effect of analogues of abscisic acid (ABA) on stomatal aperture and GUS activity driven by an ABA-responsive gene promoter (*CDeT6-19*) in guard cells was examined. The ABA analogues used were (–)-ABA, (±)-*trans*, *trans*-ABA, and (+)- and (–)-dihydroacetylenic abscisyl alcohol (PB1-63 and PBI-51, respectively). (+)-ABA and (±)-ABA were included in the investigations for comparison.

(–)-ABA, hitherto believed to have little or no effect on stomatal aperture, significantly inhibited stomatal opening in *Commelina communis*, *Vicia faba*, *Nicotiana tabacum* and *Arabidopsis thaliana.* In contrast, PBI-51 [a competitive inhibitor of ABA-induced gene expression] had no effect on ABA-induced inhibition of stomatal opening (determined in both *C. communis* and *N. tabacum*) or ABA-induced promotion of stomatal closure (determined in *C. communis* only). Differences in the effectiveness of PBI-63, PBI-51 and (–)-ABA at inhibiting stomatal opening were discovered between plant species. These data demonstrate the importance of comparing the effect of ABA analogues on stomatal opening and *CDeT6-19*/GUS activity in guard cells in a single plant species.

(±)-ABA enhanced *CDeT6-19*/GUS activity in guard cells of *A. thaliana* but not *N. tabacum*. The ABA analogues that inhibited stomatal opening in *A. thaliana* also enhanced *CDeT6-19*/GUS activity in guard cells of this species. The relative

biological activity of the ABA analogues in detached epidermis of *A. thaliana* was (+)-ABA > (±)-ABA > (–)-ABA > PBI-63. PBI-51 and (±)-*trans, trans*-ABA had no effect on stomatal opening or *CDeT6-19*/GUS activity in guard cells of *A. thaliana*. Interestingly, a differential effect of 10^{-5} M (–)-ABA on the two ABA-induced responses in *A. thaliana* was observed; 10^{-5} M (–)-ABA enhanced *CDeT6-19*/GUS activity in guard cells but had no effect on stomatal opening.

Preliminary measurements of guard cell cytosolic free calcium ($[Ca^{2+}]_i$) showed that (–)-ABA induced oscillations in $[Ca^{2+}]_i$ in guard cells of *C. communis*. In addition, these studies demonstrate the possibility of microinjecting guard cells of *A. thaliana* with fura-2 and provide a measure of resting $[Ca^{2+}]_i$ in this species.

The data presented in this thesis from *A. thaliana* suggest that the "receptor" utilized in the signalling pathway by which ABA inhibits stomatal opening is similar but not identical to that by which ABA enhances *CDeT6-19*/GUS activity in guard cells.

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Abbreviations

ABA	Abscisic acid
ABRC	Abscisic acid response complex
ABRE	Abscisic acid response element
AOV	Analysis of variance
BAPTA	1, 2-bis(o-aminophenoxy)ethane N', N', N', N' tetraacetic acid
bp	base pair
cADPR	cyclic adenosine 5'-diphosphoribose
$[Ca^{2+}]_e$	External calcium ion concentration
$[Ca^{2+}]_i$	Cytosolic-free calcium ion concentration
cAMP	cyclic-adenosine monophosphate
CAT	Chloroamphenicol acetyl transferase
cGMP	cyclic-guanine monophosphate
CICR	Calcium induced calcium release
DAG	Diacylgerol
cDNA	complementary DNA
DMSO	Dimethylsulphoxide
dsp	desiccation stress protein
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid (disodium salt)
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
FDA	Fluoroscein diacetate

FGP	Fluorescent green protein
FV	Fast-activating-vacuolar
GA_1	A form of gibberellic acid
GA_3	A form of gibberellic acid
GASP	GUS activity score per peel
GC	Gas chromatography
GCP	Guard cell protoplast
g_s	Stomatal conductance (m s^{-1} or mol m^{-2} s^{-1})
GTP	Guanine triphosphate
GUS	β-glucuronidase
HPLC	High performance liquid chromatography
IEBS	Institute of Environmental and Biological Sciences
IAA	Indole-3-acetic acid
IP_3	Inositol (1, 4, 5) trisphosphate
$[K^+]_e$	External potassium ion concentration
Lea	Late embryogenesis abundant
LSD	Least significant difference
MES	2-[N]-morpholinoethane sulphonic acid
MES/KOH	10 mM MES adjusted to pH 6.15 using KOH
MES/Tris	10 mM MES adjusted to pH 6.15 using Tris-base
mRNA	messenger RNA
4-MUG	4-methylumbelliferryl-β-glucuronide
NPT11	Neomycin phosphotransferase
PFD	Photon flux density

PIP_2	Phosphatidyl inositol (4, 5) bisphosphate
PLC	Phospholipase C
PP2C	Protein phosphatase type 2C
Rab	Responsive to abscisic acid
RIA	Radioimmunoassay
RH	Relative humidity
RNA	Ribonucleic acid
SE	Standard error
SV	Slow-vacuolar
UK	United Kingdom
V-type	Vacuolar-type
VK	Vacuolar-K^+
V_m	Membrane potential
WT	Wild-type
X-Gluc	5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid

Chapter 1

General Introduction

1.1 Signal transduction

Signal transduction is an essential phenomenon common to all biological organisms. It is the process by which an extracellular stimulus is coupled to its characteristic intracellular response. The stimulus is perceived by receptors and the response involves changes in cellular activity such as the regulation of ion channels or specific genes. Considerably more progress has been made in understanding signal transduction mechanisms in animal cells (for example see Barritt, 1992; Alberts *et al.*, 1994; Clapham, 1995; Cohen *et al.*, 1995; Divecha & Irvine, 1995; Heldin, 1995; Hill & Treisman, 1995; Hunter, 1995; Marshall, 1995; Neer, 1995; Berridge, 1997) than in plant cells. Consequently, many investigations of plant cell signal transduction have used animal paradigms of signalling pathways as frameworks within which to work (see McAinsh *et al.*, 1991b; Assmann, 1993, 1995; Drøbak, 1993; Mizoguchi *et al.*, 1993; Bolwell, 1995; Bush, 1995; Chasan, 1995; Stone & Walker, 1995; Braun & Walker, 1996; Ferl, 1996; Millner & Clausier, 1996; Smith & Walker, 1996; Webb *et al.*, 1996b). This approach is useful as long as it is employed without assuming that the mechanisms by which animal and plants cells carry out stimulus-response coupling are exactly the same.

One animal cell signalling system which has been used as a paradigm for investigating plant cell signal transduction is the phosphatidyl inositol pathway (see Berridge, 1993; Alberts *et al.*, 1994). In overview, this pathway is known to involve: (i) the binding of a stimulus (often a hormone) to a plasma membrane-bound receptor;

(ii) the coupling of the activation of the receptor to the activation of the enzyme phospholipase C by heterotrimeric G-proteins; (iii) the cleavage of the plasma membrane lipid, phosphatidyl inositol (4, 5) bisphosphate (PIP_2) to inositol (1, 4, 5) trisphosphate (IP_3) and diacylgerol (DAG) by phospholipase C; (iv) the diffusion of IP_3 through the cytoplasm to activate a calcium (Ca^{2+})-release channel in the endoplasmic reticulum, termed the IP_3 receptor, whilst DAG remains in the plasma membrane and activates protein kinase C, which in turn can regulate other proteins (see Berridge, 1993; Alberts *et al.*, 1994). An increase in the concentration of cytosolic-free Ca^{2+} ions ($[Ca^{2+}]_i$) can either directly modulate effectors such as ion channels, activate protein kinase C (see above) or act via association with the ubiquitous Ca^{2+} binding protein, calmodulin, CaM (James *et al.*, 1995). It is believed that Ca^{2+} and CaM can affect phosphorylation and dephosphorylation events by activating specific kinases and/or phosphatases which are either directly or indirectly responsible for initiating the final response (see review by Berridge, 1993). The description of the phosphatidyl inositol pathway given here is highly simplified but serves to illustrate some of the main features; the reader is referred to reviews (Tsien & Tsien, 1990; Berridge *et al.*, 1988; Berridge, 1993; Fewtrell, 1993; Clapham, 1995) for a more in-depth consideration.

In the phosphatidyl inositol signalling pathway, Ca^{2+}, IP_3 and DAG all act in the capacity of second messengers, with Ca^{2+} being the most well studied (see reviews by Berridge, 1993; Clapham, 1995; Webb *et al.*, 1996b). Second messengers are molecules whose concentration in the cell increase in response to a stimulus and subsequently this alteration triggers the cell's internal machinery to produce the response. Other second messengers identified in animal cells include cyclic-adenosine monophosphate (cyclic-AMP), cyclic-guanine monophosphate (cyclic-GMP) and also

cyclic adenosine 5'-diphosphoribose (cADPR), (see reviews by Galione, 1993; Alberts *et al.*, 1994).

Evidence is particularly plentiful for the involvement of Ca^{2+} in plant signalling pathways (see reviews by Poovaiah & Reddy, 1993; Bowler & Chua, 1994; Bush, 1993, 1995; Gilroy & Trewavas, 1994; Webb *et al.*, 1996b, McAinsh *et al.*, 1997) and Ca^{2+} has become firmly established as a ubiquitous second messenger in plant cell signal transduction (see reviews by McAinsh *et al.*, 1991b; Bush *et al.*, 1995; Webb *et al.*, 1996b). In addition, evidence is accumulating for the involvement of other players from animal cell signalling pathway paradigms in plant cell signal transduction, but more work is required to firmly establish their precise roles (see review by Webb *et al.*, 1996b; see Section 1.5.3).

1.2 The stomatal guard cell as a model system to study plant cell signal transduction pathways

The stomatal guard cell (see Figure 1.1) is proving to be invaluable in investigations of plant cell signal transduction (see reviews by Assmann *et al.*, 1993; Webb *et al.*, 1996; Willmer & Fricker, 1996; McAinsh *et al.*, 1997). Stomatal guard cells are specialized cells found in pairs in the epidermes of above ground plant parts and control the aperture of the stomatal pore (see Figure 1.1). Stoma is the Greek word for mouth and normally refers to both the stomatal pore and also the two guard cells which delimit this pore. The stomatal pore is a gateway for the entry of CO_2 into the leaf for photosynthesis and for the exit of water vapour from the transpiration stream. The role of stomatal guard cells is to optimize leaf gas exchange under changing environmental conditions

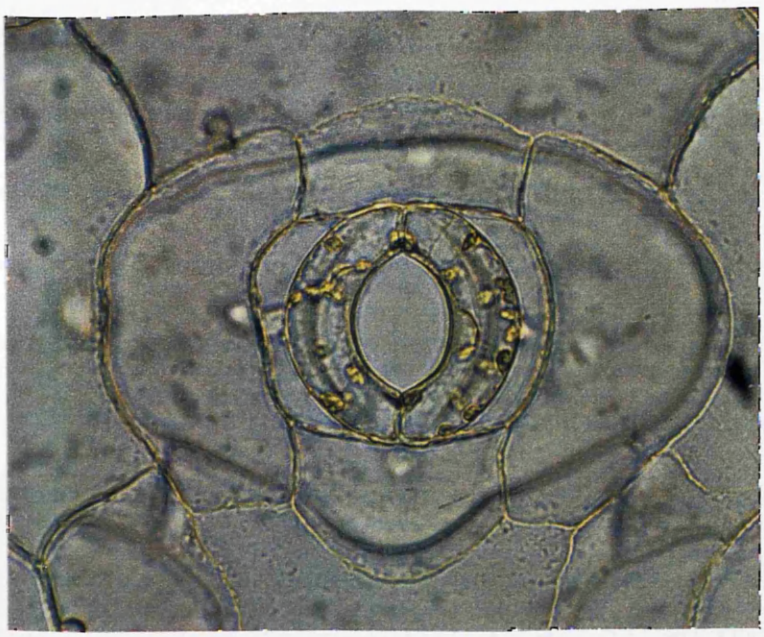


Figure 1.1. A pair of stomatal guard cells surrounding a stomatal pore in abaxial epidermis from a leaf of *Commelina communis*.

Guard cells control the size of the stomatal pore (aperture) by altering their turgor (see reviews by Assmann, 1993; Willmer & Fricker, 1996). An increase in guard cell turgor is achieved by the rapid accumulation of solutes (see Section 1.3.1). This includes the uptake of inorganic [potassium ions (K^+) and chloride ions (Cl^-)] and organic solutes (possibly sucrose - see Talbott & Zeiger, 1996) from the apoplast and/or the synthesis of osmotically active molecules within the cell (for example, the conversion of starch to malate^{2-}) (see reviews by Assmann, 1993; Willmer & Fricker, 1996). The accumulation of solutes in the guard cell increases the osmotic pressure of the guard cell (and so lowers the water potential) thus resulting in the uptake of water and an increase in the turgor pressure. This increase in turgor pressure coupled with the differential cell wall thickening, extensibility and cellulose microfibril orientation of guard cells results in a deformation in the shape of these cells such that they push apart from each other and so widen the pore between them; i.e. stomatal opening (see Section 1.3.1). A decrease in the concentrations of intracellular osmotica has the opposite effect, and water efflux results in guard cell deflation and stomatal closure (see Section 1.3.2).

There are many reasons why stomatal guard cells are particularly suitable for studying signalling pathways in plant cells. These include:

(i) Stomatal guard cells respond to a vast array of stimuli. They can respond to signals as diverse as light (red and blue), humidity, temperature and CO_2 concentration, as well as a number of plant hormones including abscisic acid (ABA) and auxins (see review by Assmann, 1993). They therefore provide an ideal model system within which to investigate the complex nature of plant cell signal transduction.

(ii) Stomatal guard cells have a rapid and easily detectable response to the array of stimuli described above in (i). This response, as indicated above, is a change in guard

cell turgor and hence stomatal aperture width. A change in stomatal aperture width has been reported to occur in response to stimuli such as ABA within minutes of application (Addicott, 1983; Raschke, 1987). Changes in stomatal aperture width can be measured easily, either directly by microscopic examination of detached or undetached epidermis or indirectly by measurements of leaf stomatal conductance (see Weyers & Meidner, 1990).

(iii) Guard cells are amenable to ion channel investigations (see reviews by Assmann, 1993; Ward *et al.*, 1995; MacRobbie, 1997; Tester, 1990, 1997). The guard cells of many plants, particularly *Vicia faba* and *Commelina communis* are readily accessible in detached epidermis (epidermal peels). The movement of ions in isolated guard cells in epidermal peels can be studied using unidirectional radioactive tracer fluxes (MacRobbie 1981; 1983; 1989, 1990; 1995a & b; Brindley 1990a & b; see review by MacRobbie, 1997). Intact guard cells can also be used for electrophysiological studies of ion fluxes across the plasma membrane (for example see Blatt, 1987, 1988, 1990; Thiel *et al.*, 1992; Armstrong & Blatt, 1995; and reviews by Tester, 1990, 1997; MacRobbie, 1997); these studies involve the impalement of the cell with a micro-electrode and subsequent clamping of the membrane potential to fixed values (voltage clamping) (see reviews by Tester, 1990, 1997; Blatt, 1991). In addition, guard cell protoplasts and isolated guard cell vacuoles make excellent subjects for studying ion fluxes across the plasma membrane and tonoplast using patch-clamp techniques (for example Schroeder *et al.*, 1987; Schroeder, 1988; Keller *et al.*, 1989; Schroeder & Hagiwara, 1989; Hedrich *et al.*, 1990; Schroeder & Keller, 1992; Ward & Schroeder, 1994; Allen & Sanders, 1995; Schulz-Lessdorf & Hedrich, 1995; see reviews by Assmann, 1993; Ward *et al.*, 1995; MacRobbie, 1997). Patch clamping involves the study of ion fluxes across membrane patches using a patch-pipette; measurements can

be made from entire cells or small excised membrane patches (see reviews by Tester, 1990, 1997; Ward, 1997). Recently, it has been shown that ion channels in the guard cell plasma membrane can also be studied using patch-clamping in intact guard cells in epidermal peels (Henrikson *et al.*, 1996 - *V. faba*). This has been made possible by the use of laser microsurgery to remove a small section of the guard cell wall thus allowing access of the patch pipette (Henrikson *et al.*, 1996).

(iv) Guard cells are amenable to microinjection techniques (for reviews see Assmann, 1993; Webb *et al.*, 1996b). The microinjection of fluorescent, Ca^{2+}- or pH-indicator dyes and the release of putative components of the ABA-signalling pathway, such as IP_3, Ca^{2+} or even ABA itself into the guard cell cytosol has provided very important clues as to the organization of ABA signalling pathways in guard cells (see Sections 1.5.1, 1.5.2 and 5.1.1) (for reviews see Assmann *et al.*, 1993; Webb *et al.*, 1996b; McAinsh *et al.*, 1997; MacRobbie, 1997).

(v) Guard cells have recently also been shown to respond to stimuli such as ABA or drought stress by alterations in gene expression (Taylor *et al.*, 1995; Shen *et al.*, 1995; Kaldenhoff *et al.*, 1995; del Mar Parra *et al.*, 1996; Hey *et al.*, 1997; Kopka *et al.*, 1997; Parcy & Giraudat, 1997) (see Sections 1.7.2, 1.7.4 and 3.1.1). Therefore, guard cells can be used as a model system to study the signalling pathways by which stimuli induce changes in gene expression as well as those by which they induce changes in guard cell turgor.

1.3 The control of stomatal aperture

1.3.1 Stomatal opening

Blue and red light, a low CO_2 concentration, the phytohormone, auxin and the fungal phytotoxin, fusicoccin all stimulate stomatal opening (see reviews by Mansfield

et al., 1990; Assmann *et al.*, 1993; Kearns & Assmann, 1993; Willmer & Fricker, 1996). It is well established that stomatal opening involves the rapid accumulation of osmotically active solutes in guard cells. (see review by Assmann, 1993; Willmer & Fricker, 1996) (see Section 1.2). It has been reported that during stomatal opening, guard cells extrude H^+, take up K^+ and Cl^- and produce $malate^{2-}$ (for reviews see Assmann, 1993; Willmer & Fricker, 1996). Osmotica are stored to a large extent in the vacuole, thus uptake and efflux across the tonoplast are integral to stomatal responses. These processes are generally poorly understood (see reviews by Raschke *et al.*, 1988; Willmer & Fricker, 1996). However, there are suggestions of the involvement of a vacuolar type (V-type) proton pump (Fricker & Willmer, 1990, Hedrich *et al.*, 1988), fast-activating-vacuolar (FV) channels (Hedrich & Schroeder, 1989) and vacuolar $malate^{2-}$ channels (see Willmer & Fricker, 1996).

The ion fluxes which occur across the guard cell plasma membrane during stomatal opening are much better understood than those which occur at the tonoplast membrane (for reviews see Assmann, 1993; Willmer & Fricker, 1996). There is a wealth of evidence which suggests that K^+ uptake generally proceeds via a chemiosmotic mechanism (Zeiger *et al.*, 1978; see reviews by Assmann, 1993; Willmer & Fricker, 1996) such that the pumping of H^+ out of the guard cell by plasma membrane H^+ATPases provides the driving force for K^+ uptake. H^+ extrusion hyperpolarizes the membrane potential (V_m) to values in the order of -150 to -250 mV (Lohse & Hedrich, 1992; Thiel *et al.*, 1992) thus creating an electrical gradient for passive K^+ uptake. The majority of this uptake occurs through inward K^+ channels (Schroeder *et al.*, 1987; Schroeder, 1988; Schroeder & Fang, 1991). The uptake of Cl^- by the guard cell requires coupling to a second system due to the opposition of uptake by the Cl^- gradient and the negative plasma membrane potential. Possible candidates for

the active uptake of Cl^- during stomatal opening are a nH^+/Cl^- symport system (see Sanders 1984; Assmann and Zeiger, 1987) or a Cl^-/OH^- antiport (Zeiger *et al.*, 1978; Assmann & Zeiger, 1987). It has been reported that despite the uptake of Cl^- (Penny & Bowling, 1974; MacRobbie, 1980; Schnabl & Raschke, 1980), and the conversion of starch to $malate^{2-}$ (for reviews see Outlaw, 1983; Robinson & Priess, 1985; Willmer & Fricker, 1996) there still seems to be a requirement for additional osmotica to account for the increase in guard cell osmotic pressure during stomatal opening (e.g. MacRobbie & Lettau 1980a & b). This role is possibly filled by sucrose (Outlaw & Manchester, 1979; Poffenroth *et al.*, 1992; Hite *et al.*, 1993; Talbott & Zeiger, 1988, 1993). Indeed, more recent work has shown that the osmotica used to maintain stomatal opening (sucrose, $malate^{2-}$ or Cl^-) may depend on the time of day and other environmental factors (Talbott & Zeiger, 1996).

1.3.2 Stomatal closure

Signals which induce stomatal closure include abscisic acid (see Section 1.4), a high CO_2 concentration, low humidity and under some conditions indoleacetic acid (IAA) (Davies & Mansfield, 1987; see reviews by Mansfield *et al.*, 1990; Assmann *et al.*, 1993; Kearns & Assmann, 1993). Stomatal closing involves loss of inorganic and/or organic solutes from the guard cell with a concomitant decrease in the guard cell osmotic pressure, thus increasing the water potential and causing loss of water until the turgor pressure decreases. However, stomatal closure is not the reverse of the processes occurring during opening; it invokes unique ion transport systems (Assmann, 1993). Events which bring about stomatal closure include the depolarization of the plasma membrane and the activation of voltage-dependent, outward K^+ channels; these events have been reported to be accompanied by anion efflux, by an increase in

cytosolic pH and by glycolytic removal of organic osmotica (see reviews by Assmann, 1993; Ward *et al.*, 1995; Willmer & Fricker, 1996) and in some cases by the elevation of $[Ca^{2+}]_i$ [see Section 1.5.2(b)]. Some of these processes will be briefly considered here, but the reader is also referred to Section 1.5 for a description of the mechanisms by which ABA may bring about stomatal closure.

Stomatal closing involves the release of K^+ from the guard cell vacuole (where the majority of it is believed to be stored in open stomata) possibly through both slow-vacuolar (SV)-type channels (Hedrich *et al.*, 1988; Amodeo *et al.*, 1994; Ward & Schroeder, 1994) and vacuolar K^+ (VK) channels (Ward & Schroeder, 1994). Stomatal closing also involves the efflux of K^+ across the plasma membrane through outward K^+ channels (see above) (Blatt 1988, 1991; see reviews Assmann, 1993; Ward *et al.*, 1995; MacRobbie 1997). The activation of these channels requires depolarization of the plasma membrane in order to remove the driving forces for K^+ uptake. This depolarization may occur through the efflux of anions across the plasma membrane down their electrochemical gradient and possibly also to a lesser extent by the inhibition of the plasma membrane H^+ATPase (see review by Assmann *et al.*, 1993; also see Kinoshita *et al.*, 1995; Goh *et al.*, 1996). The efflux of anions across the guard cell plasma membrane during stomatal closure may occur through rapid activating (R-type) anion channels (Hedrich *et al.*, 1990; Schroeder & Keller, 1992; Linder & Raschke, 1992) or slow activating (S-type) anion channels (Linder & Raschke 1992; Schroeder & Hedrich, 1989; Schroeder & Keller, 1992; Schroeder *et al.*, 1993). Because of their role in mediating anion efflux (directly) and in regulating K^+ efflux (indirectly through membrane depolarization) anion channels have been suggested as a central control mechanism for stomatal closure (Schroeder & Hagiwara, 1989). In addition to the outward K^+ channels, K^+ efflux at the plasma membrane may occur

through a hitherto unknown channel "leak" conductance; Thiel *et al.*, 1992) and stretch-activated channels (Cosgrove & Hedrich, 1991).

1.4 Abscisic acid

Abscisic acid (ABA) is a naturally-occurring phytohormone which is found in all parts of higher plants. ABA is a sesquiterpenoid which can be synthesized from mevalonic acid (see review by Milborrow, 1983). The structure of ABA is shown in Figure 1.2. It is believed that ABA is also produced in higher plants by an indirect (or C_{40}) pathway by the breakdown of xanthophylls (see reviews by Walton, 1980; Zeevaart & Creelman, 1988; Taylor, 1991; Zeevaart *et al.*, 1991; Giraudat *et al.*, 1994). Mutants deficient in ABA synthesis have contributed extensively to the elucidation of the biosynthetic pathways of ABA (see review by Giraudat *et al.*, 1994).

ABA was initially isolated in the 1960's as a compound which was involved in the regulation of abscission (see Addicott *et al.*, 1964). However, subsequently ABA has been shown to have an important role in the control of a wide range of essential physiological plant processes including seed development and plant adaptation to environmental stress (including water, salt, cold and wounding stress) (see reviews by Addicott, 1983; Davies & Jones, 1991; Hetherington & Quatrano, 1991; Bray, 1993; Chandler & Robertson, 1994; Giraudat *et al.*, 1994; Giraudat, 1995). It was suggested by Hetherington and Quatrano (1991) that a general role for ABA may be to "prepare tissue for entry into a new and different physiological state, perhaps by resetting the direction of cellular metabolism".

ABA has two distinct types of response in plants. ABA's actions at both the seed and vegetative level involve long term physiological changes and appear mainly to involve modifications in gene expression at the transcriptional level (see reviews by

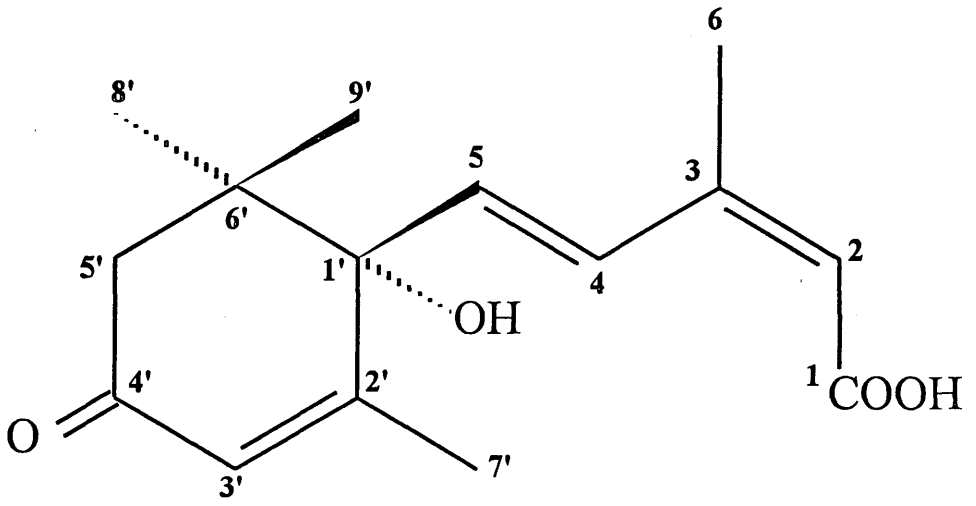


Figure 1.2 The molecular structure of the naturally occurring plant hormone ABA, (+)-(S)-ABA. The conventional numbering of the carbon atoms is shown (see Addicott, 1983).

Chandler & Robertson, 1987, Bray, 1991; Giraudat *et al.*, 1994, Giraudat, 1995) (see Section 1.7). In addition, ABA regulates guard cell turgor which involves rapid (within minutes) changes in the flux of ions across the guard cell plasma and tonoplast membranes (see reviews by Walton, 1980; Addicott, 1983; Davies & Jones, 1991; Hetherington & Quatrano, 1991; Assmann, 1993; Giraudat, 1994; Ward *et al.*, 1995; MacRobbie, 1997; McAinsh *et al.*, 1997) (see Section 1.5).

1.5 The effect of ABA on guard cell turgor

ABA is intimately involved in the regulation of guard cell turgor and hence stomatal aperture (for reviews see Walton, 1983; Zeevaart & Creelman, 1988; Davies & Jones, 1991; Hartung & Slovik, 1991; Assmann 1993; Ward *et al.*, 1995; MacRobbie, 1997). In the late 1960s and early 1970s it was discovered that ABA inhibited transpiration (Little & Eidt, 1968; Mittleheuser & van Steveninck, 1969; Jones & Mansfield, 1970) and accumulated in leaf tissue under water stress (Wright, 1969; Wright & Hiron, 1969). Several lines of evidence have since consolidated the link between water stress, ABA and the control of stomatal aperture. For example, ABA-deficient mutants display an increased tendency to wilt and/or display enhanced water loss in excised aerial parts. This is suggestive of a defect in stomatal regulation, but these mutants do close their stomata in response to exogenous ABA (for example, Koornneef *et al.*, 1982; see reviews by Giraudat *et al.*, 1994; Willmer & Fricker, 1996). In addition, in wild-type plants exogenous ABA has been shown to close stomata when applied to leaf surfaces or to the transpiration stream; it has been shown to inhibit stomatal opening and promote stomatal closure in detached epidermis; and also to prevent the swelling of guard cell protoplasts (GCPs) of *C. communis* and cause swollen GCPs of *C. communis* to shrink (see reviews by Walton, 1983; Davies &

Mansfield, 1983; Raschke, 1987; Zeevaart & Creelman, 1988; Davies & Zhang, 1991; Hartung & Slovik, 1991; Willmer & Fricker, 1996).

The effects of exogenous ABA on stomatal guard cells are documented by a wealth of electrophysiological studies and will be discussed in detail in Section 1.5.2. Briefly, at the plasma membrane ABA has been shown to: (i) inhibit the inward K^+ channel (Blatt, 1990; Thiel *et al.*, 1992) and the H^+ATPase (Goh *et al.*, 1996); (ii) activate the outward K^+ channel (Blatt, 1990; 1992; Lemitiri-Chlieh & MacRobbie, 1994; Schwartz *et al.*, 1994; Armstrong *et al.*, 1995) and anion channels (Pei *et al.*, 1997); and (iii) induce depolarization (Ishikawa *et al.*, 1983; Thiel *et al.*, 1992). Flux studies have indicated that ABA also activates tonoplast ion channels, thereby allowing the efflux of K^+ and Cl^- from the vacuole (MacRobbie 1995a & b). For some ABA-induced changes in ion channel activity, knowledge of the mechanisms of regulation of particular ion channels allows plausible hypotheses to be drawn up for the sequence of events between ABA perception and changes in ion channel activity. One such hypothesis/model is based around Ca^{2+} acting as a second messenger in such a system (McAinsh *et al.*, 1997) and is described in Section 1.5.2.

1.5.1 Perception of ABA by the guard cell

ABA can enter epidermal cells by passive diffusion of protonated ABA (ABAH) across the plasma membrane (Kaiser & Hartung, 1981) and also to some extent via saturable ABA-transporters (Daeter & Hartung, 1993). Schwartz *et al.* (1994) showed that the uptake of [^{14}C]-ABA at high pH was decreased but not eliminated, thus suggesting that guard cells of *C. communis* have a significant level of carrier mediated uptake of ABA^- in addition to diffusive uptake of ABAH.

The first event in the ABA signal transduction pathway leading to a change in guard cell turgor is that of ABA perception by the guard cell. The ABA receptor in guard cells has not been isolated and there is much controversy as to its location. Early work by Hornberg and Weiler (1984) using photoaffinity labelling suggested the presence of extracellular ABA receptors. However, this work still lacks confirmation. Evidence has accumulated for both internal and external ABA perception sites in guard cells (see below). Indeed, recently it has been proposed that ABA has multiple sites of action (Anderson *et al.*, 1994; MacRobbie *et al.*, 1995a).

1.5.1(a) Intracellular site of ABA perception

An intracellular localization of the ABA receptor and hence a requirement for ABA uptake was suggested by work showing that lowering the pH [and so increasing the concentration of ABAH which readily diffuses across the plasma membrane (Hartung & Slovik, 1991)] enhanced the effect of ABA on stomatal aperture (stomatal closure: Ogunkami *et al.*, 1973; Paterson *et al.*, 1988; Schwartz *et al.*, 1994; inhibition of stomatal opening: Anderson *et al.*, 1994). In addition, application of external ABA did not reduce the inward K^+ currents at pH 8.0 but its effectiveness increased as the pH decreased (Schwartz *et al.*, 1994).

The application of ABA directly to the guard cell cytoplasm by microinjection (Schwartz *et al.*, 1994) or release of microinjected caged ABA by UV photolysis (Allan *et al.*, 1994) results in stomatal closure, thus suggesting an intracellular ABA perception site. Schwartz *et al.* (1994) reported that the microinjection of between 2.4 to 14.7 μM ABA was more effective at promoting stomatal closure than an external application of 10 μM ABA at pH 8.0. In addition, Schwartz *et al.* (1994) using patch clamp techniques reported that a direct application of ABA to the guard cell cytosol

inhibited inward K^+ currents in the absence of external ABA (and to an equal extent at all external values of pH tested). In addition, the application of ABA to the cytoplasmic face of an isolated patch of guard cell plasma membrane activated outward K^+ channels (Schauf & Wilson, 1987).

1.5.1(b) Extracellular site of ABA perception

As stated above, early work by Hornberg and Weiler (1984) suggested the presence of extracellular ABA receptors. Evidence for an extracellular guard cell ABA binding site is also suggested by data showing that ABA induces stomatal closure at pH 8.0 to the same extent as at pH 5.0 (Hartung, 1983). In addition, ABA which was microinjected into the cytosol of closed guard cells (resulting in cytosolic concentrations of 50 to 200 μM ABA) did not inhibit light-induced stomatal opening, whereas an extracellular application of 10 μM ABA did inhibit stomatal opening by 98%. (Anderson *et al.*, 1994). This suggests that ABA has to be perceived extracellularly to inhibit stomatal opening; the presence of ABA only inside the guard cell was not sufficient to inhibit stomatal opening.

Reports of a role for heterotrimeric G-proteins (Armstrong & Blatt, 1995; Assmann, 1996) and IP_3 (Lee *et al.*, 1996) in ABA signalling pathways in guard cells [see Section 1.5.3(a)] also suggest that there is an extracellular site of ABA perception. This is because heterotrimeric G-proteins and IP_3 are associated with external, plasma membrane-bound receptors in animal cells (see Section 1.1; Alberts *et al.*, 1994).

1.5.1(c) Multiple sites of ABA perception

The study by Anderson *et al.* (1994) showed that external ABA was more effective at inhibiting stomatal opening at low pH thus suggesting an internal site of

ABA perception [see Section 1.5.1(a)]. But Anderson *et al.* (1994) also reported that an application of internal ABA in the absence of external ABA had no effect on inhibition of stomatal opening [see Section 1.5.1(b)]. These data therefore suggest that both intracellular and extracellular sources of ABA are required for ABA-induced changes in guard cell turgor. It has been suggested that the conflicting results of Anderson *et al.* (1994), which showed internal ABA had no effect on stomatal opening, and Allan *et al.* (1994) and Schwartz *et al.* (1994), which showed that internal ABA induced stomatal closure, can be reconciled by the idea that ABA-induced promotion of stomatal closure and inhibition of stomatal opening involve a differential relative stimulation of the two ABA perception sites (for more discussion, see Giraudat, 1995).

Tracer flux studies involving the measurement of [$^{86}Rb^{+}$] efflux from preloaded isolated guard cells in epidermal strips also suggest the importance of both intracellular and extracellular ABA in regulating the ionic composition of the guard cell (MacRobbie, 1995a & b; and see review by MacRobbie, 1997). It was suggested by MacRobbie (1995a & b) that there is both regulation of tonoplast ion channels by internal ABA and regulation of a channel responsive to ion content by external ABA.

In conclusion, at present the available data point to multiple sites of ABA action, inside and outside the guard cell (see above). Indeed, multiple sites of action have also been suggested for the plant hormone auxin (Barbier-Brygoo *et al.*, 1989, 1991; Thiel *et al.*, 1994; Claussen *et al.*, 1996). However, it is worth mentioning at this point that the data described above concerning the effect of extracellular pH on the biological activity of ABA may have to be reassessed. This is because recent work by Wilkinson & Davies (1997) suggests that lowering the extracellular pH (in the absence of ABA) leads to a decrease in stomatal aperture width in detached epidermis. Therefore the pH

effects described in Section 1.5.1(a) may not provide unequivocal evidence for an intracellular site of ABA perception.

1.5.1(d)Other characteristics of the ABA receptor

Results from tracer flux studies showed that short pulses of ABA (of less than 2 min) are sufficient to trigger efflux of K^+ and Cl^- from guard cells in epidermis of *C communis* (MacRobbie 1983, 1984). In addition, MacRobbie (1990) found that the putative receptors for ABA were rapidly desensitized and unable to respond to subsequent applications of ABA.

1.5.2 The role of calcium ions in the ABA signalling pathway which terminates in a change in guard cell turgor

The calcium ion (Ca^{2+}) plays an important role as second messenger in animal cell signalling (see Tsien & Tsien, 1990; Berridge *et al.*, 1988; Berridge, 1993; Fewtrell, 1993; Clapham, 1995) (see Section 1.1) and more recently this role has been extended to plant cells (for recent reviews see Johannes *et al.*, 1991; Poovaiah & Reddy, 1993; Bush , 1993; Bowler & Chua, 1994; Gilroy & Trewavas, 1994; Webb *et al.*, 1996b; McAinsh *et al.*, 1997). The role of Ca^{2+} in ABA-induced promotion of stomatal closure and inhibition of stomatal opening was initially suggested by the fact that: (i) the Ca^{2+}-chelator, EGTA and Ca^{2+}-channel blockers had an inhibitory effect on ABA-induced changes in guard cell turgor in *C. communis* (De Silva *et al.*, 1985a & b); (ii) that the Ca^{2+} ionophore, A2371, stimulated stomatal closure (De Silva *et al.*, 1985b); and (iii) that synergism was found for the effect of ABA and Ca^{2-} on stomatal opening in this species (De Silva *et al.*, 1985a). In addition, an application of exogenous Ca^{2+} induced both promotion of stomatal closure and inhibition of stomatal

opening in a similar manner to ABA (Schwartz *et al.*, 1985; Smith & Willmer, 1988). More recent and direct investigations, involving measurements of guard cell $[Ca^{2+}]_i$ by the microinjection of Ca^{2+}-indicator dyes into the cytosol of guard cells in isolated epidermis has led to the establishment of the involvement of Ca^{2+} in the ABA signalling pathway leading to changes in stomatal guard cell turgor (McAinsh *et al.*, 1990, 1992; Schroeder & Hagiwara, 1990; Irving *et al.*, 1992; Allan *et al.*, 1994; Staxén *et al.*, 1996, 1997; see reviews by Webb *et al.*, 1996 and McAinsh *et al.*, 1997).

Direct measurements of guard cell $[Ca^{2+}]_i$ have revealed that ABA stimulates an increase in $[Ca^{2+}]_i$ in guard cells (McAinsh *et al.*, 1990, 1992; Schroeder & Hagiwara, 1990; Gilroy *et al.*, 1991; Irving *et al.*, 1992; Allan *et al.*, 1994; Staxén *et al.*, 1996, 1997) which precedes stomatal closure by 5-10 min (McAinsh *et al.*, 1990). However, the percentage of guard cells which have been reported to exhibit an increase in $[Ca^{2+}]_i$ in response to ABA varies from 30% (Gilroy *et al.*, 1991) to 70-80% (McAinsh *et al.*, 1990, 1992; Irving *et al.*, 1992). Several hypotheses have been proposed to explain this variation. For example, it has been suggested that the effect of ABA on guard cell $[Ca^{2+}]_i$ may depend on the temperature at which plants are grown (Allan *et al.*, 1994). It has also been proposed that the variation may reflect the methodology used to measure $[Ca^{2+}]_i$ in guard cells (McAinsh *et al.*, 1992). Many studies of the effects of ABA on guard cell $[Ca^{2+}]_i$ report changes in average $[Ca^{2+}]_i$ and therefore, any localized changes which occur in response to ABA may be missed (McAinsh *et al.* 1992; see review by MacRobbie, 1997).

There is direct and indirect evidence to suggest that the source of the ABA-induced increase in guard cell $[Ca^{2+}]_i$ involves both influx of Ca^{2+} across the guard cell plasma membrane and the release of Ca^{2+} from the guard cell vacuole (see review by McAinsh *et al.*, 1997). The indirect evidence comes from the use of calcium channel

blockers (De Silva *et al.*, 1995a; McAinsh *et al.*, 1991a) and [$^{45}Ca^{2+}$]-tracer flux studies (MacRobbie, 1989), whereas more direct evidence comes from imaging changes in guard cell $[Ca^{2+}]_i$ in response to ABA (McAinsh *et al.*, 1992). In addition, Schroeder and Hagiwara (1990) reported that ABA-induced increases in guard cell $[Ca^{2+}]_i$ coincided with Ca^{2+} influx through ABA-activated non-selective ion channels in the plasma membrane. Further evidence has come from the fact that the microinjection of known intracellular Ca^{2+} mobilizing second messengers in plants, such as IP_3 (Gilroy *et al.*, 1990) and cADPR (McAinsh *et al.*, 1996a) into guard cells induces an elevation of $[Ca^{2+}]_i$ and a reduction in guard cell turgor.

Many different types of Ca^{2+} permeable channels have been identified in both the plasma and vacuolar membranes of guard cells which may allow such fluxes to occur in response to ABA (for a review see McAinsh *et al.*, 1997). In the plasma membrane, these include stretch-activated Ca^{2+} selective channels (Cosgrove & Hedrich, 1991), the inwardly rectifying K^+ channel which exhibits limited permeability to Ca^{2+} (Fairley-Grenot & Assmann, 1992a) and non-selective voltage-gated Ca^{2+} channels (Schroeder & Hagiwara, 1990). Ca^{2+}-mobilizing pathways characterized in the vacuolar membrane include the SV channel which exhibits Ca^{2+} selectivity (Ward & Schroeder, 1994, Allan & Sanders, 1995), cADPR-activated Ca^{2+}-permeable channels (McAinsh *et al.*, 1996a) and voltage-dependent Ca^{2+} channels (Allen & Sanders, 1995). In addition, as described above, the microinjection of IP_3 into guard cells results in an elevation of $[Ca^{2+}]_i$ and stomatal closure (Gilroy *et al.*, 1990). There is evidence of an increase in the levels of IP_3 in guard cells in response to ABA (Lee *et al.*, 1996) [see Section 1.5.3(a)] Therefore, IP_3-gated, Ca^{2+}-permeable channels in the guard cell vacuolar membrane may constitute a further Ca^{2+}-release pathway by which ABA can induce an increase in guard cell $[Ca^{2+}]_i$ (see McAinsh *et al.*, 1997).

ABA-induced changes in guard cell turgor (stomatal movements) involve changes in the activity of ion channels in both the plasma and vacuolar membranes (for reviews see Assmann, 1993; Ward *et al.*, 1995; McAinsh *et al.*, 1997). The activity of several of these channels is modulated by $[Ca^{2+}]_i$ (see reviews by Ward *et al.*, 1995; Willmer & Fricker, 1996; MacRobbie, 1997; McAinsh *et al.*, 1997). Recently, McAinsh *et al.* (1997) have described one possible model for the role of $[Ca^{2+}]_i$ in ABA-induced stomatal closure. This model is summarized in Figure 1.3 and described in more detail below [Sections 1.5.2(a) and(b)].

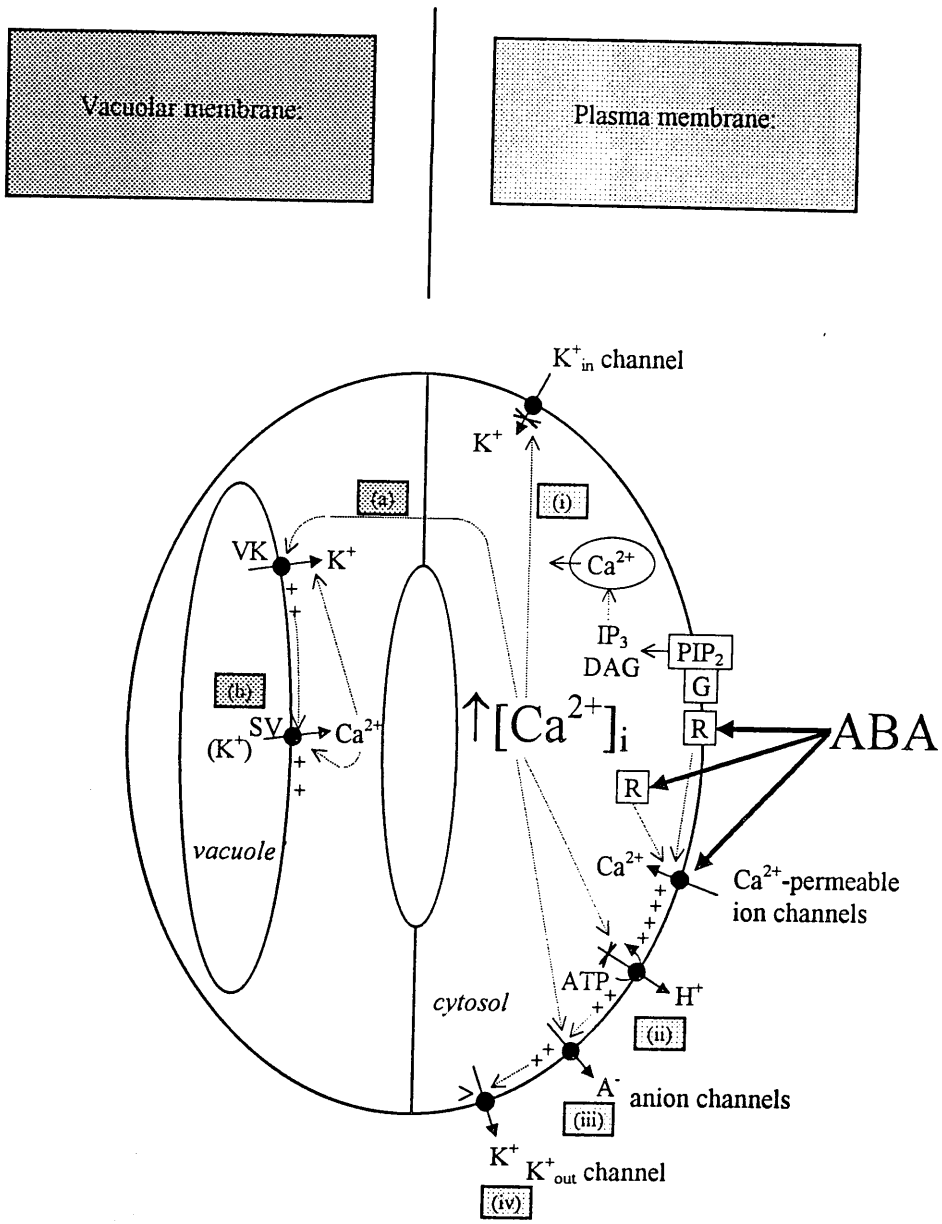
1.5.2(a) Plasma membrane ion channels

An ABA-induced increase in $[Ca^{2+}]_i$ may affect guard cell plasma membrane ion channels. These effects include:

(i) Inhibition of the inward K^+ channel. Increases in guard cell $[Ca^{2+}]_i$ have been reported to inhibit the inward K^+ channel in the guard cell plasma membrane (Schroeder & Hagiwara, 1989; Blatt *et al.*, 1990; Fairley-Grenot & Assmann 1992b; Lemtiri-Chlieh & MacRobbie 1994). This inhibition would prevent an accumulation of K^+ in the guard cell which occurs during stomatal opening (Schroeder *et al.*, 1987; Schroeder & Hagiwara, 1989; Thiel *et al.*, 1992). Consistent with this, is the fact that ABA has been shown to inhibit inward K^+ currents in the guard cell (Blatt 1990; Thiel *et al*, 1992). Despite this correlation, the ABA-induced inhibition of the guard cell inward K^+ current may be of secondary importance in stomatal closure (see review by Ward *et al.*, 1995).

(ii) Inhibition of the plasma membrane H^+-ATPase. As described in Section 1.3.2 the plasma membrane H^+-ATPase has been reported to be reversibly inhibited by physiological concentrations of Ca^{2+} (Kinoshita *et al.*, 1995) and more recently it has

Figure 1.3 A possible model of ABA signal transduction during stomatal closure. Perception of ABA by an extracellular or an intracellular receptor (R) causes an increase in $[Ca^{2+}]_i$ through Ca^{2+} influx and release of Ca^{2+} from intracellular stores. The latter may occur via the action of G proteins (G) and the cleavage of phosphatidylinositol (4, 5) bisphosphate (PIP_2) to inositol (1, 4, 5) trisphosphate (IP_3) and 1, 2-diacylglycerol (DAG) or through Ca^{2+}-induced Ca^{2+} (CICR) release at the vacuolar membrane (see below). The effects of an ABA-induced increase in guard cell $[Ca^{2+}]_i$ on ion channel activity in the plasma membrane ▒ [(i) to (iv)] and vacuolar membrane ▒ [(a) and (b)] are shown. At the guard cell plasma membrane an ABA-induced increase in $[Ca^{2+}]_i$ could inhibit directly the inward rectifying K^+ channel (i) and the H^+-ATPase (ii). It could activate anion channels (iii) both directly and through plasma membrane depolarization. The plasma membrane depolarization needed to activate the anion channels could be induced directly by an increase in $[Ca^{2+}]_i$ and also indirectly by the inactivation of the H^+-ATPase [see (ii)]. Activation of the anion channels leads to anion efflux (and hence contributes to a reduction on guard cell turgor) and sustained depolarization of the plasma membrane. The sustained depolarization could activate the outward K^+ channel in the plasma membrane (iv). This would lead to K^+ efflux and consequently a reduction in guard cell turgor. At the guard cell vacuolar membrane an ABA-induced increase in $[Ca^{2+}]_i$ could activate directly the vacuolar K^+ channel (VK channel) (a) thus allowing K^+ efflux from the vacuole. It could also activate the SV channels both directly and indirectly (b). The indirect activation could occur through the shifting of the vacuolar membrane potential to less negative values on the cytosolic side due to the Ca^{2+}-stimulated K^+ efflux through the VK channels [see (a)]. The activation of the SV channels results in an efflux of Ca^{2+} and also K^+ out of the vacuole. This CICR [see Section 1.5.2(b)] can further fuel the Ca^{2+}-dependent changes, which occur in the plasma and vacuolar membranes. The efflux of K^+ from the vacuole through both the VK and SV channels is an essential component of the efflux of K^+ out of the guard cell, which is required to reduce guard cell turgor. (Model modified from McAinsh *et al.*, 1997).


Vacuolar membrane:
Plasma membrane:
K^+_{in} channel
K^+
(a)
(i)
VK
K^+
Ca^{2+}
IP_3
DAG
PIP_2
G
R
ABA
(b)
SV
(K^+)
Ca^{2+}
$\uparrow[Ca^{2+}]_i$
R
vacuole
Ca^{2+}
Ca^{2+}-permeable
ion channels
ATP
H^+
cytosol
(ii)
A^-
anion channels
(iii)
K^+
K^+_{out} channel
(iv)

been reported that ABA inhibits this pump (Goh *et al.*, 1996). Inhibition of the plasma membrane H^+-ATPase would lead to depolarization of the plasma membrane.

(iii) Activation of the S-type anion channels. Recently, it has been shown that ABA directly activates S-type anion channels in guard cells of *Arabidopsis thaliana* (Pei *et al.*, 1997). These channels have also been reported to be activated directly by an increase in guard cell $[Ca^{2+}]_i$ and indirectly by depolarization (Schroeder & Hagiwara, 1989; Schroeder & Keller, 1992) (see Section 1.3.2). Thus an ABA-induced increase in guard cell $[Ca^{2+}]_i$ may activate these channels directly and also indirectly through membrane depolarization. Membrane depolarization may be induced directly by an increase in guard cell $[Ca^{2+}]_i$ and indirectly through Ca^{2+}-induced inhibition of the H^+-ATPase as described above in *(ii)*. Anion efflux through the activated anion channels leads to a sustained depolarization of the plasma membrane (Schroeder & Hagiwara, 1989; Linder & Raschke, 1992; Schroeder & Keller, 1992) which is essential for stomatal closure (Schroeder & Hagiwara, 1989; see below). It also contributes directly to a reduction in guard cell turgor through the loss of Cl^- from the guard cell (see Section 1.3.2). As stated earlier, it has been suggested that anion channels in the guard cell plasma membrane are a central control mechanism for stomatal closure (Schroeder & Hagiwara, 1989; see Section 1.3.2).

(iv) Indirect activation of the outward K^+ channel. The outward K^+ channel is not directly activated by an increase in guard cell $[Ca^{2+}]_i$, rather it can be activated by long term depolarization of the plasma membrane which can be brought about by an ABA-induced increase in guard cell $[Ca^{2+}]_i$ as described in *(ii)* and *(iii)* above. It has been proposed that the S-type anion channels, unlike the R-type anion channels (see Section 1.3.2) can produce the sustained depolarization required to drive K^+ efflux via voltage gated K^+ channels (Schroeder & Hagiwara, 1989). The resultant efflux of K^+ from the

guard cell, along with the efflux of anions described in *(iii)*, is essential for a reduction in guard cell turgor (see Section 1.3.2). As well as being activated by a long term depolarization of the plasma membrane (which is brought about by Ca^{2+}) the outward K^+ channel in the guard cell plasma membrane is pH sensitive (Blatt & Armstrong, 1993) [see Section 1.5.3(b)]. It has been proposed by Blatt and Armstrong (1993) that the ABA-induced rise in cytoplasmic pH is responsible for the activation of the outward K^+ channel [see Blatt & Grabov, 1997a & b, and Section 1.5.3(b) for a more detailed discussion]. One possible framework for the involvement of an increase in pH in ABA-induced stomatal closure in relation to a Ca^{2+}-based signalling pathway has been described by McAinsh *et al.* (1997) [also see Section 1.5.3(b)]. During ABA-induced stomatal closure the long-term release of K^+ through VK channels [see Section 1.5.2(b)] could be driven by the activity of vacuolar H^+ pumps (Ward *et al.*, 1995). Increased pumping of H^+ into the vacuole would lead to the alkalinization of the cytosol which has been observed in response to ABA (Irving *et al.*, 1992) and has been suggested to activate the outward K^+ channel in the guard cell plasma membrane (Blatt 1990, 1992, Lemitiri-Chlieh & MacRobbie, 1994, Schwartz *et al.*, 1994; Armstrong *et al.*, 1995). In addition, it would cause a shift in the vacuolar membrane potential to more negative potentials on the cytosolic side. Voltage-gated Ca^{2+}-release channels in the vacuolar are activated under these conditions (Allen & Sanders, 1994). Therefore, this provides an additional pathway for Ca^{2+} release from the vacuole which may also contribute to ABA-induced increases in $[Ca^{2+}]_i$ during stomatal closure.

An addition to the model discussed by McAinsh *et al.* (1997) is the involvement of plasma membrane, stretch-activated channels in the ABA response, as proposed recently by MacRobbie (1997). This idea has come from observations made during

tracer flux experiments (MacRobbie, 1995a & b) and suggests that ABA induces a change in a regulatory system in the guard cell which controls cell turgor; i.e. ABA changes the "set-point" of such a system. It was suggested that an initial reaction involving a stretch-sensitive plasma membrane channel can lead to the release of internal Ca^{2+} which then operates in the signalling cascade which leads to an alteration in guard cell turgor (see review by MacRobbie, 1997).

1.5.2(b) Vacuolar membrane ion channels

An ABA-induced increase in $[Ca^{2+}]_i$ may also affect guard cell vacuolar ion channels. These effects include:

(i) Activation of the VK channel. The highly selective VK channels in the vacuolar membrane (see Section 1.3.2) are rapidly activated by physiological increases in $[Ca^{2+}]_i$ (Ward & Schroeder, 1994). This suggests that an ABA-induced increase in $[Ca^{2+}]_i$ would allow efflux of K^+ out of the vacuole. As was stated above (see Section 1.3.2), K^+ efflux from the vacuole is an essential component of the efflux of K^+ out of the guard cell required to reduce guard cell turgor (MacRobbie, 1981). In addition, K^+ efflux from the vacuole would lead to a shift of the vacuolar membrane potential to less negative values on the cytosolic side This alteration in vacuolar membrane potential is believed to be important for the activation of SV channels [see *(ii)*, below].

(ii) Activation of the SV channels. SV channels are activated directly by elevated $[Ca^{2+}]_i$ (Hedrich & Neher, 1987; Ward & Schroeder, 1994; Ward 1995). In addition, the shift of the vacuolar membrane potential to less negative values on the cytosolic side via the Ca^{2+}-induced K^+ efflux from the vacuole [see *(i)*] has been proposed to activate the SV channels (Ward & Schroeder, 1994; Ward *et al.*, 1995). The activation of the SV channels results in an efflux of Ca^{2+} and K^+ out of the vacuole (Ward &

Schroeder, 1994; Ward *et al.*, 1995). As described above in Section 1.5.2(b)*(i)*, release of K^+ from the vacuole is an essential component of the efflux of K^+ out of the guard cell required to reduce guard cell turgor (MacRobbie, 1981). The release of Ca^{2+} from the vacuole leads to an increase in $[Ca^{2+}]_i$ and this may fuel the additional release of Ca^{2+} from the vacuole through SV channels. This process is termed Ca^{2+}-induced Ca^{2+} release (CICR) (Ward & Schroeder, 1994; Ward *et al.*, 1995). During ABA-induced stomatal closure, an initial Ca^{2+} influx through Ca^{2+}-permeable channels in the plasma membrane or Ca^{2+} release from intracellular stores via the action of IP_3 (see Section 1.1) or cADPR (McAinsh *et al.*, 1996a) could trigger CICR through SV channels. The resultant increase in $[Ca^{2+}]_i$ could contribute to the regulation of plasma membrane ion channels required for stomatal closure (see Figure 1.3).

1.5.3 Other putative components of the signalling pathway(s) by which ABA controls guard cell turgor

1.5.3(a) Ca^{2+}-based components

There is a growing body of evidence that the link between the ABA receptor(s) and the Ca^{2+}-dependent part of the signalling pathway in guard cells is mediated by the phosphoinositide cycle (see reviews by Webb *et al.*, 1996b; Willmer & Fricker, 1996; McAinsh *et al.*, 1997; MacRobbie *et al.*, 1997) (see Section 1.1). The data which support this possibility will be considered briefly here, but the reader is advised to consult recent reviews by Webb *et al.*, (1996a), Willmer & Fricker (1996), McAinsh *et al.* (1997) for a more in depth discussion.

There is some evidence to suggest that heterotrimeric G-proteins have a role in the ABA signalling pathway(s) in guard cells which leads to stomatal closure (Fairley-Grenot & Assmann, 1991; Kelly *et al.*, 1995; Assmann, 1996), although the activation

of plant phospholipase C by a G-protein (see Section 1.1) in guard cells has not been demonstrated. Additionally, there are data to suggest that the involvement of G-proteins in the signalling pathway(s) which control guard cell turgor may also involve coupling to a seven-trans-membrane span (7TMS) receptor (Armstrong & Blatt, 1995), as reported in animal cell signalling systems (see Alberts et al., 1994).

A wide range of phosphoinositides have been characterized in guard cells (Parmar & Brearly, 1993) and their turnover has recently been reported to be stimulated by ABA (Lee *et al.*, 1996). However, there is only circumstantial evidence to link an ABA-induced increase in IP_3 levels to release of Ca^{2+} from internal stores via an IP_3 receptor and to consequent stomatal closure (Gilroy *et al.*, 1990; Johannes *et al.*, 1991). In addition, 3-phosphorylated phosphatidyl inositols are rapidly metabolized in guard cells (Parmar & Brearly, 1995), although any possible role for these lipids in signalling remains to be established (in either plant or animal cells) (see MacRobbie, 1997). There are also data which point to an involvement of free fatty acids (Willmer *et al.*, 1978; Lee *et al.*, 1994) and diacylglycerols (Lee & Assmann, 1991) in the regulation of guard cell turgor.

As discussed in Section 1.5.2, the second messenger cADPR could be a component of the ABA signalling pathway which leads to a reduction in guard cell turgor (McAinsh *et al.*, 1996a). In animal cells, cADPR mobilizes Ca^{2+} from internal stores by activating ryanodine receptors (Galione, 1993; Sitsapesan *et al.*, 1994). This provides a Ca^{2+} release pathway which is discrete from IP_3-elicited Ca^{2+} mobilization. In guard cells, cADPR has been reported recently to activate Ca^{2+} permeable channels in the vacuolar membrane, increase guard cell $[Ca^{2+}]_i$ and induce stomatal closure (McAinsh *et al.*, 1996a; Leckie *et al.*, 1997).

There is much evidence to suggest that CaM (see Section 1.1) plays an important role in higher plant signal transduction (see reviews by Poovaiah & Reddy, 1993; Bowler & Chua, 1994). More specifically, CaM is present in high levels in guard cells and a number of calmodulin-binding proteins are also detectable in guard cells (Ling & Assmann, 1992; Cotelle *et al.*, 1996). Several lines of evidence have suggested that CaM plays a role in the ABA signalling pathway which leads to stomatal closure (De Silva *et al.*, 1985b; Donovan *et al.*, 1985). Some of the proteins activated by Ca^{2+}-CaM are likely to be protein kinases and phosphatases (see below) which modulate the activity of other enzymes by covalent phosphorylation. There is some evidence of both Ca^{2+}/CaM-mediated and also Ca^{2+}-independent phosphorylation and dephosphorylation events which could regulate guard cell ion channel activity in response to ABA. (Luan *et al.*, 1993; Thiel & Blatt, 1994; Cousson *et al.*, 1995; Cotelle *et al.*, 1996; Pei *et al.*, 1997; Mori & Muto, 1997).

Electrophysiological studies indicate that inward K^+ channels, outward K^+ channels and anion channels may be modulated by protein phosphorylation and dephosphorylation (Luan *et al.*, 1993; Li *et al.*, 1994a; Thiel & Blatt, 1994; Armstrong *et al.*, 1995; Schmidt *et al.*, 1995; Pei *et al.*, 1997; see review by MacRobbie, 1997). For example, recent evidence for the involvement of protein phosphatases in the ABA signalling pathway which terminates in a reduction in guard cell turgor comes from studies using the *abi1* and *abi2* mutants of *A. thaliana* (for more details of these mutants see Section 4.1.1). These mutants are wilty and unresponsive to ABA (Koornneef *et al.*, 1984). It has been reported that the *ABI1* and more recently that the *ABI2* gene encodes a serine/threonine phosphatase type 2C protein (Meyer *et al.*, 1994; Leung *et al.*, 1994; Leung *et al.*, 1997). The ABA-induced activation of slow anion channels in guard cells and ABA-induced stomatal closure was reported to be

abolished in these *abi* mutants of *A. thaliana* thus suggesting an important role for phosphatases in ABA-regulated guard cell turgor (Pei *et al.*, 1997). These data have led to the proposal of new models for ABA signalling in guard cells (Pei *et al.*, 1997), although the precise roles of protein kinases and phosphatases in such pathways are far from understood (see Merlot & Giraudat, 1997; Pei *et al.*, 1997; MacRobbie, 1997). In addition, recently it has been shown that inhibitors of protein kinases and phosphatases reduced the effectiveness of ABA at stimulating changes in guard cell turgor in epidermal peels of *Pisum sativum L. Argenteum* (Hey *et al.*, 1997). Interestingly, although okadaic acid, an inhibitor of protein phosphatases type 1 and 2A, reduced the inhibitory effects of ABA on stomatal opening it enhanced the effect of ABA on stomatal closure (Hey *et al.*, 1997). This suggests the involvement of multiple protein phosphorylation/dephosphorylation steps in the ABA signalling pathways terminating in changes in guard cell turgor.

1.5.3(b) Other components

Evidence exists for a role for cyclic AMP (cAMP) (Curvetto & Delmastro, 1990; Morsucci *et al.*, 1991, 1992; Assmann, 1995) and pH (Blatt 1990, 1992, Irving *et al.*, 1992; Blatt & Armstrong, 1993; Lemitiri-Chlieh & MacRobbie, 1994, Schwartz *et al.*, 1994; Armstrong *et al.*, 1995; Blatt & Grabov, 1997a & b) in the control of guard cell turgor. ABA has been shown to increase the cytosolic pH of guard cells by about 0.04 to 0.3 units with a lag time of 2 min (Irving *et al.*, 1992). As stated in Section 1.5.2(a), the outward K^+ channel in the guard cell plasma membrane is pH sensitive (Blatt & Armstrong, 1993). Therefore, it has been proposed by Blatt and Armstrong (1993) that the ABA-induced rise in cytoplasmic pH is responsible for the activation of the outward K^+ channel. Indeed there are several reports which suggest

that ABA-induced alkalinization of the guard cell cytosol plays a role in the ABA-induced enhancement of the activity of the outward K^+ channel (Blatt 1990, 1992, Lemitiri-Chlieh & MacRobbie, 1994, Schwartz *et al.*, 1994; Armstrong *et al.*, 1995). This may constitute a further mechanism by which ABA may stimulate K^+ efflux from guard cells, in addition to the Ca^{2+}-based mechanism described in Section 1.5.2 (see McAinsh *et al.*, 1997). In addition, the inward K^+ channel and possibly anion channels in the plasma membrane have been reported to be pH sensitive (Blatt & Armstrong, 1993). The mechanism by which ABA induces an increase in cytoplasmic pH is unclear but a possible framework for the involvement of pH as a second messenger in ABA-induced stomatal closure has been proposed by Blatt and Grabov (1997a & b).

1.6 The effect of ABA on gene expression

1.6.1 ABA-responsive gene expression in plants

Over 150 ABA-responsive genes have been isolated from a variety of plant species (see reviews by Skriver & Mundy, 1990; Quatrano *et al.*, 1992; Bray, 1993; Hull *et al.*, 1993; Chandler & Robertson, 1994; Delseny *et al.*, 1994). Most of these genes were originally identified as being expressed during late seed development and/or in the vegetative tissues of plants exposed to environmental stress. ABA-responsive genes whose expression is up-regulated by ABA include *Lea* (late embryogenesis abundant) and *Rab* (responsive to ABA) genes isolated from several plant species (see reviews by Mundy & Chua, 1988; Chandler & Robertson, 1994), *CDeT* (*Craterostigma* desiccation tolerance) or dsp (desiccation stress protein) genes, isolated from the resurrection plant *Craterostigma plantagineum* (Bartels *et al.*, 1990; Piatkowski *et al.*, 1990), and dehydrin (dehydration induced) genes (Close *et al.*, 1989; Dure, 1993; Chandler & Robertson, 1994). The precise function of the products of

these genes is as yet unknown although some of them code for hydrophilic proteins (which contain a stretch of serines and conserved lysine-rich repeat motifs) which may be important in desiccation tolerance (Skriver & Mundy, 1988; Bray, 1991; Piatkowski *et al.*, 1991; Chandler & Robertson, 1994). Although the majority of reported ABA-responsive genes are positively regulated by ABA, there are also genes which are negatively regulated by ABA (Quatrano *et al.*, 1983; Jacobsen & Beach, 1985; Zwar *et al.*, 1986; Jacobsen & Close, 1991). For example, ABA suppresses the gibberellic acid (GA_3)-induced increase in transcription of the α-amylase gene in cereal aleurone (Zwar *et al.*, 1986; Jacobsen & Beach, 1985). Negative regulation of gene expression by ABA may be equally important as positive regulation when the plant is preparing to cope with entry into a new physiological state (Hetherington & Quatrano, 1991; Chandler & Robertson, 1994; Kopka *et al.*, 1997).

A number of genes have been isolated that show expression in correlation with the occurrence of ABA and/or stress such as osmotic stress or cold. Studies on the regulation and expression of these genes make clear that ABA is involved in at least part of the complex regulation of stress tolerance (Chandler & Robertson, 1994). However, the responsiveness of these genes to applied ABA does not necessarily imply that all of them are primarily regulated by endogenous ABA *in vivo* (Chandler & Robertson, 1994). Nevertheless, these target genes represent promising tools for investigating the cellular components that relay the ABA signal to the nucleus in plant cells (see Section 1.6.3, below).

1.6.2 ABA-responsive gene expression in guard cells

In recent years there have been an increasing number of studies which have examined gene expression in guard cells (Terryn *et al.*, 1993; Müller-Röber *et al.*, 1994; Kaldenhoff *et al.*, 1995; Nakamura *et al.*, 1995; Shen *et al.*, 1995; Taylor *et al.*,

1995; Wang & Cutler, 1995; del Mar Parra *et al.*, 1996; Hentzen *et al.*, 1996; Hey *et al.*, 1997; Kopka *et al.*, 1997; Parcy & Giraudat, 1997) including those which have investigated the effect of ABA on guard cell gene expression (Kaldenhoff *et al.*, 1995; Shen *et al.*, 1995; Taylor *et al.*, 1995; del Mar Parra *et al.*, 1996; Hey *et al.*, 1997; Parcy & Giraudat, 1997) (see Table 1.1). Taylor *et al.* (1995) were the first to report that the guard cell was competent to relay an ABA signal from the site of perception to the nucleus. They showed that GUS expression driven by the promoter of the *CDeT6-19* gene (a desiccation and ABA-responsive gene from *C. plantagineum*) (see Sections 1.6.1 and 3.1.2) was responsive to both ABA and drought in guard cells of *N. tabacum* (tobacco) and *A. thaliana*. They also showed that GUS activity in guard cells driven by the promoters of the ABA-responsive genes, *CDeT27-45* (from *C. plantagineum*) and *Em* (from wheat; predominately seed specific) were unaffected by a ABA or drought treatment. They suggested that this differential ABA responsiveness may be due to: (i) the presence or absence of specific regions in the gene promoters which are required to be active for effective transduction of the signal; or (ii) that guard cells lack all or part of the specific transduction apparatus required to couple the ABA signal to all these promoters (Taylor *et al.*, 1995). More recently, it has been shown that ABA-induced GUS activity driven by the seed specific *Em* gene from *A. thaliana* (*AtEm1*) in guard cells of vegetative tissue of transgenic *A. thaliana* required the ectopic expression of the ABI3 gene from *A. thaliana* (Parcy & Giraudat, 1997). The ABI3 gene product has been identified as a seed specific transcriptional activator [see Section 1.6.3(b)] and suggests that guard cells lack endogenous levels of this signalling component.

ABA has also been shown to enhance gene expression in guard cells in a number of other homologous and heterologous plant systems (see Table 1.1). ABA increased the accumulation of transcripts of dehydrin-like genes in guard cells of both

Name of gene	Putative function of gene product	Investigative system	Factors regulating expression	Reference
CDeT6-19 (*Craterostigma plantagineum* desiccation tolerance gene)	Desiccation tolerance	*CDeT6-19*/GUS in tobacco & *A. thaliana*	ABA & desiccation	Taylor *et al.*, 1995
Dehydrin gene from *Vicia faba* with similarity to *Psdhn1*	Desiccation tolerance	mRNA accumulation in *V. faba*	ABA	Shen *et al.*, 1995
AthH2 (Blue-light responsive gene from *A. thaliana*)	Plasma membrane channel protein with water transport capacities	*AthH2*/GUS & *in situ* hybridization of transcript in *A. thaliana*	ABA & blue light; most responsive in younger tissues	Kaldenhoff *et al.*, 1995
tas14 (Dehydrin gene from tomato)	Protection against dehydration	*tas14*/GUS in tobacco	ABA, salt & mannitol; most responsive in mature plants	del Mar Parra *et al.*, 1996
Dehydrin gene from *Pisum sativum L. Argenteum*	Desiccation tolerance	mRNA accumulation in *P. sativum L. Argenteum*	ABA	Hey *et al.*, 1997
AtEm1 (*Arabidopsis thaliana Em* gene)	Involved in seed development	*AtEm1*/GUS in *A. thaliana*	ABA-induced expression in the presence of ectopic expression of ABI3	Parcy & Giraudat, 1997

Table 1.1 A summary of reported ABA-responsive gene expression in guard cells, to date.

V. faba and *Pisum sativum L. Argenteum* (Shen *et al.*, 1995; Hey *et al.*, 1997). ABA enhanced GUS activity driven by the blue-light responsive *AthH2* gene promoter in guard cells of *A. thaliana* (Kaldenhoff *et al.*, 1995). Interestingly, it has been proposed that the *AthH2* gene codes for a plasma membrane channel which has water transport capacity. This putative function of the *AthH2* protein correlates well with fact that both blue light and ABA have been shown to affect guard cell turgor which involves the movement of water in and out of guard cells (see Section 1.3). In addition, the promoter of the *tas14* gene from tomato has been shown to drive GUS activity in guard cells of transgenic tobacco in response to ABA, as well as salt and mannitol suggesting a role in desiccation tolerance (del Mar Parra *et al.*, 1996).

1.6.3 Mechanism(s) by which ABA affects gene expression

The mechanism(s) by which ABA regulates gene expression may involve transcriptional events and/or a collection of post-transcriptional events such as transcript processing, mRNA stability, translational control, and protein activity and turnover (see reviews by Hetherington & Quatrano, 1991; Chandler & Robertson, 1994; Giraudat, 1995). In many cases, ABA-responsiveness was monitored by Northern blot analysis of steady-state mRNA levels (see reviews by Hetherington & Quatrano, 1991; Chandler & Robertson, 1994; Giraudat, 1995). More recently, interest in understanding the molecular basis of gene regulation by ABA has focused on the mechanism(s) involved in transcriptional activation using a general model in which a *trans*-acting factor interacts with an element in the promoter region of a gene (Ptashne, 1988; see reviews by Hetherington & Quatrano, 1991; Chandler & Robertson, 1994; Giraudat, 1995). This type of analysis requires the identification of an ABA-responsive *cis*-acting element within the promoter of an ABA-regulated gene and the

characterization of the *trans*-acting factor. In addition it also requires the functional analysis of the *trans*-acting factor and *cis*-acting element to test whether ABA regulated gene expression is conferred by their interaction *in vivo* (see reviews by Hetherington & Quatrano, 1991; Chandler & Robertson, 1994; Giraudat, 1995).

1.6.3(a) *Cis*-acting elements

Cis-acting elements are sequences in a gene promoter which are important for the regulation of gene expression by various stimuli. The molecular dissection of ABA-regulated promoters has revealed a diversity of *cis*-acting sequences that represent likely end-points of ABA-regulatory pathways (see Hetherington & Quatrano, 1991; Chandler & Robertson, 1994; Giraudat, 1994; Quatrano, 1997). The best characterized class of *cis*-acting ABA responsive elements (ABREs) is that which contains a G-box motif. This class is exemplified by the Em1a element from the wheat *Em* gene (which is a member of a set of genes that is expressed exclusively in maturing embryos in response to elevated levels of ABA) (Marcotte *et al.*, 1989) and the Motif I element from the rice *rab16A* gene (Mundy *et al.*, 1990), although the induction of other genes by ABA, such as the *rab28* gene (Pla *et al.*, 1993) also involves a *cis*-acting element containing the G-box motif.

Several other *cis*-acting elements involved in ABA-responsive gene expression which are distinct from the G-box related sequences have been identified (Lam *et al.*, 1991; Hattori *et al.*, 1992; Nelson *et al.*, 1994; Isawaki *et al.*, 1995; Kao *et al.*, 1996; Shen *et al.*, 1995b, 1996; Busk *et al.*, 1997; also see Quatrano *et al.*, 1997). These include the coupling elements (CEs) identified by Shen *et al.* (1995b, 1996) and a novel cis-acting element in the *CDeT*27-45 gene (see Section 1.7.2) which is necessary but alone insufficient for ABA responsiveness (Nelson *et al.*, 1994).

In addition to the activation of transcription by ABA, a few sequence elements are also known for transcriptional repression by ABA. These include the GA_3 response elements (GAREs) which are essential for the GA_3-inducible, ABA-repressible expression of the barley α-amylase *Amy1/6-4* (Skriver *et al.*, 1991) and *Amy32b* (Rogers & Rogers, 1992) genes.

1.6.3(b) *Trans*-acting factors

Trans-acting factors are nuclear proteins that bind to *cis*-acting regulatory elements in DNA. Promoter sequences that are known to mediate ABA responses at the transcriptional level have been used as probes for the identification of *trans*-acting factors (Nantel & Quatrano, 1996). Complementary DNA clones have been isolated that encode proteins with binding affinity for the Em1a (wheat EmBP-1 protein [Guiltinan *et al.*, 1990]) and Motif 1 (tobacco TAF-1 protein [Oeda *et al.*, 1991]) elements, respectively. The EmBP-1 and TAF-1 proteins display the two adjacent domains which are characteristic of the basic region-leucine zipper (bZIP) transcription factor family (see Armstrong *et al.*, 1992; Schindler *et al.*, 1992; Izawa *et al.*, 1993). However, the binding of *trans*-acting factors to the G-box related Em1a and Motif 1 elements was not shown to be induced by ABA (Guiltinan *et al.*, 1990; Oeda *et al.*, 1991). ABA-inducible binding of *trans*-acting factors to the ABA-responsive *cis*-acting elements has been reported for the *CDeT27-45* gene (Nelson *et al.*, 1994) and the *Arabidopsis RD22* gene (see Quatrano *et al.*, 1997).

Mutational analysis has led to the identification of novel transcription factors which are involved in the regulation of gene expression by ABA (McCarty *et al.*, 1989a; 1991; Giraudat *et al.*, 1992). Mutations in the *viviparous* (*vp1*) regulatory locus in maize and its equivalent in *Arabidopsis*, *ABA-insensitive 3* (*abi3*) have pleiotropic

effects during seed maturation, one of which is to control the sensitivity of cells to ABA (see review by Giraudat *et al.*, 1994). Genes closely related to the *VP1/ABI3* gene have also been isolated from other species (Hattori *et al.*, 1994; Bobb *et al.*, 1995) The VPI/ABI3 protein which is expressed only during seed development (McCarty *et al*, 1991; Parcy *et al.*, 1994), may be the factor most directly responsible for the strict regulation of genes that are expressed exclusively in seeds, for example the ABA-responsive *Em* gene. Support for this comes from the elegant work of Parcy and Giruadat, (1997) who demonstrated that in transgenic *Arabidopsis* plants overexpression of *ABI3* confers the ability of ABA to induce the ectopic expression of the seed-specific, endogenous *Em* gene (*AtEm1*) in vegetative tissue (see Section 1.6.2). However, data suggest that the VPI protein may be involved in the regulation of a number of diverse genes and response pathways, only one of which is embryo-specific ABA-regulated gene expression (McCarty *et al.*, 1989b; Hattori *et al.*, 1992; Dooner, 1985). More recently, it has been shown that VP1 enhances the binding of EmBP-1 to the *Em* gene (Hill *et al.*, 1996). In addition, mutations in the Em1a region of the *Em* gene [see Section 1.6.3(a)] have revealed that this region and the EmBP-1 are sites through which ABA and VP1 enhance transcription (Hattori *et al.*, 1995; Vasil *et al.*, 1995).

Recently, VP1 has been shown to have a transcription repressor function (Hoecker *et al.*, 1995) as well transcription activating function (i.e. its regulation of the *Em* gene) (McCarty *et al.*, 1989a, 1991; Hattori *et al.*, 1992). VP1 represses the expression of germination specific α-amylase genes in aleurone cells (Hoecker *et al.*, 1995); ABA also has this effect (Huttly & Baulcombe, 1989; Jacobsen & Close, 1991; Skriver *et al.*, 1991; Salinas *et al.*, 1992) [see Section 1.6.3(a)]. Work by Suzuki *et al.* (1997) suggests that different domains of the VP1 protein appear to separately regulate

processes involved in embryo maturation (i.e. up-regulation of the *Em* gene) and the repression of germination (i.e. inhibition of enzymes such as α-amylase [McCarty *et al.*, 1991; Vasil *et al.*, 1995; Kao *et al.*, 1996]). These characteristics suggest that VP1 represents a new class of DNA binding proteins that have critical functions in development (Suzuki *et al.*, 1997).

Very recently, the protein G-14 (de Vetten *et al.*, 1992), which is a member of a family of regulatory proteins called 14-3-3s (Ferl, 1996), has been shown to interact with VP1 on the *Em* promoter (see Quatrano *et al.*, 1997). It has been proposed that GF-14 and VP1 might interact *in vivo* with a G-box binding factor to stabilize and/or activate the regulatory complex responsible for the ABA-mediated induction of *Em* transcription (see Quatrano *et al.*, 1997).

1.6.3(c) Different modes of ABA-responsive gene expression

Evidence suggests that it is likely that there is more than one pathway through which ABA regulates gene expression (see Giraudat *et al.*, 1994; Shen *et al.*, 1995, 1996; Shinozaki & Yamaguchi-Shinozaki, 1996; Quatrano *et al.*, 1997). The ABA-mediated regulation of the *Catalase 1* (*Cat 1*) (Williamson & Scandalios, 1992) and *Rab28* (Pla *et al.*, 1991) genes does not require the transcription factor VP1, whereas VP1 enhances the expression of the ABA-responsive *Em* gene (Hattori *et al.*, 1995; Hill *et al.*, 1996; Vasil *et al.*, 1995) [see Section 1.6.3(b)]. Similarly, the presence of VP1 and ABA has a synergistic effect on the ABA-responsive complex (ABRC) in the *HVA1* barley gene but not on that of *HVA22* barley gene (Shen *et al.*, 1996). In addition, the induction of the *Arabidopsis* ABA-inducible gene *RD22* [see Section 1.6.3(b)], unlike *Em* induction, is dependent on protein synthesis (Isawaki *et al.*, 1995). Two distinct modes for the regulation of gene expression by ABA have been recently

suggested (see Quatrano *et al.*, 1997). One mode, which is represented by the primary ABA-inducible genes typified by *Em*, involves the G-box and pre-existing transcription factors of the basic leucine zipper type (bZIP) type (Nantel & Quatrano, 1996). The other mode may require *de novo* synthesis of stress- and/or ABA-inducible factors, as appears to be the case for the *Arabidopsis RD22* gene (see Quatrano, 1997).

1.6.3(d) Components other than *cis* and *trans*-acting elements involved in ABA-responsive gene expression

A bottom-up approach to investigating the pathways by which ABA regulates gene expression has led to the identification of many *cis*-acting elements and *trans*-acting factors [see Sections 1.6.3(a) and (b)]. Other putative components of ABA signalling pathways terminating in a change in gene expression, which lie upstream of the *cis*-acting elements and *trans*-acting factors, are beginning to emerge. Some evidence suggests that Ca^{2+} plays a role in ABA-responsive gene expression in chick pea seeds (Colorado *et al.*, 1991, 1994). Studies of ABA- and GA_3-induced changes in gene expression in barley aleurone have revealed putative components of the pathways by which these two phytohormones control gene expression. A GA_3-induced increase in CaM mRNA levels, which only occurs in the presence of Ca^{2+} and precedes a GA_3-induced increase in α-amylase synthesis and secretion, was prevented by ABA (Schuurinck *et al.*, 1996). The existence of both Ca^{2+}/CaM-dependent and -independent ABA and GA3-induced signalling pathways in aleurone cells has also been suggested (Gilroy, 1996). Gilroy (1996) reported that the induction of the *Em* gene by ABA and α-amylase gene by GA_3 appear to operate via a Ca^{2+}/CaM independent pathway, whereas the GA_3-induction and ABA-induced repression of α-amylase secretion is Ca^{2+}-dependent (Gilroy, 1996). cGMP (see review by Alberts *et al.*, 1994;

Section 1.1) does not appear to be involved in the ABA-induced enhancement of the transcript levels of the ABA-responsive *Rab21* gene in barley aleurone layers, whereas it has been reported to play an important role in GA_3-induced increases in α-amylase synthesis in this system (Penson *et al.*, 1996). Recently, the differential activation of transcription factors in animal cells (B lymphocytes) has been shown to be induced by the amplitude and duration of increases in $[Ca^{2+}]_i$ (Dolmetsch *et al.*, 1997; also see Section 5.1.2). Whether this occurs in plant cells is an intriguing question worthy of future investigation.

In addition to Ca^{2+} and CaM, there is evidence to suggest that protein dephosphorylation is involved in the pathway by which ABA enhances the levels of mRNA of a wheat gene (Kuo *et al.*, 1996), although the involvement of Ca^{2+} in this pathway needs to be determined. There is evidence for the involvement of tyrosine dephosphorylation in ABA-induced gene expression (Heimovaara-Dijkstra *et al.*, 1996). In addition, there is evidence that the activation of a mitogen-activated protein (MAP) kinase (see Nishida & Gotoh, 1993; Nishihama *et al.*, 1995) via a tyrosine phosphatase is a prerequisite for the induction of *rab16* gene expression by ABA in barley aleurone protoplasts (Knetsch *et al.*, 1996). Another source of evidence for the involvement of protein phosphatases in the regulation of gene expression by ABA comes from studies using the *abi1* and *abi2* mutants of *A. thaliana* [see Sections 1.5.3(b) and 4.1.1]. As described earlier [see Section 1.5.3(b)], it has been reported that the *ABI1*, and more recently, that the *ABI2* gene encodes a serine/threonine phosphatase type 2C protein (Meyer *et al.*, 1994; Leung *et al.*, 1994, 1997). The presence of the *abi1* mutation and in some cases *abi2* mutation (for example, de Bruxelles *et al.*, 1996) has been shown to interfere with ABA-induced mRNA accumulation (Gilmour & Thomashow, 1991; Nordin *et al.*, 1991, 1993; Lang &

Palva, 1992; Yamaguchi-Shinozaki & Shinozaki, 1993; Gosti *et al.*, 1995; de Bruxelles *et al.*, 1996; Söderman *et al.*, 1996), proline accumulation and changes in protein synthesis (Finkelstein & Sommerville, 1990) in *A. thaliana*.

Protein phosphorylation may also play a role in ABA-regulated gene expression. The involvement of Ca^{2+}-dependent protein kinases in ABA-induced activation of the barley *HVA1* gene promoter has been reported (Sheen, 1996). However, much work still needs to be done to firmly establish the components of the ABA signalling pathway which leads to a changes in gene expression upstream of the *trans*-acting factors and *cis*-acting elements. However, it seems as if common players, such as Ca^{2+}, CaM and protein phosphatases and kinases, are involved in the pathways by which ABA regulates gene expression and guard cell turgor (see Section 1.5.4).

1.6.4 Mechanism of ABA-responsive gene expression in guard cells

What is known about the pathway(s) by which ABA regulates gene expression in guard cells? To date, little is known about the *cis*-acting elements and *trans*-acting factors which are specifically involved in ABA-responsive gene expression in guard cells. However, it is likely that similar elements and components to those described in Sections 1.6.3(a) and 1.6.3(b) will also be present in guard cells. Indeed, the work by Parcy and Giraudat (1997) (see Sections 1.6.2 and 1.6.3) shows that overexpressing the seed-specific transcriptional activator *ABI3 gene* throughout the whole plant conferred to ABA the ability to induce the ectopic expression of the seed-specific, endogenous *Em* gene (*AtEm1*) in guard cells (as well as other vegetative tissue; see Section 1.6.2). The work by Taylor *et al.* (1995) (see Section 1.6.2) also suggests that the components (including *cis* and *trans* acting factors) of the signalling pathway(s) by which ABA regulates gene expression in guard cells, in common with other vegetative

tissues, differ from those by which ABA regulates gene expression in seeds and vegetative tissues of the resurrection plant, *C. plantagineum*.

Studies on the role of Ca^{2+} in ABA-regulated gene expression in guard cells are in their infancy. To date, only two studies have specifically addressed this issue. The ABA-responsive gene transcript with sequence similarity to pea dehydrin was not induced by external $[Ca^{2+}]_e$ in guard cells of *V. faba* (see Section 1.6.2 and Table 1.1) (Shen *et al.*, 1995a), whereas, cyclopsorin A (which is known to inhibit Ca^{2+}-activated protein phosphatase 2B enzymes in eukaryotic cells) inhibited the accumulation of mRNA encoding the ABA-inducible dehydrin in guard cells of *P. sativum L. Argenteum* investigated by Hey *et al.* (1997) (see Section 1.6.2 and Table 1.1). In addition, Hey *et al.* (1997), using protein kinase and phosphatase inhibitors, reported that both protein phosphorylation and dephosphorylation were required for ABA-induced dehydrin gene expression. They also showed that these inhibitors affected ABA-induced changes in stomatal aperture (Hey *et al.*, 1997) [see Section 1.5.3(a)]. This suggests that there are similar components in the pathway by which ABA affects guard cell gene promoter activity and turgor.

1.7 ABA analogues as tools for investigating ABA signalling pathways in plants

1.7.1 Why study ABA analogues?

ABA analogues are ABA-like molecules which are structurally similar to the naturally-occurring plant hormone, (+)-(S)-ABA (see Figure 1.2). In this thesis, the term "ABA analogue" is taken to include isomers of (+)-(S)-ABA [see Sections 1.7.4(a) and (b); Walton, 1983]. In 1983, it was reported that over 100 different ABA analogues had been tested in an array of plant systems (Walton, 1983). The biological activities of these ABA analogues were summarized (Walton, 1983) and the one over-

riding conclusion that emerged was that most alterations to the structure of the naturally-occurring ABA molecule result in a decrease in biological activity. Since then, many more ABA analogues have been synthesized and tested in a wealth of different plant systems (see Section 1.7.2). The ABA analogues used in early investigations were often those with drastic alterations to the structural features of the naturally-occurring ABA molecule (Walton, 1983), whereas the analogues used in more recent studies involve more subtle changes, often concentrating on one carbon atom (Churchill *et al.*, 1992; Walker-Simmons., 1992).

Many of the early studies of ABA analogues followed on from the discovery that (+)-(S)-ABA acts as an antitranspirant (Wright & Hiron, 1969; Mittleheuser & Van Steveninck, 1969) (see Section 1.5). These early studies were concerned with synthesizing and testing ABA-like molecules to be used in the field to control transpiration and therefore improve crop efficiency and stress tolerance (Orton & Mansfield, 1974; Malloch & Fenton, 1979). This research was spurred on by the fact that the naturally-occurring antitranspirant, (+)-(S)-ABA, was expensive to synthesize, rapidly inactivated by sunlight and degraded by plants (Davies & Mansfield, 1983). More recently, similar studies have been carried out in the continuing quest for the ideal, synthetic antitranspirant (Blake *et al.*, 1990a & b; Schubert *et al.*, 1991; Hite *et al.*, 1994). Very recently, a possible candidate for an ideal, synthetic ABA-like antitranspirant has emerged (Abrams *et al.*, 1997). 8'-methylene ABA is metabolized more slowly than ABA and so results in a ABA analogue which is longer-lasting and more biologically active relative to ABA. 8'-methylene ABA has been shown to be more active at reducing transpiration in wheat seedlings than ABA (Abrams *et al.*, 1997), but awaits field trials. In addition, a potentially photo-stable ABA analogue, 2-fluoroabscisic acid, has been recently synthesized (Kim *et al.*, 1997).

ABA analogues have also been used as tools to elucidate ABA perception mechanism(s) in plants. One way one of elucidating ABA perception mechanisms is to study the structural features of the ABA molecule required for biological activity. Systematic modification of the ABA molecule, using ABA analogues helps to identify both the spatial and electronic regions that elicit signal recognition. For example, the ABA analogues chosen to investigate the induction of gene expression in barley aleurone protoplasts were those in which the polar groups of ABA were either removed or changed (Van der Meulen *et al.*, 1993). These changes may affect potential hydrogen bonding between ABA and its receptor and/or the conformational changes that the ABA molecule may adopt upon binding to its receptor (Van der Meulen *et al.*, 1993). Such studies thus provide information on the structural features of the ABA perception mechanism(s)/receptor(s) (Walker-Simmons *et al.*, 1992; Perras *et al.*, 1994).

Another way of elucidating ABA perception mechanisms is to use ABA analogues to identify carbon atoms in the ABA molecule to which photoaffinity ligands can be attached without affecting biological activity. Such ABA analogues (with photoaffinity ligands incorporated into their structure) can potentially be used to identify the elusive ABA receptor(s) (Hite *et al.*, 1994; Todoroki *et al.*, 1996; see Quatrano *et al.*, 1997). In a similar manner, ABA analogues can be used as tools to make anti-idiotypic antibodies, which are again designed to identify and isolate the ABA receptor (Walker-Simmons *et al.*, 1991, 1992; Perras *et al.*, 1994). At a time when there is evidence of both intracellular and extracellular sites of ABA perception (Allan *et al.*, 1994; Anderson *et al.*, 1994; MacRobbie *et al.*, 1995a & b; Schwartz *et al.*, 1994) (see Section 1.5.1) the importance of identifying and characterizing such ABA receptors cannot be overemphasized.

In addition, studies using ABA analogues as tools are providing suggestions of the existence of multiple ABA perception mechanisms and ABA signalling pathways in plants (Walker-Simmons *et al.*, 1992; Perras *et al.*, 1994; Hill *et al.*, 1995; Hays *et al.*, 1996) (see Section 1.7.2).

1.7.2 Advances made in understanding ABA signalling pathways in plants using ABA analogues

The equal or almost equal activity of the enantiomers of ABA in some plant assays [see Section 1.7.4(a)] has resulted in models of the ABA receptor being put forward (Milborrow, 1978, 1986). These models are based on the fact that both of the ABA enantiomers can fit into its active site (Milborrow, 1978, 1986). More recently, additional evidence [from determinations of the importance of the orientation of the ring methyl groups of (+)-(S)-ABA for biological activity] has arisen for one of these models (Nakano *et al.*, 1995).

Suggestions of multiple ABA perception mechanisms and signalling pathways in plants have arisen from a number of ABA analogue studies. Milborrow (1980) put forward the idea of "fast" (stomatal closure) and "slow" responses (germination and growth) to ABA having different receptors. This was based on the fact that the biological activity of the unnatural enantiomer of ABA, (–)-(R)-ABA [see Section 1.7.4(a)], appeared to be a function of the biological response studied [see Section 1.7.4(a)]. More recent data also suggest that ABA-induced stomatal closure occurs via a different receptor/signalling pathway than other plant responses to ABA such as inhibition of germination and growth (Yamamoto *et al.*, 1995; Yamamoto & Oritani, 1995; Rose *et al.*, 1996b; Todoroki *et al.*, 1995). For example, the ABA analogue (–)-C-1'-O-methyl ABA was more active than the (+)-form in a wheat embryo germination

assay, whereas the reverse was true in a wheat seedling transpiration assay (Rose *et al.*, 1996). The idea that there are different receptors for different ABA responses mirrors recent data on the localization of the receptor(s) of a different plant hormone, namely auxin. (see discussion by Claussen *et al.*, 1996). There is evidence to suggest that the auxin receptor for growth is located intracellularly (Claussen *et al.*, 1996), whereas, the auxin receptor involved in electrophysiological responses in guard cells is located at the outer surface of the plasma membrane (Thiel *et al.*, 1994).

Differences in the biological activity of ABA analogues have been reported between other types of ABA responses in plants. For example, the ABA-induced inhibition of germination and induction of two genes (oleosin and napin) in microspore embryos of *B. napus* (Hays *et al.*, 1996) had different stereochemical requirements. Similarly, the induction of the *Em* gene promoter in barley aleurone protoplasts had stricter stereochemical requirements than the reversal of GA_3-induced α–amylase synthesis (Hill *et al.*, 1995). The differential ABA antagonistic activity of the ABA analogue PBI-51 [see Section 1.7.4(c)] suggests that there may be stricter stereochemical requirements for the activation of sucrose transporters than for the induction of ABA-responsive heat stable proteins in bromegrass culture cells during the acquisition of freezing tolerance (Wilen *et al.*, 1996). ABA analogues have also been used to examine the stereochemical requirements of ABA in the desiccation tolerance response of *C. plantagineum* (Chandler *et al.*, 1997) [see Sections 1.7.4 and 4.1.2]. The effects of a number of ABA analogues on the viability of callus and on the expression of four desiccation-induced late embryogenesis abundant (*lea*)-type transcripts and sucrose synthase in the callus, after a drying treatment were measured. One of the ABA analogues (PBI-89, a dihydro-derivative of ABA) had a differential effect on the two processes. It induced all the transcripts, but callus treated with it was

not viable after drying. This suggests that, in this species, more than one desiccation-induced pathway is stimulated by ABA.

The ABA uptake carriers studied in barley and carrot suspension-cultured cells have less strict stereochemical requirements than ABA-induced gene expression (Perras *et al.*, 1994 - barley, *WCS 120* gene) or the inhibition of growth (Windsor *et al.*, 1994 - carrot). More specifically, the 7'-methyl group of ABA was critical for wheat embryo germination, the induction of the *Em* gene promoter in barley protoplasts and the inhibition of α-amylase activity in barley aleurone layers (Walker-Simmons *et al.*, 1994; Hill *et al.*, 1995) but not important for uptake by carriers (Perras & Abrams, 1993, Perras *et al.*, 1994; Windsor *et al.*, 1994). These results suggest that ABA uptake carriers are not directly involved in ABA signalling pathways (Perras *et al.*, 1994).

Interestingly, there are differences in specificity within one type of ABA-induced response, namely in ABA-regulated gene expression. The expression of the wheat *Em* gene has stricter stereochemical requirements for induction by ABA than two other wheat genes [*dhn* (*rab*) and wheat group 3 *lea*] (Walker-Simmons *et al.*, 1992). In addition, a differential regulation by ABA analogues has been shown for the napin and oleosin genes in *Brassica napus* (Hays *et al.*, 1996), suggesting different modes of ABA-regulated gene expression.

1.7.3 Assumptions made when using ABA analogues as tools to investigate ABA signalling pathways in plants

As described above ABA analogues can be used as tools to study the structure-activity relationships of ABA in plants. However, the results from such studies must be considered with caution and a number of assumptions must be borne in mind

(Milborrow, 1978, 1985; Walton, 1983). The assumptions include: (i) the compounds compared must act at the same site and with a similar mode of action; (ii) uptake and transport of the compounds to the receptor sites is comparable; and (iii) the compounds being considered must not be differentially metabolized to compounds of greater or lesser activity.

There is generally no evidence in many reported studies, especially the earlier ones, to support any of these assumptions. However, some assays, such as rapid stomatal bioassays using detached epidermis provide a more direct measure of the activity of ABA analogues. This is due to the fact that there is less time (usually a maximum of about 3-4 h) and fewer cell types to take up or metabolize ABA analogues than in assays of a longer time duration and in which actively growing tissue is present (Cummins & Sondheimer, 1973; Hite *et al.*, 1994).

1.7.4 The biological activity of the ABA analogues used in the present study

As described already, a vast number of ABA analogues which contain alterations to many of the structural features of the naturally-occurring ABA molecule are available as tools for studying ABA signalling pathways in plants. However, an attempt will not be made here to describe and discuss the importance of all the different structural features of (+)-(S)-ABA for biological activity. For such information, the reader is referred to Walton (1983) and Table 1.2, in which studies involving ABA analogue after 1983 have been listed in an order dependent upon the alteration(s) to the structure of (+)-(S)-ABA apparent in the ABA analogues used. However, the effects on biological activity of the alterations to the structure of (+)-(S)-ABA which manifest themselves in the ABA analogues used in the present study will be considered. This description will include the effect of such ABA analogues in all biological systems so

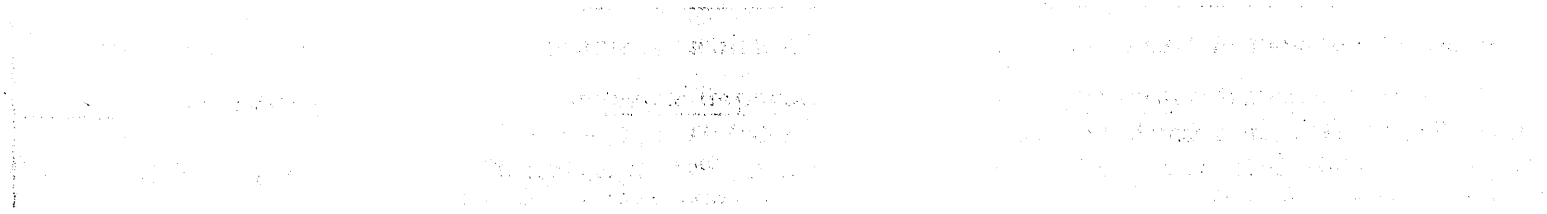


Table 1.2 Studies that have used ABA analogues as tools to investigate the structural features of (+)-(S)-ABA that are important for biological activity in a plethora of different plant systems. The studies are listed according to which structural feature(s) of the (+)-(S)-ABA molecule that the ABA analogues have been used to investigate. The studies are all post 1983. For information on studies prior to 1983 see Walton (1983).

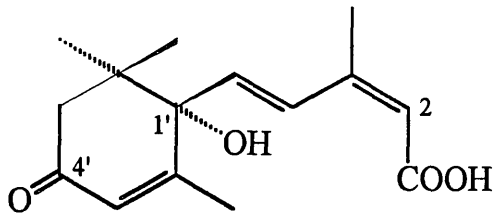
Structural features of (+)-(S)-ABA	Studies in which ABA analogues have been used as tools to investigate the biological importance of the different structural features of (+)-(S)-ABA
Chirality at C-1' (optical isomerism)	Abrams *et al.*, 1993; Balsevich *et al.*, 1994b; Bianco-Colomas *et al.*, 1991; Chandler *et al.*, 1997; Churchill *et al.*, 1992; Dunstan *et al.*, 1991, 1992; Gusta *et al.*, 1992; Hill *et al.*, 1995; Hornberg & Weiler, 1984; Milborrow & Rubery, 1985; Perras *et al.*, 1994; Robertson *et al.*, 1994; Walker-Simmons *et al.*, 1992; Wilen *et al.*, 1993, 1996; Windsor *et al.*, 1994.
Changes to the side chain: (i) isomerism and length	Abrams & Milborrow, 1991; Chandler *et al.*, 1997; Churchill *et al.*, 1992; Hornberg & Weiler, 1984; Milborrow & Abrams, 1993; Milborrow & Rubery, 1985; Perras & Abrams, 1993; Schubert *et al.*, 1991; Walker-Simmons *et al.*, 1991, 1992; Windsor *et al.*, 1992, 1994.
(ii) C-1 functional group	Gusta *et al.*, 1992; Hays *et al.*, 1996; Hite *et al.*, 1994; Perras & Abrams, 1993; Robertson *et al.*, 1994; Schubert *et al.*, 1991; Suttle & Abrams, 1993; van der Meulen, 1993; Windsor *et al.*, 1994.
Changes to the ring: (i) ring double bond	Chandler *et al.*, 1997; Dong *et al.*, 1994; Gusta *et al.*, 1992; Hill *et al.*, 1995; Hornberg & Weiler, 1984; Perras *et al.*, 1994; Robertson *et al.*, 1994; Suttle & Abrams, 1993; Walker-Simmons *et al.*, 1992; Yamamoto & Oritani, 1995; Yamamoto *et al.*, 1995.
(ii) C-1' functional group	Hornberg & Weiler, 1984; Rose *et al.*, 1996a & b; van der Meulen, 1993.
(iii) C-4' functional group	Hite *et al.*, 1994; Hornberg & Weiler, 1984; Schubert *et al.*, 1991; van der Meulen, 1993; Windsor *et al.*, 1994; Yamamoto & Oritani, 1995; Yamamoto *et al.*, 1995.
(iv) ring methyl groups	Abrams *et al.*, 1997; Hill *et al.*, 1995; Lamb *et al.*, 1993, 1996; Nakano *et al.*, 1995; Perras & Abrams, 1993; Perras *et al.*, 1994; Todoroki *et al.*, 1994, 1995; Walker-Simmons *et al.*, 1993, 1994; Windsor *et al.*, 1994.
Multiple changes	Blake *et al.*, 1989, 1990a & b; Chandler *et al.*, 1997; Dong *et al.*, 1994; Hays *et al.*, 1996; Hill *et al.*, 1995; Hornberg & Weiler, 1984; Lamb *et al.*, 1993; Perras et al, 1994; Reaney *et al.*, 1990; Rose *et al.*, 1996a; Suttle & Abrams, 1993; van der Meulen, 1993; Walker-Simmons *et al.*, 1991, 1992; Wilen *et al.*, 1993, 1996; Yamamoto & Oritani, 1995; Yamamoto *et al.*, 1995.
ABA metabolites	Balsevich *et al.*, 1994a & b; Gusta, 1992; Hill *et al.*, 1995; Lin & Ho., 1986; Robertson *et al.*, 1994; Todoroki *et al.*, 1994; Zou *et al.*, 1995.

far tested. For a summary of all of the structural features of the ABA molecule which have been reported to be important for ABA-regulated changes in guard cell turgor and ABA-regulated changes in gene expression the reader is referred to Sections 2.1.2 and 4.1.2, respectively.

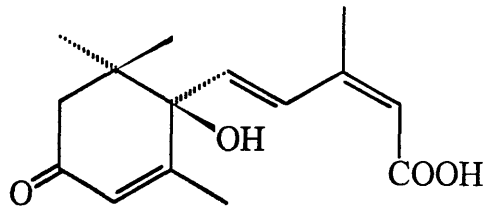
The ABA analogues used in the present study include: (**a**) the optical isomer of (+)-(S)-ABA, (–)-(R)-ABA; (**b**) the geometric isomer of (±)-(RS)-ABA, (±)-(RS)-*trans, trans*-ABA; and (**c**) the optical isomers of 2', 3'-dihydroacetylenic abscisyl alcohol. The (+)-isomer of 2', 3'-dihydroacetylenic abscisyl alcohol has been designated PBI-63 and the (–) isomer, PBI-51 (Wilen *et al.*, 1993). In addition, the effect of naturally-occurring ABA, (+)-(S)-ABA and the commonly used synthetic form of ABA, namely racemic ABA [(±)-(RS)-ABA] was studied. The molecular structure of ABA and the ABA analogues is given in Figure 1.4.

1.7.4(a) (–)-(R)-ABA

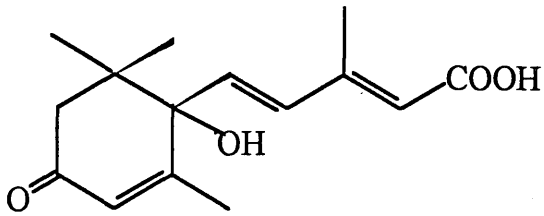
One of the simplest changes to the shape of the ABA molecule is that of altering the chirality at the C-1' carbon atom. ABA possesses optical activity due to the asymmetric carbon atom at position C-1'. This asymmetry results in the existence of two ABA enantiomers, (+)-(S)-ABA and (–)-(R)-ABA (see Figure 1.4). A molecule with a chiral centre is called S or R depending on whether a group attached to the chiral centre protrudes forwards (S) or backwards (R). In plants ABA occurs only as the S enantiomer (i.e. with the side chain attached to the C-1' chiral centre protruding forwards; see Willmer & Fricker, 1996). The asymmetric C-1' also confers optical activity to ABA which results in its ability to rotate polarized light to the right (clockwise) or to the left (anticlockwise). In the former condition the molecule is called the (+)-enantiomer, in the latter condition, the (–)-enantiomer. The enantiomer that



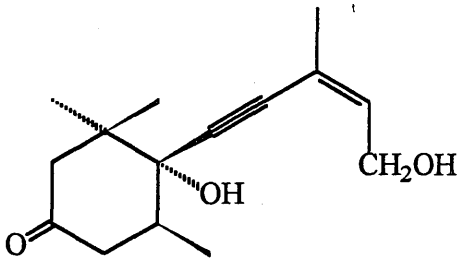
(+)-(S)-ABA



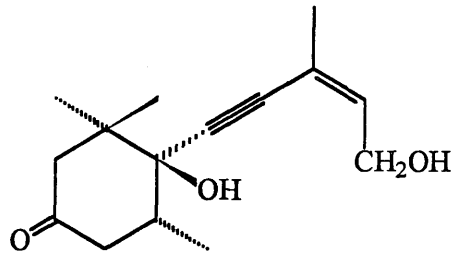
(–)-(R)-ABA



(±)-(RS)-*trans, trans*-ABA



PBI-63



PBI-51

Figure 1.4 The molecular structures of (+)-(S)-ABA, (–)-(R)-ABA, (±)-(RS)-*trans, trans*-ABA, PBI-63 and PBI-51.

occurs naturally in plants is (+)-(S)-ABA (see reviews by Milborrow, 1978; Dörffling & Tietz, 1983; Willmer & Fricker, 1996). Until recently, the only commercially available form of ABA was racemic ABA [(±)-(RS)-ABA], which is a 1:1 mix of (+)-(S)-ABA and (–)-R-ABA. From this point on, (–)-ABA is taken to mean (–)-(R)-ABA; (+)-ABA, (+)-(S)-ABA and (±)-ABA, (±)-(RS)-ABA.

Both enantiomers of ABA have been obtained in optically pure form by total synthesis (Kienzle *et al.*, 1978) and by a number of chromatographic methods for resolving racemic ABA and ABA derivatives (Zeevaart & Creelman, 1988). However, (+)-ABA and (–)-ABA only became commercially available in 1996 (Sigma Chemical Co., UK) and are considerably more expensive to synthesize than (±)-ABA. Therefore, the majority of work carried out with ABA in the last 30 years has used (±)-ABA rather than (+)-ABA (Walker-Simmons *et al.*, 1992). This makes many of the results difficult to interpret (Balsevich, 1994) for reasons which will become apparent (see below).

In order to investigate the specificity of the ABA binding site much research has compared the effects of the two ABA enantiomers on plant physiology, biochemistry and more recently plant gene expression (see Table 1.3), as a reversal in the chirality of ABA is a simple way to change the shape of the molecule. Usually, only the natural mirror-image forms of molecules are acceptable to active sites, for example, inversion of just one of the eight optical centres of the plant hormone gibberellic acid (GA_1) renders the molecule inactive (Milborrow, 1978). However, the findings are not so clear cut for the ABA enantiomers; the biological activities of which seem to depend upon both the type of response studied and the species and/or tissue in which it is studied (see Table 1.3). This has led to suggestions of multiple ABA receptors and signal transduction pathways in plants (see Section 1.7.3).

Table 1.3 The reported biological activity of (–)-ABA, relative to (+)-ABA, in a diverse array of plant processes and in a number of different systems. The biological activity of (–)-ABA was described in the following manner: "=" and "–" indicate that (–)-ABA was equally as active or less active than (+)-ABA, respectively; "0" indicates that (–)-ABA had no biological activity.

Plant process affected by (+)-ABA	Biological activity of (−)-ABA	System (species/cell type)	References
Abscission	=	Cotton explants	Sondheimer *et al.*, 1971
Germination	=	excised bean axes	Sondheimer *et al.*, 1971
		wheat embryos	Walker-Simmons *et al.*, 1992
		barley embryos	Abrams *et al.*, 1993; Milborrow, 1978
	−	*Fraxinus americana*	Sondheimer & Galson, 1966
		root growth of barley seeds	Milborrow, 1978
		cress seed	Gusta *et al.*, 1992
Growth	−	maize cells	Balsevich *et al.*, 1994b
	0	white spruce somatic embryos	Dunstan *et al.*, 1991, 1992
Stomatal closure	−	excised leaves of *Hordeum vulgare*	Cummins & Sondheimer, 1973
	0	leaves of *Xanthium pennysylvanicum*	Kriedman *et al.*, 1972
		epidermis of *C. communis, Tropaeolum majus & V. faba*	Hornberg & Weiler, 1984; Milborrow, 1980
Gene expression	=	wheat seeds	Milborrow, 1980
		barley seeds/aleurone	Hill *et al.*, 1995; Jones & Varner, 1967; Walker-Simmons *et al.*, 1992
		bromegrass cell cultures	Robertson *et al.*, 1994
		Craterostigma plantagineum callus tissue	Chandler *et al.*, 1997
	−	*Em* in wheat seeds, white spruce & barley aleurone	Dong *et al.*, 1994; Hill *et al.*, 1995; Walker-Simmons *et al.*, 1992;
		barley cells	Perras *et al.*, 1994
		Brassica napus microspore-derived embryos	Wilen *et al.*, 1993
Freezing	−	bromegrass cell cultures	Churchill *et al.*, 1992; Robertson *et al.*, 1994;
		Brassica napus microspore-derived embryos	Wilen *et al.*, 1996
Desiccation	−	*Craterostigma plantagineum* callus tissue	Chandler *et al.*, 1997
(+)-ABA absorption and uptake	−	bean axes	Milborrow & Rubery, 1985; Sondheimer *et al.*, 1971
		bromegrass cell culture	Abrams *et al.*, 1989
		carrot cell suspension cultures	Windsor *et al.*, 1994
	=	sunflower embryos	Barthe & Le Page-Degivry, 1991
		Amaranthus ticolor and barley cells	Bianco-Colomas *et al.*, 1991; Perras *et al.*, 1994

One explanation for the biological activity of (–)-ABA has been the suggestion that it is converted to (+)-ABA or it increases endogenous (+)-ABA levels. However, no detectable increases in (+)-ABA were found in wheat embryos when treated with (–)-ABA (Walker -Simmons *et al.*, 1992). Milborrow (1980) suggested that it was very unlikely that a racemase could convert (–)-ABA to (+)-(S)-ABA, and no racemization at C-1', as tested by HPLC and GC, was seen for (+)- or (–)-ABA when supplied to cultured maize cells (Balsevich *et al.*, 1994b).

1.7.4(b) *Trans, trans* -ABA

As well as optical isomerism, the ABA molecule displays geometrical isomerism due to the double bonds in the side chain. The "*cis, trans*" description of naturally-occurring ABA refers to the orientation of the various groups attached to the carbon atoms forming the C-2, C-3, and C-4, C-5 double bonds, respectively. Thus naturally-occurring ABA is C-2 *cis*, C-4 *trans*. A geometric isomer of ABA is C-2 *trans*, C-4 *trans* ABA, which is known as *trans, trans*-ABA (see Figure 1.4), 2-*trans*-ABA or *t*-ABA. *Trans, trans*-ABA occurs naturally in plants (Milborrow, 1970; Broquedis & Bouard, 1993; Li *et al.*, 1994b; Christmann *et al.*, 1995; Ferreres *et al.*, 1996), although the quantity isolated has varied from trace amounts (*Rosa arvensis*: Milborrow, 1970) to slightly less than the concentration of *cis, trans*-ABA [Li *et al.*, 1994b -*Marchantia polymorpha* (Hepataphyta)].

When *cis, trans*-ABA is exposed to light, especially ultra-violet (UV) light, it is isomerized to give a 1:1 equilibrium mixture with its *trans, trans*-isomer (Mousseron-Canet *et al.*, 1968; Plancher, 1979). Therefore, it is vital to test the biological activity of *trans, trans*-ABA in the dark, to avoid contamination with the biologically active *cis, trans*-isomer. Some of the earlier studies which have attributed biological activity

to *trans, trans*-ABA did not taken account of this fact (Walton, 1983). The biological activity of *trans, trans*-ABA seen in bean axes, rice seedling, and duckweed growth assays (Sondheimer & Walton, 1970; Oritani & Yamashita, 1970; Roberts *et al.*, 1968) and the closure of stomata in bean seedlings (Cummins *et al.*, 1971) was, therefore, likely to be due to the presence of the *cis, trans*-isomer. In other systems, where exposure to light was minimal or non-existent, *trans, trans*-ABA was inactive. Thus, *trans, trans*-ABA was unable to close stomata of *Xanthium* or sugar beet when tested in the dark in the presence of CO_2-free air (Kriedman *et al.*, 1972) and had no effect on stomata of *V. faba* on isolated epidermal strips during a bioassay of a relatively short time duration (Hornberg & Weiler, 1984). In addition, it did not affect the ABA uptake carrier in either hopbush or carrot suspension culture cells (Windsor *et al.*, 1992; 1994) or the carrier in young root segments of *Phaseolus coccineus* (Milborrow & Rubery, 1985).

1.7.4(c) (S)-(+)-dihydroacetylenic abscisyl alcohol (PBI-63) and **(R)-(-)-dihydroacetylenic abscisyl alcohol (PBI-51)**

The ABA analogue, (+)-dihydroacetylenic abscisyl alcohol (PBI-63) has multiple structural alterations to the structure of (+)-ABA (see Figure 1.4). These include: (i) an acetylenic group in the side chain; (ii) an alcoholic group at C-1; and (iii) lack of a C-2', C-3' ring double bond. PBI-51, the optical isomer of PBI-63, has all the above structural alterations and also has altered chirality at C-1' (see Figure 1.4).

The presence of an acetylenic group (triple bond) in the side-chain results in C-1', C-3 and C-5 being colinear and therefore the side-chain becomes perpendicular to the ring (Walker-Simmons *et al.*, 1991). However, the presence of an acetylenic group in the side-chain has no effect on the overall geometry of the ABA molecule, i.e. the

relative spatial position of the C-1 functional group relative to the ring (Schubert *et al.*, 1991). The addition of two hydrogen atoms to the 2', 3' double bond of the ring results in the formation of a dihydroABA.

Interesting findings have resulted from studying the effect of PBI-63 and PBI-51, (see Figure 1.4) on ABA-sensitive expression of storage protein genes (oleosin and napin) in microspore-derived embryos of *B. napus* (Wilen *et al.*, 1993). PBI-63 increased gene expression in a dose-dependent manner, being slightly less effective than ABA whereas, PBI-51 was inactive until applied at high concentrations (40-50 μM) and at these concentrations induced only a weak response. Concurrent application of PBI-63 with ABA had no effect on ABA-induced gene expression whereas, excitingly, PBI-51 acted as a competitive, reversible inhibitor of ABA.

ABA uptake by microspore-derived embryos of *B. napus* was not reduced by either PBI-63 or PBI-51 (Wilen *et al.*, 1993). In fact, application of both these ABA analogues resulted in an increase in endogenous ABA pools, possibly due to the competition for ABA catabolic enzymes caused by the presence of the molecules. The fact that PBI-51 inhibits the action of ABA despite also leading to an increase in endogenous ABA levels, strongly suggests that PBI-51 is competing with ABA at the hormone recognition site (see Wilen *et al.*, 1993).

Using the extensively studied bromegrass cell culture, the effect of PBI-51 and PBI-63 on ABA-induced freezing tolerance and associated processes has also been investigated (Wilen *et al.*, 1996). Applied individually, PBI-51 had no effect on freezing tolerance, whereas PBI-63 showed a small level of induction. However, when applied concurrently with ABA, PBI-51 inhibited the induction of maximum freezing tolerance by ABA. It also inhibited the ABA-induced accumulation of heat stable and dehydrin-like proteins but had no effect on ABA-induced uptake of sucrose. The

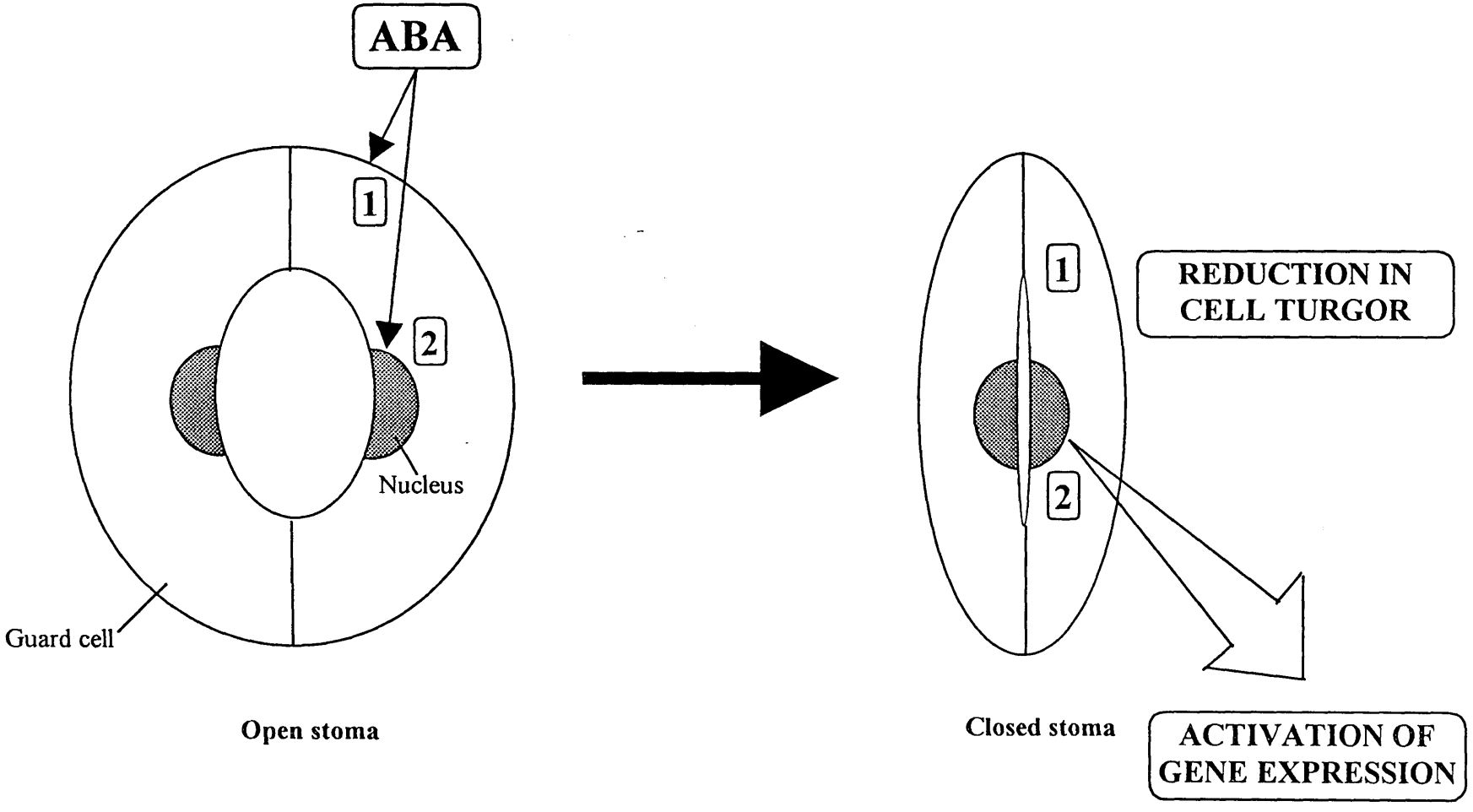
differential antagonistic activity of PBI-51 suggests that there may be stricter stereochemical requirements for the activation of sucrose transporters than for the induction of ABA-responsive heat stable proteins in bromegrass culture cells during the acquisition of freezing tolerance (Wilen *et al.*, 1996; see Section 1.7.3). PBI-63 showed no such antagonistic activity.

In addition to its effect on the accumulation of storage proteins and the acquisition of freezing tolerance, PBI-51 has also been shown to act as reversible inhibitor on the action of ABA on cress seed germination (Reaney *et al.*, 1990). The existence an ABA analogue which acts as a reversible competitive inhibitor of ABA (Reaney *et al.*, 1990; Wilen *et al.* 1993, 1996) may help in the struggle to understand the precise role of ABA in plant processes which have been reported to involve ABA (see for example Chandler & Robertson, 1994; Wilen *et al.*, 1993). A reversible competitor of ABA could be used to help consolidate work using the carotenoid biosynthetic inhibitor (and hence ABA synthesis inhibitor), fluridone (Bray & Beachy, 1985; Finklestein & Crouch, 1986; Fischer *et al.*, 1987) and also more recent work using mutants of ABA synthesis or action (for example Koorneef *et al.*, 1989; Giraudat *et al.*, 1994; Giraudat, 1995).

1.8 Aims of the present study

As described above, ABA affects both turgor (see Section 1.5) and gene expression (see Section 1.6) in stomatal guard cells (see Figure 1.5). The present study aimed to investigate the ABA signalling pathways by which these two responses are regulated. Therefore, the effects of ABA and ABA analogues on stomatal aperture (guard cell turgor) (Chapters 2 and 4) and GUS activity driven by the ABA-responsive *CDeT6-19* gene promoter (see Section 1.6.2) in guard cells (Chapters 3 and 4) were

Figure 1.5 Simple schematic diagram to show that ABA affects both guard cell turgor (promotion of stomatal closure illustrated; ABA also inhibits stomatal opening; see Section 1.5) and guard cell gene expression (see section 1.6).


ABA
1
2
Nucleus
Guard cell
Open stoma
1
REDUCTION IN CELL TURGOR
2
Closed stoma
ACTIVATION OF GENE EXPRESSION

investigated with a view to elucidating whether the two pathways are initiated in a similar manner. In addition, it was of interest to use ABA analogues to study ABA-induced increases in guard cell $[Ca^{2+}]_i$ reported to be involved in the ABA signalling pathway which terminates in a change in guard cell turgor (see Section 1.5.1); and also to determine the role of Ca^{2+} in the ABA signalling pathway which leads to change in *CDeT6-19* gene promoter activity in guard cells. Therefore, investigations were initiated on the effect of ABA analogues on $[Ca^{2+}]_i$ in guard cells (Chapter 5).

As described previously (see Section 1.7.4), the ABA analogues chosen include the optical isomer of ABA, (–)-ABA; the geometric isomer of ABA, *trans, trans*-ABA and the optical isomers of 2', 3'-dihydroacetylenic abscisyl alcohol, PBI-63 and PBI-51 (see Figure 1.4). The unnatural enantiomer of ABA, (–)-ABA, was chosen because: (i) it represents a simple change to the shape of the molecule; (ii) there is a wealth of research which suggests that (–)-ABA can distinguish between different ABA perception mechanisms (Milborrow, 1980; see Sections 1.7.3 and 1.7.4(a)] and; (ii) it is important to determine the activity of the enantiomers of ABA before studying the enantiomers of an ABA analogue with multiple changes (i.e. PBI-51 and PBI-63). The geometrical isomer, *trans, trans*-ABA, was chosen as a molecule which had another simple change to the shape of naturally-occurring ABA, but one that rendered it inactive [see Section 1.7.4(b)]. The impetus behind using the optical isomers of 2', 3'-dihydroacetylenic abscisyl alcohol, PBI-63 and PBI-51 was related firstly to the desire to determine whether PBI-51 would act as an ABA antagonist in the responses of guard cells to ABA, as it had in many other systems [see Section 1.7.4(c)]. An antagonist of ABA-induced responses in guard cells would be a powerful tool in investigations of the role of ABA in guard cell stimulus-response coupling, for example in the CO_2 signalling pathway (see Webb *et al.*, 1996a; Webb & Hetherington, 1997).

Secondly it was of interest to determine and compare the structural specificity of the two types of ABA response in the guard cell to ABA analogues which were structurally very different from natural (+)-ABA.

The data gained from the investigations described above aim to provide information concerning early events in the ABA signalling pathways in guard cells which lead to a change in guard cell turgor and guard cell gene promoter activity. This information will help answer fundamental questions concerning the points of convergence and divergence of these two signalling pathways. These answers may not only help establish a clearer picture of plant signal transduction mechanisms but may also offer exciting opportunities for the manipulation of ABA/stress-induced responses of plants in the future.

Chapter 2

The Effects of ABA Analogues on Guard Cell Turgor

2.1 Introduction

2.1.1 The use of ABA analogues as tools to investigate ABA-induced changes in guard cell turgor

The effects of ABA analogues on guard cell turgor (measured as changes in transpiration or stomatal aperture) have been investigated for two main reasons, as described in Section 1.7.1. In summary, firstly, many different ABA analogues have been synthesized with a view to discovering an ideal, synthetic, ABA-like antitranspirant for use in the field (Blake *et al.*, 1990a & b; Malloch & Fenton, 1979; Orton & Mansfield, 1974; Raschke *et al.*, 1975; Schubert *et al.*, 1991; Abrams *et al.*, 1997; see Section 1.7.1). Secondly, several studies have been concerned with using ABA analogues to probe the structural features of the ABA molecule important in the control of guard cell turgor (Abrams & Milborrow, 1991; Hite *et al.*, 1994; Hornberg & Weiler, 1984; Milborrow, 1980; Rose *et al.*, 1996b; Todoroki *et al.*, 1996; Uehara *et al.*, 1975; Yamamoto & Oritani, 1995). All of the studies described above play a role in the identification of the structural features of the ABA perception mechanism/"receptor" involved in the control of guard cell turgor. They also help in the development of photoaffinity probes and anti-idiotypic antibodies for use in the

isolation of the ABA "receptor" associated with changes in guard cell turgor (Hite *et al.*, 1994; Todoroki *et al.*, 1996; see Section 1.7.1).

2.1.2 Structural features of the ABA molecule which are important for the regulation of guard cell turgor

ABA analogue studies have shed light on the structural features of the (+)-ABA molecule which are important for evoking a change in guard cell turgor (see Figure 2.1 and Table 2.1). Essential features of the ABA molecule for the regulation of guard cell turgor include the (+)-orientation at C-1' and the C-2 *cis* orientation in the side-chain (see Table 1.3 and Section 1.7.4). All but one study (Oritani & Yamashita, 1982), showed that there was a requirement for the ring double bond at C-2', C-3'. There is also some requirement for a hydroxyl group at C-1', although limited research has been carried out on this group in isolation (Rose *et al.*, 1996 a & b). The requirement for a C-4' carbonyl group is unclear because it varies between plant species (Hite *et al.*, 1994; Hornberg & Weiler, 1984). Data suggest that the oxidation state of the C-1 group (i.e. alcohol, aldehyde or acid) is not as important as other changes to the ABA molecule (Uehara *et al.*, 1975; Orton & Mansfield, 1974; Addicott, 1983; Schubert *et al.*, 1991). Alterations to the 8' methyl group of the ABA molecule led to an enhancement of activity (Todoroki *et al.*, 1995) or a reduction in activity (as did altering the 9' group) (Todoroki *et al.*, 1994). The effect was dependent upon the replacement groups, fluorine groups and a methoxy group rendering the molecule more and less active, respectively (Todoroki *et al.*, 1994, 1995).

The biological activities of ABA analogues with multiple changes to the structure of (+)-ABA have also been investigated. An ABA analogue without the C-1, C-1', C-4' groups and the ring double bond of (+)-ABA resulted in a molecule with no

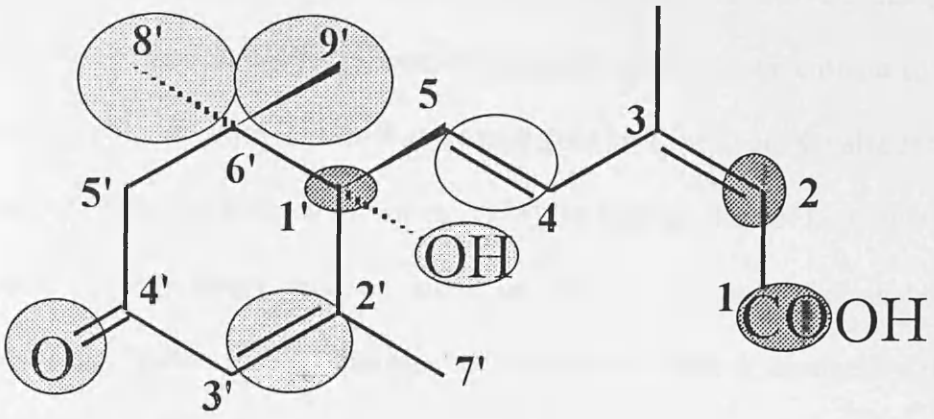


Figure 2.1 Structure of (+)-ABA. The structural features of (+)-ABA shaded dark, light or unshaded (but circled) represent those which are important, have a variable degree of importance or are less important, respectively, for the regulation of guard cell turgor (see Table 2.1).

IMPORTANT	VARIABLE	LESS IMPORTANT
(+)-orientation at C-1'	C-2', C-3' ring double bond	*trans* C-4, C-5 double bond in the side chain
C-2, *cis* orientation	C-1' hydroxyl group	C-1: oxidation state
C -1: C and O atoms	C-4' carbonyl group	
	C-6' methyl groups	

Table 2.1 A summary of the important structural features of the (+)-ABA molecule for the regulation of guard cell turgor, based on the literature to date. The structural areas described are judged to be important, have a variable degree of importance (dependent on the system under study), or to be less important (see text).

biological activity (Orton & Mansfield, 1974). Slightly less extreme changes, for example loss of the C-1' hydroxyl group in combination with drastic changes to the C-1 group (i.e. replacement of the whole carboxylic group with -OH or =O) also resulted in an inactive molecule (Malloch & Fenton, 1979). In contrast, loss of both the C-1' and C-4' groups, surprisingly, had no effect on activity (Orton & Mansfield, 1974; Hornberg & Weiler, 1984), although in combination with a change to the C-1 functional group did lead to a reduction in activity (Raschke *et al.*, 1975), or a loss of activity (Malloch & Fenton, 1979). The presence of a nitrogen atom in the C-1 replacement group resulted in injury to the stomata (Malloch & Fenton, 1979) and it was suggested by Orton and Mansfield (1974) that there is a requirement for a group containing a carbon and oxygen atom at C-1. On the other hand, the presence of an acetylenic group at C-4, C-5 in the side chain had no effect on activity of ABA aldehyde (Orton & Mansfield, 1974) or alcohol (Blake *et al.*, 1990a & b). In addition, it has been reported that the conformation of the (+)-ABA molecule required to inhibit stomatal opening on epidermal strips of spiderwort (*Tradescantia reflexa* Rafin) was close to the favoured half-chair with the side chain pseudo-axial (for details see Todoroki *et al.*, 1996).

2.1.3 Structural features of the ABA molecule relevant to this study

The ABA analogues used in the present study were (–)-ABA, (±)-*trans, trans*-ABA, PBI-63 and PBI-51; in addition (+)-ABA and (±)-ABA were used (see Figure 1.4 and Section 1.7.4 for detailed descriptions of these molecules). Several studies have either directly examined the effect of these molecules on guard cell turgor or examined the effect of the alterations to the structure of (+)-ABA apparent in these molecules (see below).

2.1.3(a) (–)-ABA

(–)-ABA (Figure 1.4) has very little, or no effect on stomatal closure (Walton, 1983) (see Section 1.7.4). (–)-ABA showed very little effect on stomatal conductance (measured by monitoring leaf temperature) of excised leaves of *Hordeum vulgare* (Cummins & Sondheimer, 1973). It was suggested that the small amount of activity that was detected could be attributed to slight contamination of the (–)-ABA with (+)-ABA (Cummins & Sondheimer, 1973). It was shown that (±)-ABA had approximately twice the activity of (+)-ABA in the closure of stomata (measured by monitoring leaf CO_2 and water exchange) on leaves of *Xanthium pennsylvanicum* thus suggesting that (–)-ABA was inactive (Kriedman *et al.*, 1972). In addition, (–)-ABA did not alter stomatal aperture in detached epidermis from the leaves of *C. communis*, *Trapaeolum majus* (Milborrow, 1980) or *V. faba* (Hornberg & Weiler, 1984).

2.1.3(b) (±)-*trans, trans*-ABA

Altering the side chain from C-2 *cis*, C-4 *trans* to C-2 *trans*, C-4 *trans* to form *trans, trans*-ABA (Figure 1.4) resulted in a loss of activity in the stomatal system (Kriedmann *et al.*, 1972 - *Xanthium pennsylvanicum* leaves; Hornberg & Weiler, 1984 - *V. faba*, detached epidermis). The low level of biological activity reported for *trans, trans*-ABA in one experiment (Cummins *et al.*, 1971 - barley seedlings) was later attributed to light-induced conversion of *trans, trans*-ABA to the active *cis, trans*-ABA (Milborrow, 1970; Walton, 1983).

2.1.3(c) PBI-63 and PBI-51

The effect of PBI-63 and PBI-51 on guard cell turgor has not been investigated previously. However, the individual changes to the structure of (+)-ABA apparent in

these molecules [see Figure 1.4 and Section 1.7.4(c)] have been investigated. The individual changes include:

(i) Saturation of the ring double bond. The ABA analogue [(+)- or (±)-] dihydro ABA, formed by the saturation of the ring double bond, was active (Oritani & Yamashita, 1982), had one tenth of the activity of ABA (Yamamoto & Oritani, 1995 - *C. communis*, detached epidermis) or was inactive (Hornberg & Weiler, 1984 - *V. faba*, detached epidermis; Orton & Mansfield, 1974 - *C. communis*, detached epidermis) at promoting stomatal closure.

(ii) Changing the C-1 group to an alcoholic group. Altering the C-1 carboxylic group of ABA to an alcoholic group resulted in no loss of activity (Uehara *et al.*, 1975 - *B. napus* leaves; Orton & Mansfield, 1974 - *C. communis* detached epidermis).

(iii) *Changing the C4, C-5 double bond to an acetylenic linkage.* To date, the effect of acetylenic ABA on stomatal closure has not been measured directly; changing the C4, C5 bond to a acetylenic linkage has always been accompanied by other changes. An ABA analogue with an acetylenic link in the side-chain and a C-1 alcohol group was active as an antitranspirant (Blake *et al.*, 1990a & b - three coniferous species, leaves).

2.1.4 Aims

This study aimed to investigate the effects of several ABA analogues (see Figure 1.4 and Section 2.1.3) on stomatal aperture with a view to using the structure-activity data, in conjunction with similar data for ABA-induced changes in guard cell gene expression (Chapter 4), to probe ABA perception in stomatal guard cells (see Montgomery *et al.*, 1996a & b). It was envisaged that if the "receptors" for the two ABA-induced responses (control of guard cell turgor and regulation of gene expression in guard cells) differed, for example in either structure or location, then their

responsiveness to a range of ABA analogues may vary (see Sections 1.7.2 and 1.8). In addition it was of interest to determine whether one of the ABA analogues, PBI-51 (Figure 1.4) would act as an ABA antagonist in the guard cell system as it does in other plant systems [see Section 1.7.4(c); Wilen *et al.*, 1993, 1996].

The effects of the ABA analogues described in Figure 1.4 on stomatal opening were tested in several plant species. For *C. communis*, the effects of the ABA analogues on stomatal opening and on stomata which were already open were investigated bearing in mind that ABA inhibits stomatal opening and promotes closure (Assmann, 1993; and see Section 1.5). The mechanisms by which ABA inhibits stomatal opening differ from those by which it promotes stomatal closure (Assmann, 1993; Giraudat, 1995; Willmer & Fricker, 1996; Hey *et al.*, 1997; see Sections 1.3 and 1.5). Therefore, a study of the effects of the ABA analogues on these two processes may help to determine whether they have similar ABA perception mechanisms.

C. communis and *V. faba* were chosen as the plant material in the investigations because they have easily detachable epidermis and a well-characterized and well-defined stomatal response to ABA (Weyers & Meidner, 1990), *C. communis* being the more sensitive to ABA of the two species (Weyers & Meidner, 1990). *Nicotiana tabacum* (tobacco) was chosen because it is a species in which ABA regulated gene promoter activity in the guard cell has been reported (Taylor *et al.*, 1995; see Section 1.7.2). It is thus a potential candidate for the investigation of two types of response to ABA (turgor changes and gene expression changes) in guard cells of a single species. Other reasons for choosing tobacco include the fact that some characterization of the effect of ABA on guard cells of tobacco has been carried out (Thomas, 1970; H. Clayton, personal communication). In addition, tobacco was the preferred choice of the plants which were available for guard cell gene expression studies (Taylor *et al.*, 1995).

This was because tobacco leaf epidermis was more suitable for detached epidermal work than that from *A. thaliana* (see Chapter 4). The reasons for this include: (i) the abaxial epidermis was easier to detach from the leaf; (ii) there was a higher percentage of viable epidermal cells (other than guard cells) in the detached epidermis; and (iii) the stomata and stomatal apertures were larger and therefore easier to measure than those of *A. thaliana*.

2.2 Materials and Methods

2.2.1 Chemicals

All the chemicals described were from Sigma Chemical Co. (UK) unless otherwise stated.

2.2.2 Growth of plants

C. communis, *V. faba* and *N. tabacum* cv. Samsun were grown from seed in Fisons M3 general purpose compost in a greenhouse maintained at a 25±1°C day and a 20±1°C night, a relative humidity (RH) of 65%, and with supplementary lighting provided by mercury vapour lamps (Osram, UK) to give a photoperiod of 16 h and a minimum photon flux density of 100 μmol m^{-2} s^{-1} (400-700 nm). Seedlings were transplanted to individual pots, 1-2 weeks after the seed were sown. At all stages of their development the plants were kept free from water deficit stress.

Plants of the correct age for experimentation (see Section 2.2.3) were placed in a controlled environment room, maintained under the same conditions as the greenhouse except that all the light was supplied by "daylight" fluorescent tubes (Thorn EMI, UK), 3-4 days before use in experiments. Plants were kept in this room on self-watering gravel trays or damp capillary matting.

2.2.3 Removal of epidermis

The method of removal of epidermis from the abaxial (lower) surface of the leaves was species specific (see below). Material was obtained from six to twelve plants for each experiment, unless otherwise stated.

For *C. communis*, epidermal peels were taken from the abaxial surface of the youngest, fully expanded leaf (the third or fourth leaf from the shoot apex) of 4-5-week-old plants as described by McAinsh *et al.* (1991a).

For *V. faba*, the abaxial epidermis was removed from first pair of fully expanded leaves of 4-6-week-old plants according to the methods of Wardle and Short (1981). In summary, the leaf was held with the abaxial epidermis uppermost and using a pair of fine forceps one side of the leaf was slowly pulled away from the tip at an acute angle, thus leading to the removal of a sheet of abaxial epidermis.

For *N. tabacum*, the epidermis was removed from leaf 6 (counted down from the shoot apex, leaf 1 being the youngest leaf > 2 cm) of 6-8-week-old plants. Using fine forceps, and with the abaxial surface of the leaf uppermost, a horizontal tear was made above a side vein, within the margins of the leaf. Using the forceps, this piece of leaf material was slowly and gently pulled up towards the tip of the leaf at an angle between 45-90°. This resulted in the removal of a triangular piece of abaxial epidermis. The peeling technique was devised in collaboration with Dr. Helen Clayton (Biological Sciences, I.E.B.S., Lancaster University).

Epidermis from the species above was peeled and subsequently floated at room temperature on a solution of 10 mM MES (2-[N]-morpholinoethane sulphonic acid) adjusted to pH 6.15 using KOH (hereafter known as MES/KOH) for *C. communis* and *N. tabacum* and Tris-base (henceforth known as MES/Tris) for *V. faba*. [A different pH adjuster was used for the incubation solution for epidermis of *V. faba* because approximately 5 mM KOH (K^+ OH^-) is required to adjust the pH of MES to pH 6.15 (Weyers & Meidner, 1990) and as 10 mM KCl was used in the epidermal bioassay incubation solution for *V. faba* (see Table 2.2), the addition of 5 mM K^+ (in the KOH added) would have increased the K^+ concentration in the buffer by 50%.]

Plant species	Buffer/pH adjuster	[KCl] mM	Reference
C. communis	10 mM Mes/KOH (MES/KOH)	50	De Silva *et al.*, 1985
V. faba	10 mM Mes/Tris-base (MES/Tris)	10	Wardle & Short, 1981
N. tabacum	MES/KOH	100	*

Table 2.2 The incubation solutions used for each plant species in the epidermal strip bioassay.

[* Optimization of pH and [KCl] for stomatal opening in epidermal strips of *Nicotiana tabacum* cv. Samsun were determined in collaboration with Dr. Helen Clayton (Biological Sciences, I.E.B.S., Lancaster University)].

Any mesophyll tissue was cut off and the epidermal peels were cut into pieces (approximately 5 x 10 mm). The epidermis was removed from all the plants used in each experiment prior to the incubation of epidermis on treatment solutions.

2.2.4 The epidermal strip bioassay

The epidermal pieces were incubated under conditions which promoted stomatal opening (De Silva *et al.*, 1985a & b). They were floated cuticle uppermost on 10 ml of the incubation medium (see Table 2.2) in 5 cm "deep form" plastic Petri dishes (Sterilin, UK) which were held partially submerged on a perspex plate in a glass water bath with the temperature maintained at 25±1°C (see Weyers & Meidner, 1990). The epidermis was illuminated from below by a bank of Phillips (UK) "daylight" fluorescent tubes providing a photon flux density (PFD) of 150 μmol m^{-2} s^{-1}. The dishes were aerated at a rate of 100 cm^{-3} min^{-1} via hypodermic needles dipping into the incubation medium with CO_2-free air obtained by passing laboratory air over soda lime.

The effect of (±)-ABA, (±)-*trans, trans*-ABA, (+)-ABA, (–)-ABA, PBI-63 and PBI-51 on stomatal function was investigated by dissolving them in the incubation medium. (+)-ABA, (–)-ABA, PBI-63 and PBI-51 were kindly supplied by Dr. S. Abrams (Plant Biotechnology Institute, Saskatoon, Canada). All the molecules used were stored as 10^{-2} M stock solutions in absolute ethanol, in the dark, at -20°C. The highest ethanol concentration in the treatment solution was 1%; this concentration of ethanol did not affect stomatal aperture (see Appendix A). Four main protocols were employed during the epidermal strip experiments:

(i) Inhibition of stomatal opening. The effect of various compounds on stomatal opening was investigated by floating epidermal pieces with "closed" (see below) stomata on the incubation medium (see Table 2.2) containing the test compounds and

under conditions promoting stomatal opening (see above) for 3 h. The removal of epidermis and subsequent flotation on MES/KOH (or MES/Tris) resulted in "closed" stomata. For *C. communis* and *N. tabacum*, "closed" stomata had a stomatal aperture width of between 0-2 μm. For *V. faba*, closed stomata had a stomatal aperture width of between 3-5 μm. In experiments designed to measure the effect of (±)-*trans, trans* - ABA, the epidermal pieces were moved to fresh solutions (kept in blackened Petri dishes prior to use) every 30 min, in an attempt to minimize light-induced isomerization of (±)-*trans, trans*-ABA.

(ii) Promotion of stomatal closure. The effect of various treatments on stomatal closure was investigated by pre-incubating epidermal pieces in the incubation medium (see Table 2.2) and under conditions promoting stomatal opening (see above) for 3 h to open the stomata and then transferring the epidermal pieces to incubation medium containing the test compounds and continuing the incubation under conditions promoting stomatal opening for a further hour.

(iii) Kinetics of stomatal aperture changes. The effect of (+)-, (±)- and (–)-ABA on stomatal opening was investigated in more detail, by making measurements of stomatal apertures, for each treatment, every 20 min for a time period of 3 h and 40 min.

(iv) Investigating a potential ABA antagonist. The effect of a potential ABA antagonist, PBI-51 [(–)-dihydroacetylenic abscisyl alcohol] was investigated on ABA-induced changes on stomatal aperture of *C. communis* and *N. tabacum*. The effects were investigated by either a concurrent application of 10^{-5} M PBI-51 and (±)-ABA, or a pre-incubation in 10^{-5} M PBI-51 followed by the addition of (±)-ABA. For *C. communis*, the pre-incubation in 10^{-5} M PBI-51 was carried out for 3 h in the light and then, after the addition of (±)-ABA, incubation continued for 1 h as described in *(ii)*, above. The effect of 10^{-5} M PBI-63, the (+)-enantiomer of dihydroacetylenic abscisyl

alcohol was investigated in a similar manner. For *N. tabacum,* the pre-incubation in 10^{-5} M PBI-51 was carried out in MES/KOH at 25±1°C for 1 h in the dark (in blackened Petri dishes) to prevent any stomatal opening before adopting a concurrent application with (±)-ABA under normal opening conditions, as described in *(i)* above. A pre-incubation in 10^{-5} M PBI-51, in the dark, had no effect on the control stomatal aperture after 3 hours under stomatal opening conditions (Appendix B).

All solutions were allowed to come to equilibrium under the conditions described for stomatal opening for at least 1 h before use, except that the solutions used in experiments with (±)-*trans, trans*-ABA were kept free from light during this period. The start time of incubation for each treatment was staggered by 10 min intervals to allow time for the measurement of stomatal apertures. Each experiment was repeated at least three times; the order in which treatments were measured was randomized within each replicate experiment so as to rule out order effects.

2.2.5 Determination of stomatal aperture width

Stomatal aperture widths were measured at the end of the incubation period by mounting epidermal pieces on a microscope slide with a coverslip in a drop of the appropriate treatment solution and measuring the aperture width at 400 x magnification using a Leitz "Labovert" (Leica, Milton Keynes, UK) microscope fitted with a projection eyepiece. In each experiment forty stomata per treatment (10, chosen at random from each of 4 epidermal pieces) were measured, except that: (i) ten stomata per treatment (5, chosen at random from each of 2 epidermal pieces) were measured in the experiment examining the kinetics of stomatal aperture changes (*C. communis*); and (ii) twenty stomata per treatment (5, chosen at random from each of 4 epidermal

pieces) were measured in the experiments examining the effect of (±)-*trans, trans*-ABA (*C. communis*) and in the experiments examining the effect of PBI-51 on (±)-ABA induced inhibition of stomatal opening in tobacco epidermis.

2.2.6 Statistics

Statistical analysis was carried out using Minitab Release 7.0 on the university's mainframe computing facilities. Each data point represents the mean of 120 stomatal aperture widths unless otherwise stated. The data were analyzed statistically using one way analysis of variance (AOV) and least significant difference (LSD) tests Significance was accepted at the 5% level unless otherwise stated.

2.3 Results

2.3.1 The effects of the optical isomers of ABA, (+)- and (–)-ABA on stomatal aperture

The effects of (+)- and (–)-ABA (see Figure 1.4) on stomatal aperture were measured in detached epidermis of *C. communis*. The unnatural enantiomer of ABA, (–)-ABA, inhibited stomatal opening and promoted stomatal closure in this species. However, (–)-ABA was not as effective as either the natural form of ABA, (+)-ABA, or synthetic (±)-ABA (Figure 2.2) (+)-ABA started to inhibit stomatal opening at 10^{-8} M and saturated the response at 10^{-6} M (Figure 2.2.A); it started to promote stomatal closure at $5x10^{-10}$ M and saturated the response at $5x10^{-8}$ M (Figure 2.2.B). (–)-ABA was not active until supplied at approximately a 10-fold higher concentration than that of (+)-ABA, and did not saturate either response at the maximum concentrations investigated (Figure 2.2). For inhibition of stomatal opening, the (±)-ABA dose-response curve lay closer to the (+)-ABA dose response curve than would be predicted if the (–)-ABA in the racemic mix were inactive (Figure 2.2.A). If (–)-ABA were inactive, it could be predicted that (+)-ABA would be twice as effective as (±)-ABA. Interestingly, for promotion of stomatal closure the dose-response curves for (+)- and (±)-ABA lay very close together, unlike the distinct separation seen for inhibition of stomatal opening (Figure 2.2).

The kinetics of (–)-ABA-induced inhibition of stomatal opening in detached epidermis of *C. communis* were examined. It was shown that (–)-ABA inhibited stomatal opening by approximately 40 % relative to control values after 20 minutes of incubation and continued to inhibit stomatal opening throughout the duration of the experiment (Figure 2.3).

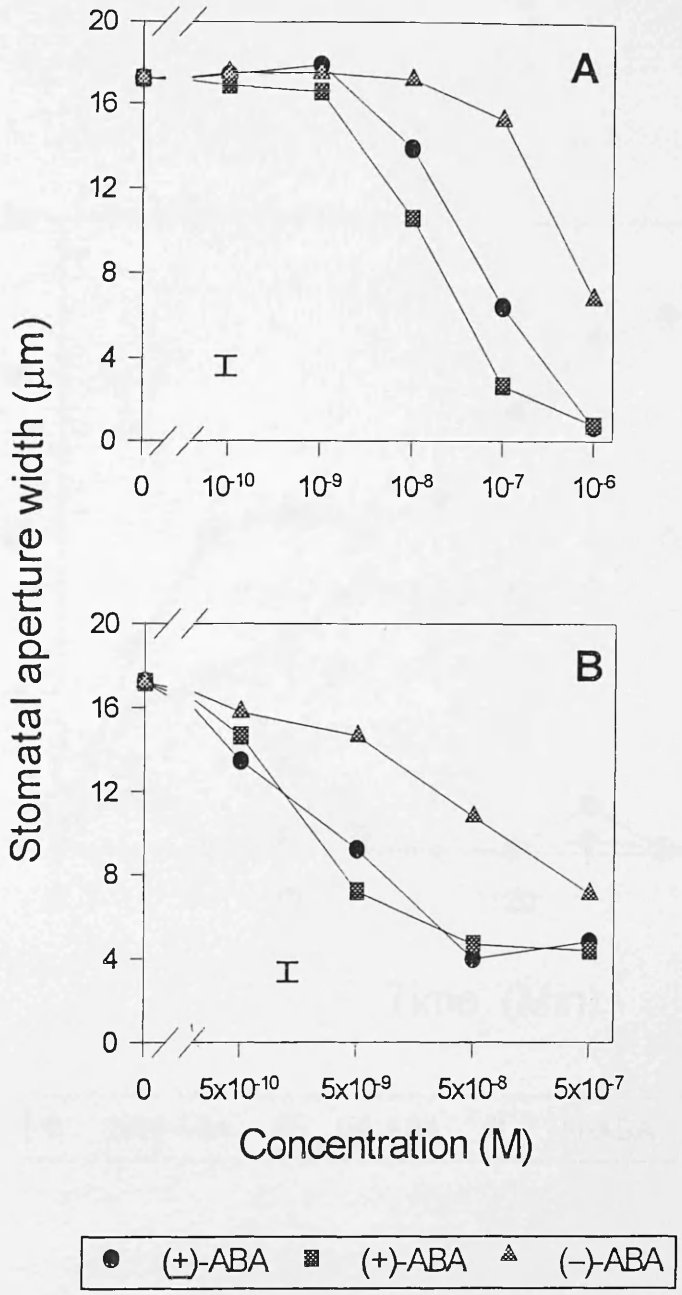


Figure 2.2 The effect of the optical isomers of ABA [(+)-ABA and (–)-ABA] on stomatal aperture of detached epidermis of *Commelina communis*. **(A)** Inhibition of stomatal opening; **(B)** Promotion of stomatal closure. Each point represents an average of 120 measurements, the vertical bar represents the LSD. [Positive and negative controls are (±)-ABA and zero ABA, respectively].

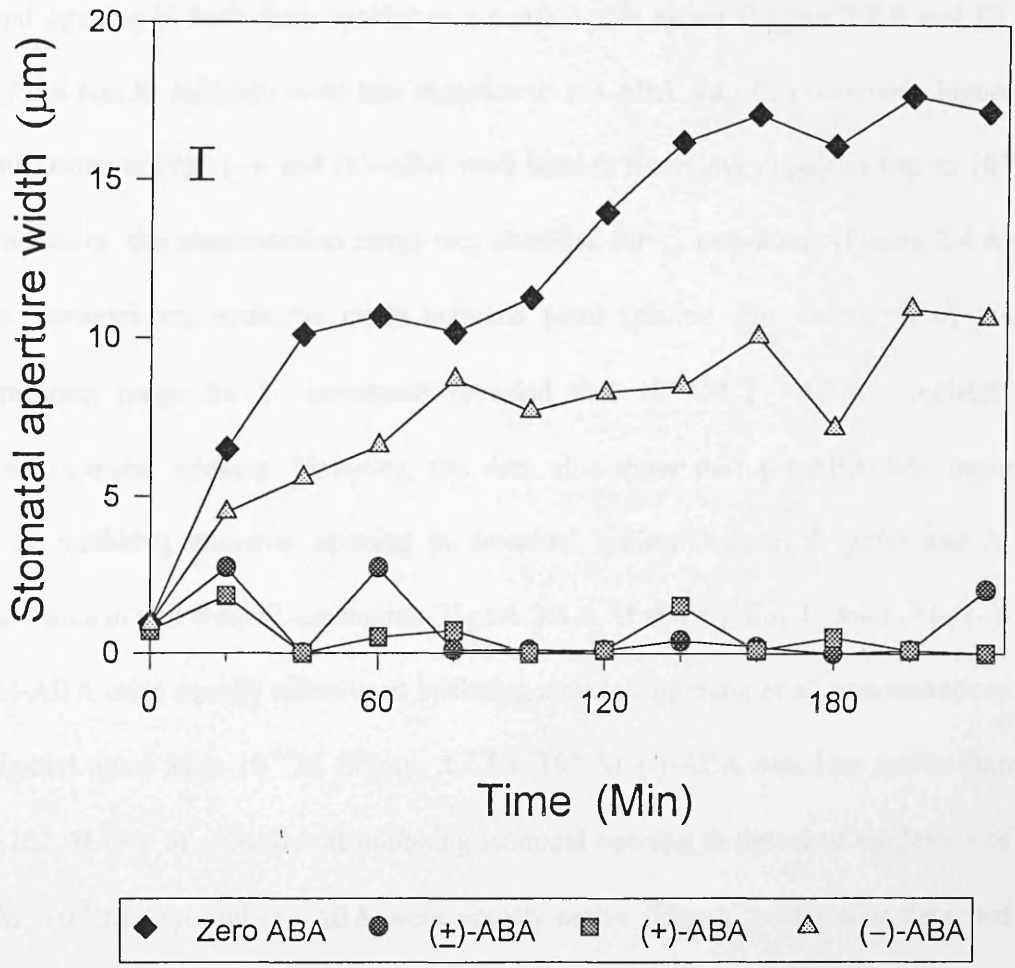


Figure 2.3 Time course of the effect of 10^{-6} M (+)- and (–)-ABA on stomatal opening in detached epidermis of *Commelina communis*. Each point represents an average of 40 measurements; the vertical bar represents the LSD. [Positive and negative controls are (±)-ABA and zero ABA respectively].

In order to determine whether the biological activity of (–)-ABA in the stomatal bioassay was specific to *C. communis*, the effect of (–)-ABA on stomatal opening in detached epidermis from *V. faba* and *N. tabacum* was investigated. (–)-ABA inhibited stomatal opening in both these species to a considerable extent (Figure 2.4.B and C). As *V. faba* and *N. tabacum* were less sensitive to (±)-ABA than *C. communis,* higher concentrations of (+)-, (–)- and (±)-ABA were used in these investigations (up to 10^{-4} M). Therefore, the concentration range was extended for *C. communis* (Figure 2.4.A) so that comparisons could be made between plant species. The extension of the concentration range for *C. communis* revealed that 10^{-5} M (–)-ABA completely inhibited stomatal opening. However, the data also show that (–)-ABA was more active at inhibiting stomatal opening in detached epidermis from *V. faba* and *N. tabacum* than in that from *C. communis* (Figure 2.4.A, B and C). For *V. faba* (+)-, (–)- and (±)-ABA were equally effective at inhibiting stomatal opening at all concentrations investigated apart from 10^{-6} M (Figure 2.4.B). 10^{-6} M (-)-ABA was less active than either 10^{-6} M (+)- or (±)-ABA at inhibiting stomatal opening in detached epidermis of *V. faba*; 10^{-6} M (+)- and (±)-ABA were equally active (Figure 2.4.B). For detached epidermis of *N. tabacum* the (-)-ABA dose-response curve lay closer to the (+)- and (±)-ABA dose-response curves than for detached epidermis of *C. communis* (Figure 2.4.A and C). However, the (–)-ABA dose-response curve for *N. tabacum* did not lie as close to the (+)- and (±)-ABA dose-response curves as seen for detached epidermis of *V. faba* (Figure 2.4.B and C). At 10^{-6} M the order of effectiveness of the molecules at inhibiting stomatal opening in detached epidermis of *N. tabacum* was: (+)-ABA> (±)-ABA>(-)-ABA (Figure 2.4.C). At 10^{-5} M, (+)- and (±)-ABA were more effective than

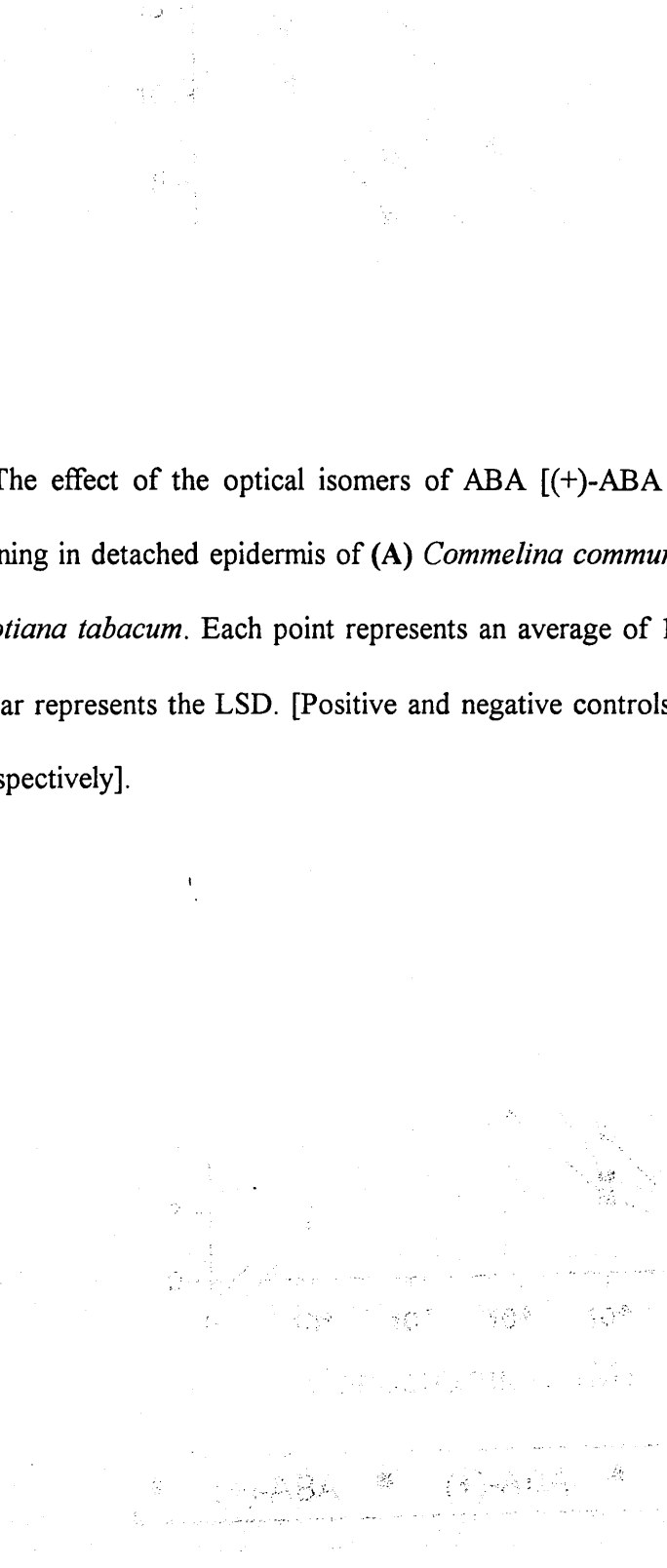
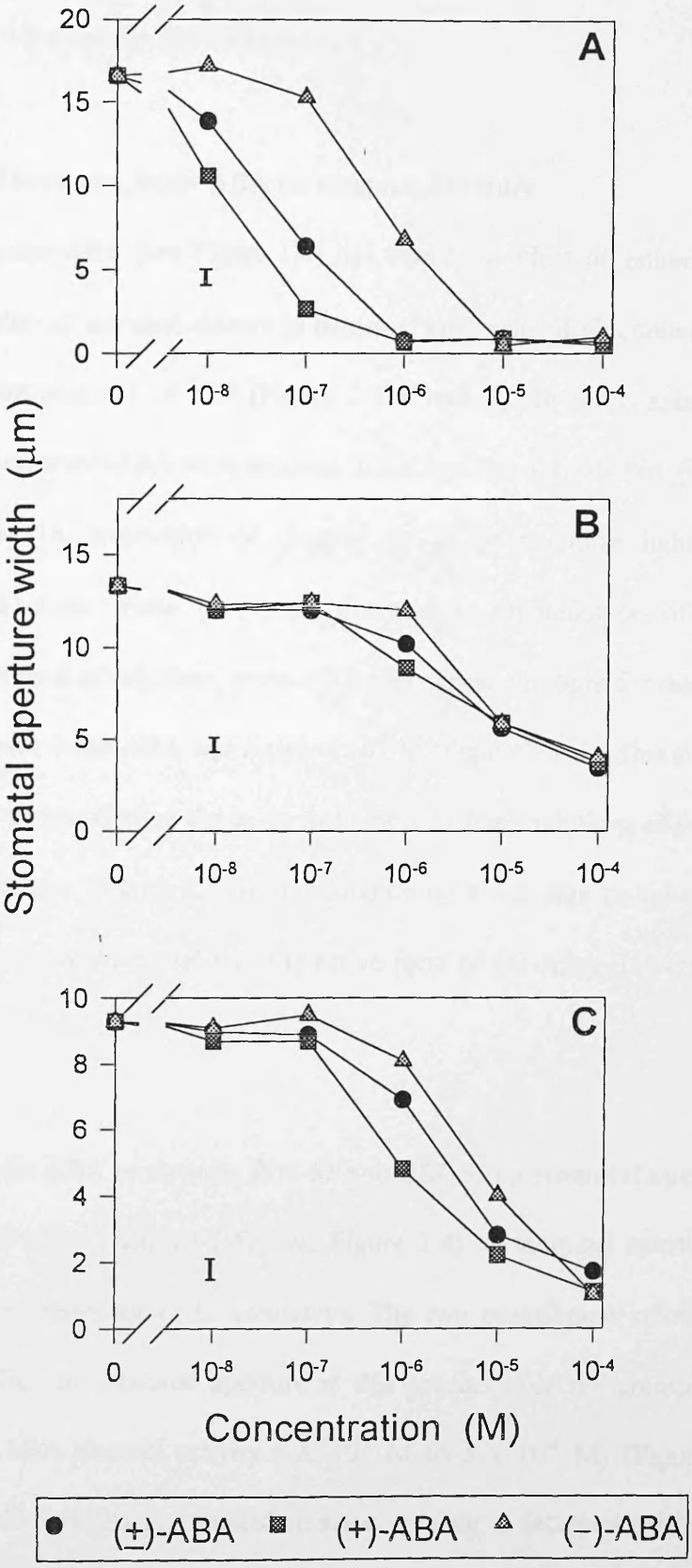


Figure 2.4 The effect of the optical isomers of ABA [(+)-ABA and (–)-ABA] on stomatal opening in detached epidermis of **(A)** *Commelina communis,* **(B)** *Vicia faba* and **(C)** *Nicotiana tabacum*. Each point represents an average of 120 measurements; the vertical bar represents the LSD. [Positive and negative controls are (±)-ABA and zero ABA respectively].


A
B
C
20
15
10
5
0
10
8
6
4
2
0
I
0
10-8
10-7
10-6
10-5
10-4
Stomatal aperture width (μm)
Concentration (M)
(±)-ABA
(+)-ABA
(−)-ABA

(-)-ABA at inhibiting stomatal opening in detached epidermis of *N. tabacum*; 10^{-5} M (+)- and (±)-ABA were equally active (Figure 2.4.C).

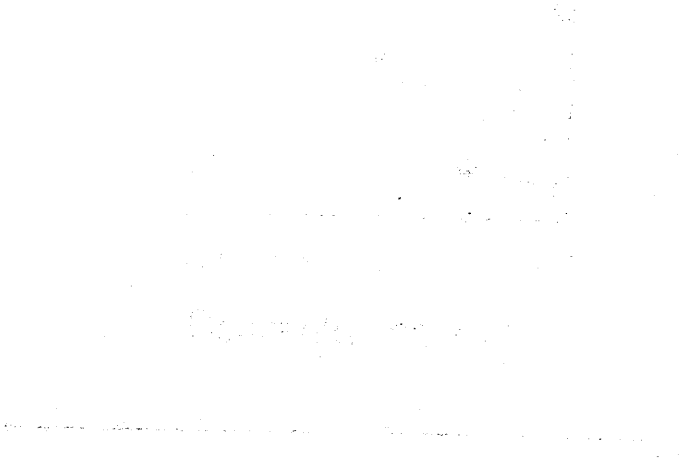
2.3.2 The effect of (±)-*trans, trans*-ABA on stomatal aperture

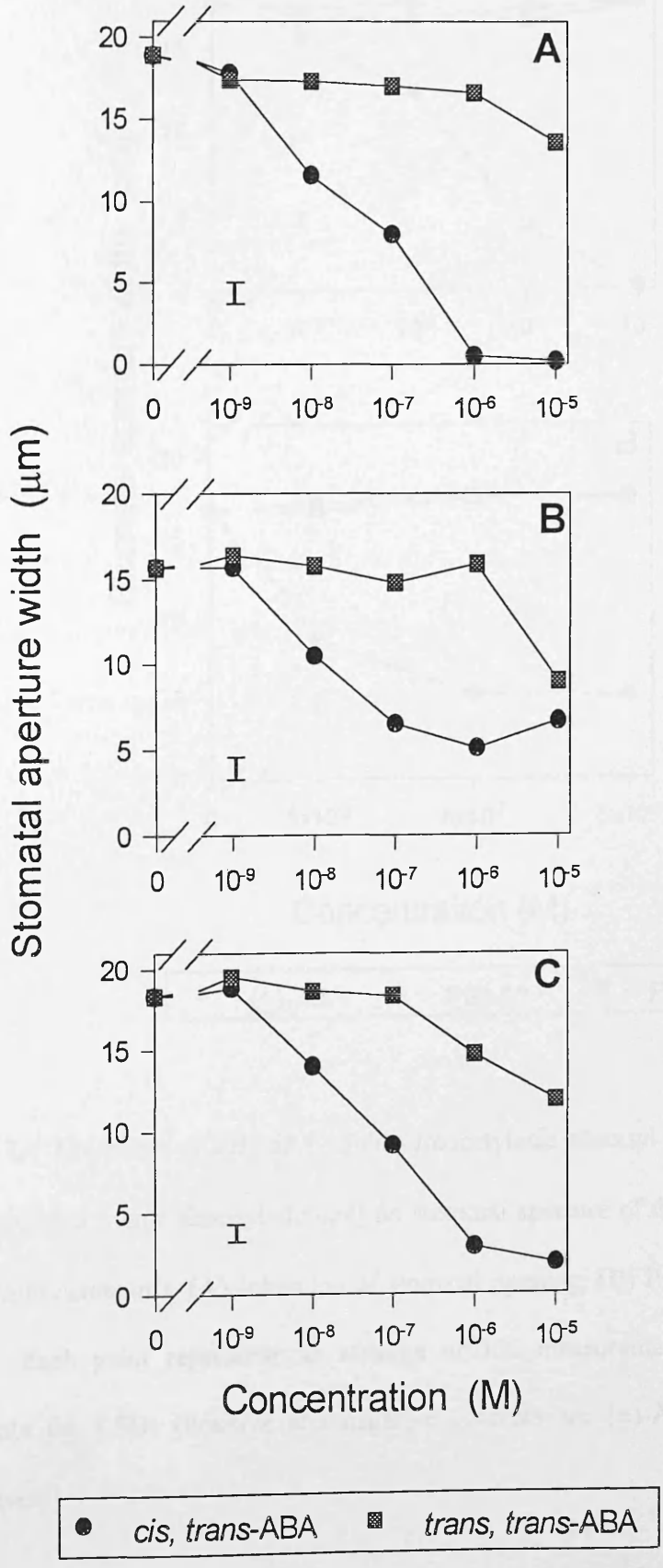
(±)-*trans, trans*-ABA (see Figure 1.4) had very little effect on either stomatal opening or promotion of stomatal closure in detached epidermis of *C. communis* until applied at a concentration of 10^{-5} M (Figure 2.5.A and B). In these assays, fresh solutions of (±)-*trans, trans*-ABA were exposed to the light for only 30 min. (inhibition of opening) or for 1h (promotion of closure) so as to minimize light-induced isomerization of (±)-*trans, trans*- to (±)-*cis, trans*-ABA. An inhibition of opening assay, in which a solution of (±)-*trans, trans*-ABA was exposed to light for the full 3 h, revealed that (±)-*trans, trans*-ABA was active at 10^{-6} M (Figure 2.5.C). This activity could be eliminated by transferring the epidermal pieces to fresh solutions of (±)-*trans, trans*-ABA every 30 min. (Figure 2.4.A), thus suggesting it was due to light-induced isomerization of (±)-*trans, trans*-ABA to the active form of (±)-ABA [(±)-*cis, trans*-ABA].

2.3.3 The effect of the ABA analogues, PBI-63 and PBI-51 on stomatal aperture

The effect of PBI-63 and PB1-51 (see Figure 1.4) on stomatal aperture was measured in detached epidermis of *C. communis*. The two enantiomers of the ABA analogue had no effect on stomatal aperture in this species over the concentration range at which (±)-ABA showed activity (i.e. 10^{-8} M to 5 x 10^{-6} M) (Figure 2.6). However, both PBI-63 and PBI-51 inhibited stomatal opening in detached epidermis of *C. communis* to a similar degree (approximately 65%) when supplied at a 10^{-4} M (Figure 2.7.A). The same was true in detached epidermis from *V. faba*; PBI-51 and

Figure 2.5 The effect of (±)-*trans, trans*-ABA on stomatal aperture of detached epidermis of *Commelina communis*. (**A**) Inhibition of stomatal opening with fresh solutions every 30 minutes; (**B**) Promotion of stomatal closure; (**C**) Inhibition of stomatal opening (in which the incubation medium was not changed during the 3 h incubation period). Each point represents an average of 80 measurements; the vertical bar represents the LSD. [Positive and negative controls are (±)-*cis, trans*-ABA and zero ABA, respectively].




A
B
C
20
15
10
5
0
0
10-9
10-8
10-7
10-6
10-5
Stomatal aperture width (μm)
Concentration (M)
cis, trans-ABA
trans, trans-ABA

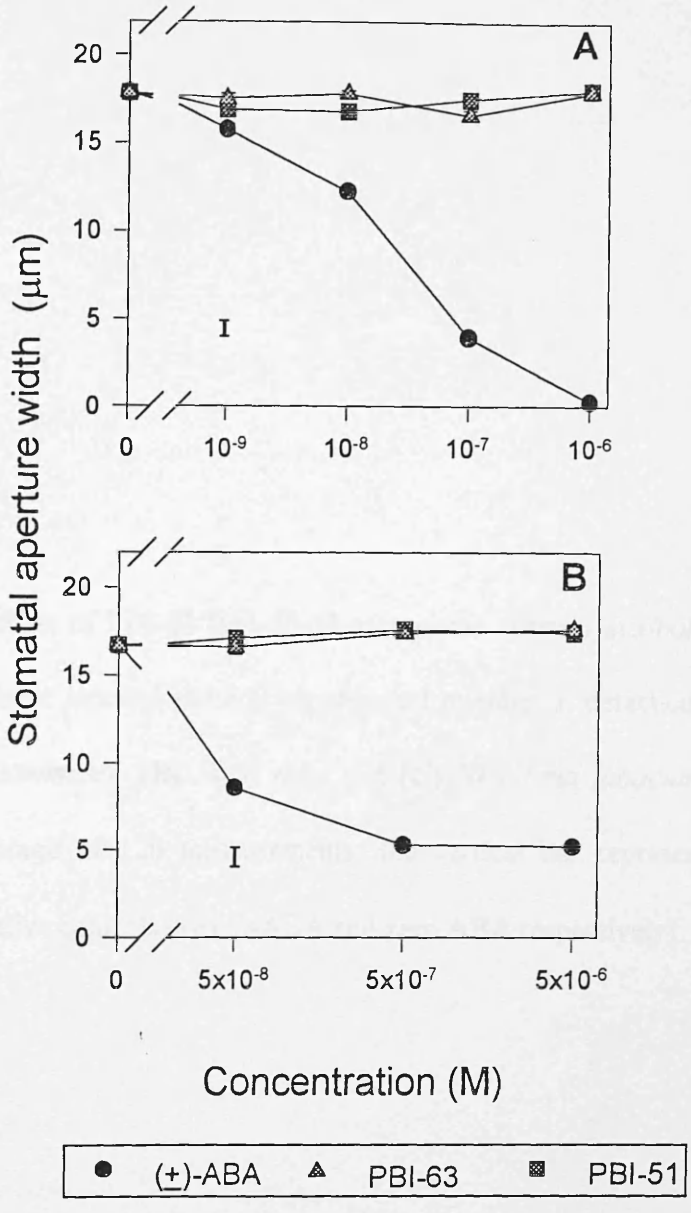
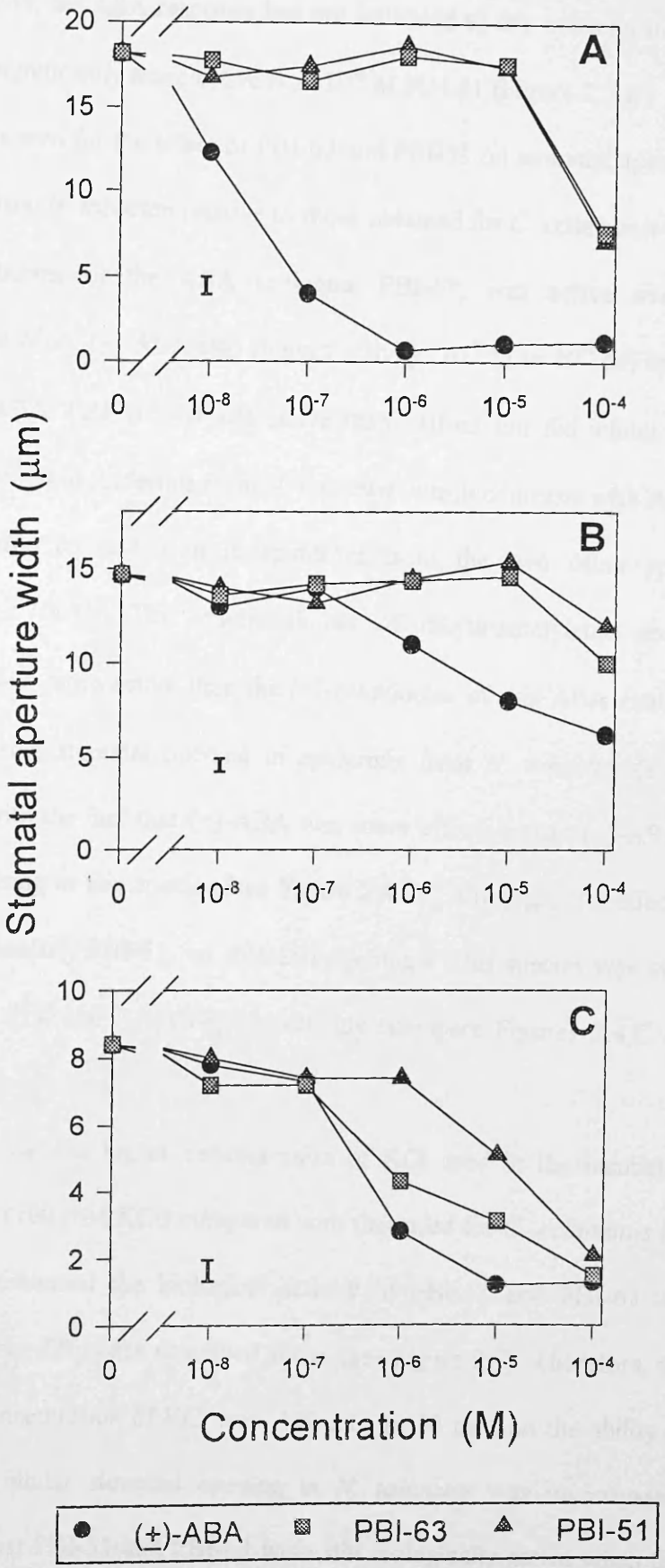


Figure 2.6 The effect of PBI-63 [(+)-dihydroacetylenic abscisyl alcohol] and PBI-51 [(−)-dihydroacetylenic abscisyl alcohol] on stomatal aperture of detached epidermis of *Commelina communis.* (**A**) Inhibition of stomatal opening; (**B**) Promotion of stomatal closure. Each point represents an average of 120 measurements; the vertical bar represents the LSD. [Positive and negative controls are (±)-ABA and zero ABA respectively].

Figure 2.7 The effect of PBI-63 [(+)-dihydroacetylenic abscisyl alcohol] and PBI-51 [(–)-dihydroacetylenic abscisyl alcohol] on stomatal opening in detached epidermis of **(A)** *Commelina communis,* **(B)** *Vicia faba* and **(C)** *Nicotiana tabacum*. Each point represents an average of 120 measurements; the vertical bar represents the LSD. [Positive and negative controls are (±)-ABA and zero ABA respectively].


A
B
C
Stomatal aperture width (μm)
Concentration (M)
(±)-ABA
PBI-63
PBI-51

PBI-63 were inactive until applied at 10^{-4} M (Figure 2.7.B). However, for detached epidermis from *V. faba*, the ABA response had not saturated at this concentration and 10^{-4} M PBI-63 was significantly more active than 10^{-4} M PBI-51 (Figure 2.7.B).

A different result was seen for the effect of PBI-63 and PBI-51 on stomatal aperture in detached epidermis from *N. tabacum* relative to those obtained for *C. communis* and *V. faba*. The (+)-enantiomer of the ABA analogue, PBI-63, was active over the concentration range at which (±)-ABA also showed activity (10^{-6} M to 10^{-4} M) but was less active than (±)-ABA. PBI-51 was less active than PBI-63 but did inhibit some stomatal opening at 10^{-5} M in epidermis from *N. tabacum*, which contrasts with its lack of activity at the same concentration in epidermis from the two other species investigated (Figure 2.7.A-C). The (+)-enantiomer of dihydroacetylenic abscisyl alcohol (i.e. PBI-63) was more active than the (–)-enantiomer of this ABA analogue (i.e. PBI-51) at inhibiting stomatal opening in epidermis from *N. tabacum* (Figure 2.7.C). This concurs with the fact that (+)-ABA was more effective than (–)-ABA at inhibiting stomatal opening in this species (see Figure 2.4.C). Although, the effect of PBI-63, and more particularly PBI-51, on stomatal opening in this species was much less than that seen for (+)- and (–)-ABA, respectively (compare Figures 2.4.C and 2.7.C).

It was possible that the higher concentration of KCl used in the incubation medium for *N. tabacum* (100 mM KCl) compared with that used for *C. communis* (50 mM KCl) could have enhanced the biological activity of PBI-51 and PBI-63 and resulted in the plant species difference described above (see Figure 2.7). Therefore, the effect of reducing the concentration of KCl from 100 mM to 50 mM on the ability of PBI-51 and PBI-63 to inhibit stomatal opening in *N. tabacum* was investigated. However, it was found that PBI-51 and PBI-63 were still biologically active when the

concentration of KCl used in the incubation medium was reduced from 100 to 50 mM (Figure 2.8).

2.3.4 Does PBI-51 antagonize ABA-induced changes in stomatal aperture?

The effect of PBI-51 on (±)-ABA-induced changes in stomatal aperture was investigated in detached epidermis from *C. communis* and *N. tabacum* in order to determine whether PBI-51 would act as an (±)-ABA antagonist in this system. It has been reported that PBI-51 acts as an ABA antagonist in other plant systems [see Section 1.7.4(c); Wilen *et al.*, 1993, 1996]. However, 10^{-5} M PBI-51 was without effect on (±)-ABA-induced inhibition of stomatal opening or promotion of stomatal closure in *C. communis* when it was applied concurrently with (±)-ABA (Figure 2.9.A and B). A 3 h incubation in 10^{-5} M PBI-51 prior to concurrent application with (±)-ABA (for 1 hour in the promotion of stomatal closure protocol), revealed that PBI-51 still did not antagonize the ABA-induced response (Figure 2.9.C). A concentration of 10^{-5} M PBI-51 was used in the experiments with detached epidermis from *C. communis* because it was the highest concentration at which an individual application of PBI-51 showed no effect on the stomatal aperture width (see Figure 2.7.A). Concurrent application of 10^{-5} M PBI-63 [(+)-dihydroacetylenic abscisyl alcohol] and (±)-ABA resulted in a small, but significant enhancement of activity in the inhibition of stomatal opening assay (Figure 2.9.A) and in the promotion of stomatal closure assay (with a 3 h pre-incubation in 10^{-5} PBI-63) but only at the lowest (±)-ABA concentration (5×10^{-9} M), in the latter case (Figure 2.9.C).

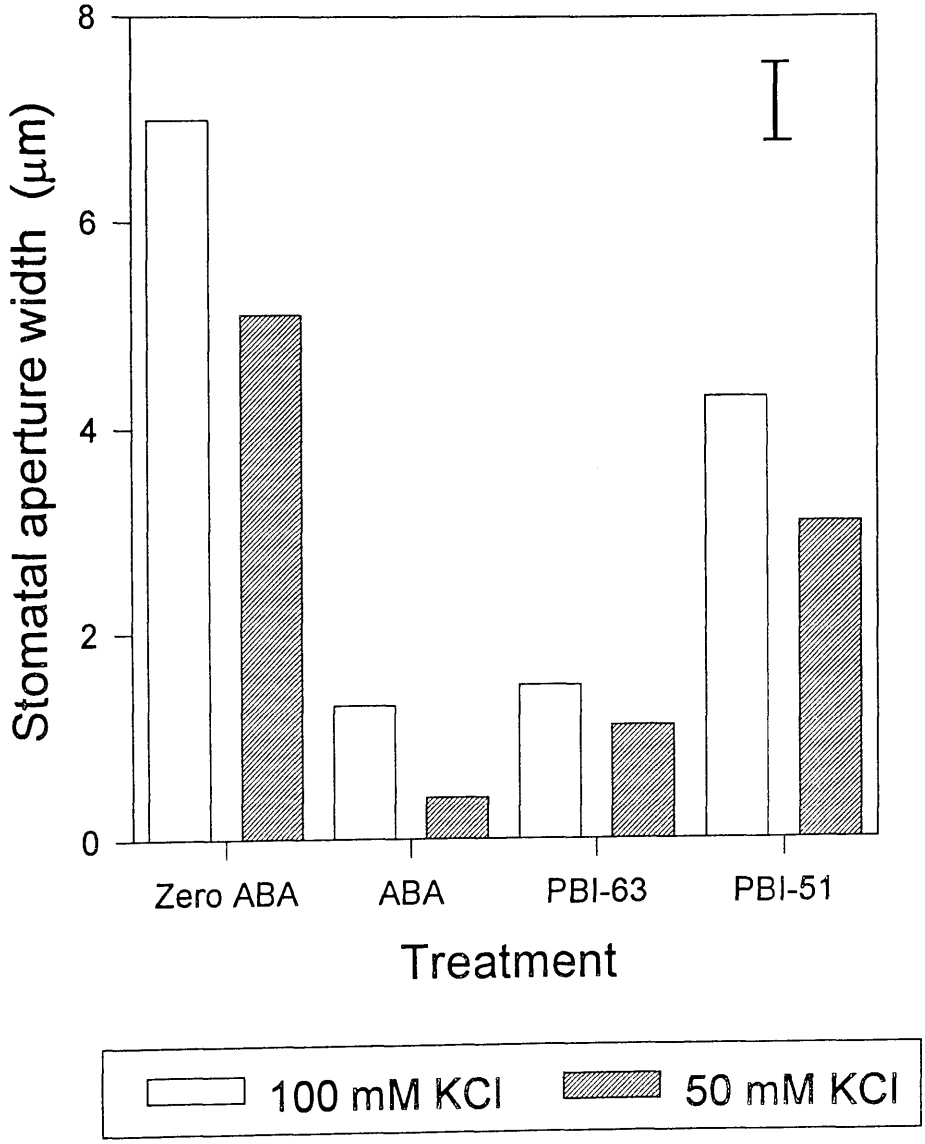
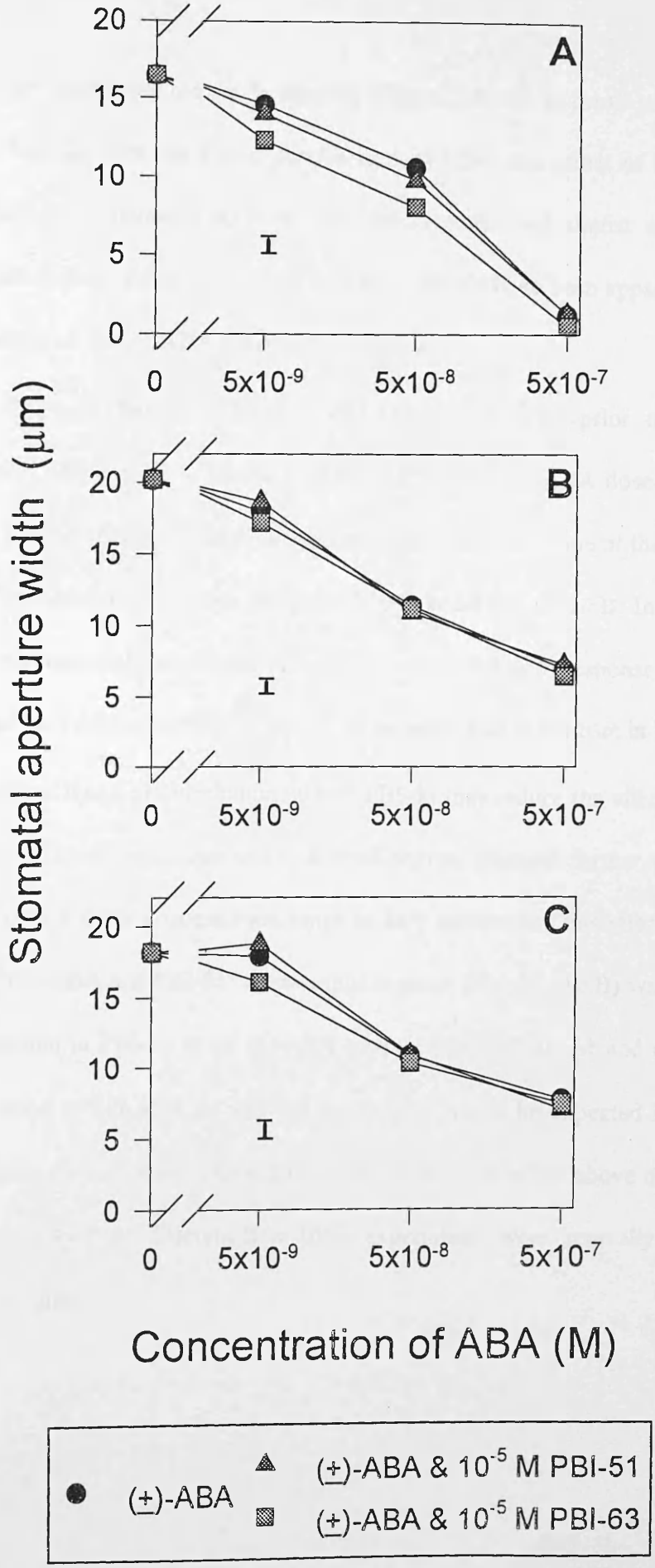


Figure 2.8 The effect of two KCl concentrations on inhibition of stomatal opening by 10^{-5} M PBI-63 and PBI-51 in detached epidermis of *Nicotiana tabacum*. Each point represents an average of 120 measurements; the vertical bar represents the LSD. [The positive and negative controls are 10^{-5} M (±)-ABA and zero ABA, repectively].

Figure 2.9 The effect of 10^{-5} M PBI-63 and PBI-51 on (±)-ABA-induced changes in stomatal aperture in detached epidermis of *Commelina communis.* (**A**) Concurrent application with (±)-ABA:- inhibition of stomatal opening; (**B**) Concurrent application with (±)-ABA:- promotion of stomatal closure; (**C**) Pre-incubation in PBI-51 then as for (B). Each point represents an average of 120 measurements; the vertical bar represents the LSD. [Positive and negative controls are (±)-ABA and zero ABA resp ectively].


A
B
C
20
15
10
5
0
0
5x10-9
5x10-8
5x10-7
Stomatal aperture width (µm)
Concentration of ABA (M)
(±)-ABA
(±)-ABA & 10-5 M PBI-51
(±)-ABA & 10-5 M PBI-63

application experiment did not lie together (Figure 2.10.A), as for *C. communis* (Figure 2.9.A). Instead, they ran almost parallel to each other, the effect of PBI-51 and (±)-ABA being approximately additive, until the response was almost saturated. It was envisaged that any ABA-antagonism by PBI-51 would have been apparent as a change in the shape of the (±)-ABA dose-response curve.

A pre-incubation in 10^{-5} M PBI-51 (in the dark), prior to a concurrent application with (±)-ABA did not drastically affect the (±)-ABA dose-response curve (Figure 2.10 B). However, there is a difference between the shape of the "(±)-ABA and PBI-51" dose-response curves in Figure 2.10.A and Figure 2.10.B. In Figure 2.10.B, there is no statistically significant difference between the dose response curves for (±)-ABA and "(±)-ABA and PBI-51" at 10^{-6} M, whereas this is not true in Figure 2.10. A. This suggests that a pre-incubation in 10^{-5} PBI-51 may reduce the effect of "(±)-ABA and PBI-51" on stomatal aperture to a small degree, although further work is needed possibly over a wider concentration range to fully substantiate this effect. It is unusual that the "(±)-ABA and PBI-51" dose-response curve (Figure 2.10.B) was affected by a pre-incubation in PBI-51 at an (±)-ABA concentration of 10^{-6} M and not at a lower concentration of (±)-ABA as well (or instead) as would be expected if PBI-51 was antagonizing the response. It should be noted, in the light of the above discussion, that the stomatal aperture differences in these experiments were generally small, in the order of ≤ 2 μm.

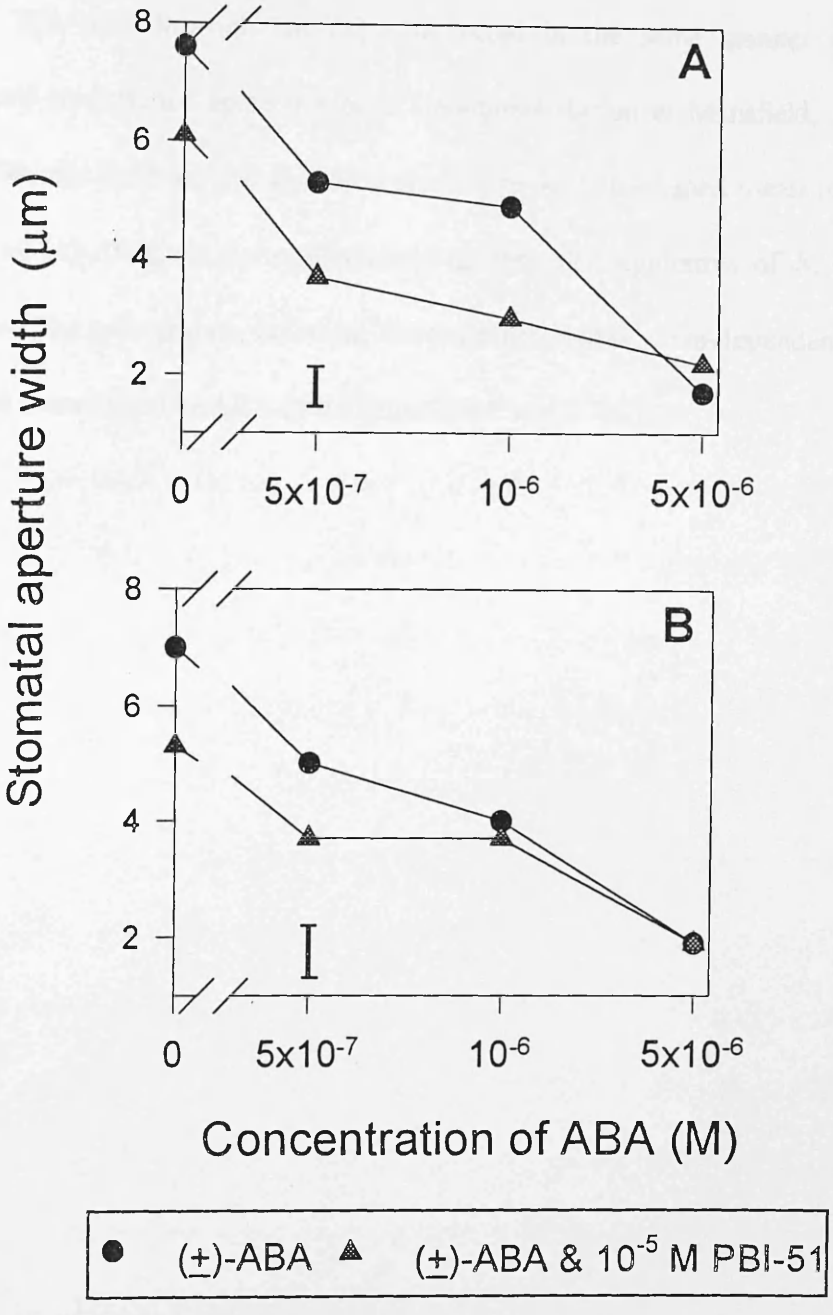


Figure 2.10 The effect of 10^{-5} M PBI-51 on (±)-ABA-induced inhibition of stomatal opening in detached epidermis of *Nicotiana tabacum*. (**A**) Concurrent application with (±)-ABA; (**B**) Pre-incubation in PBI-51 then as for (A). Each point represents an average 80 measurements; the vertical bar represents the LSD. [Positive and negative controls are (±)-ABA and zero ABA respectively].

2.3.5 The effect of (±)-ABA on stomatal aperture

The data showed that (±)-ABA acted in the same manner as previously described in detached epidermis of C. communis (Orton & Mansfield, 1974) and *V. faba* (Wardle & Short, 1981). There are no previous, published measurements of the effect of (±)-ABA on stomatal aperture in detached epidermis of *N. tabacum* cv. Samsun. The present data, however, show a reproducible, dose-dependent response of tobacco stomata to (±)-ABA (see Figures 2.4.C and 2.7.C) .

2.4 Discussion

Optically pure ABA analogues (see Figure 1.4) were used as tools to investigate the perception of ABA in the signalling pathways involved in the control of guard cell turgor. It was found that (–)-ABA, the unnatural optical isomer of (+)-ABA, inhibited stomatal opening and promoted stomatal closure in detached epidermis from *C. communis* (Figure 2.2) and inhibited stomatal opening in detached epidermis from two other species, *V. faba* and *N. tabacum* (Figure 2.4). The level of activity attributed to (–)-ABA in this study was a great deal higher than that previously reported for (–)-ABA in the stomatal system (Walton, 1983). One possible explanation for this discrepancy is the less direct approach of the earlier studies which determined the effect of (–)-ABA on stomatal aperture by measuring stomatal conductance in whole leaves (Kriedmann *et al.*, 1972; Cummins & Sondheimer, 1973). However, studies in the 1980s (Milborrow, 1980; Hornberg & Weiler, 1984), using the more direct and sensitive detached epidermal strip bioassay still showed (–)-ABA to be inactive. The apparent inability of 10^{-5} M (–)-ABA to promote stomatal closure in detached epidermis from *C. communis* and *Tropaeolum majus* (Milborrow, 1980) may, however, have been due to the relatively small aperture widths and range of aperture widths over which changes were measured. By way of contrast, in the present study the average control aperture in detached epidermis of *C. communis* was approximately six times wider than that in Milborrow's (1980) study and 5×10^{-7} M (–)-ABA and (+)-ABA promoted stomatal closure by approximately 60% and 70%, respectively (Figure 2.2.B). In addition, the present study showed that both 10^{-5} M (–)-ABA and (+)-ABA inhibited stomatal opening in detached epidermis from *C. communis* by approximately 100% (Figure 2.4.A).

It has been suggested that ABA-induced promotion of closure and inhibition of stomatal opening are evoked by different ABA perception mechanisms (see review by Giraudat, 1995; see Section 1.5.1). However, in the present study (–)-ABA both inhibited stomatal opening and promoted stomatal closure in detached epidermis from *C. communis* (Figures 2.2 and 2.4.A). This suggests that there are similarities in the stereochemical requirements of the putative ABA "receptor(s)" for the chiral centre of the ABA molecule in both these ABA-induced responses. It was interesting to note that (+)- and (±)-ABA promoted stomatal closure to a very similar extent but that (+)-ABA inhibited stomatal opening to a greater extent than (±)-ABA at all concentrations tested (Figure 2.2). Whether this reflects a difference in the perception mechanisms of the two ABA-induced responses, or the differing length of time to which the epidermal pieces were exposed to the molecules (1 or 3 h) remains to be determined.

The data suggest that (–)-ABA has intrinsic activity because the effect of (–)-ABA on stomatal opening in detached epidermis from *C. communis* was detected after 20 minutes and continued for the duration of the experiment (Figure 2.3). However, the possibility that (–)-ABA could have affected a secondary process, for example, release or synthesis of endogenous (+)-ABA in the epidermis within the first twenty minutes of the experiment cannot be ruled out. More detailed investigations, including the determination of endogenous (+)-ABA levels in (–)-ABA-treated epidermis would help to clarify the situation.

Although (–)-ABA was biologically active, it was generally less active than (+)- or (±)-ABA. This suggests that the stereochemistry at C-1' is important for ABA-induced changes in guard cell turgor, but not as important as previously believed (see Table 2.1).

The discovery that (–)-ABA is more effective in the stomatal system (in terms of turgor regulation) than previously believed, may affect the hypothesis that there are two types of ABA "receptor" depending on whether the response is classified as "fast" or "slow" (Milborrow, 1980). This model was based on the fact (–)-ABA affected plant processes such as growth (slow response) but had no effect on stomatal aperture (fast response) (Milborrow, 1980). The model may not be valid for species such as *V. faba* and *N. tabacum* because the present study has shown that (–)-ABA does inhibit stomatal opening and in some cases was as biologically active as (+)-ABA in this system (Figure 2.4.B and C). This suggests that for the guard cells of these species (–)-ABA can fit into the active site of the putative ABA "receptor" (associated with bringing about changes in stomatal aperture) normally occupied by (+)-ABA. This may be achieved in a similar manner to that put forward by Milborrow (1978) for the putative ABA growth "receptor" (see Section 1.7.2). The fact that (–)-ABA was needed at over a tenfold higher concentration than (+)-ABA in order to affect stomatal aperture in detached epidermis from *C. communis* to the same degree (Figure 2.4.A) suggests that the two receptor model idea may still hold true for this species if "slow" plant processes such as growth are equally affected by the two enantiomers. Further work is needed to confirm this. However, the biological activity of (–)-ABA reported in the present study does suggest that there may not be such a sharp distinction between the receptors for the "fast" and "slow" responses to ABA (see Section 1.5) in *C. communis* as Milborrow's (1980) general model proposed.

The effect of (±)-ABA [a 1:1 mix of (+)-ABA and (–)-ABA] on stomatal aperture was investigated because until recently it was the only form of ABA available commercially. Consequently, a large body of data exists on the effects of (±)-ABA on plant processes, including changes in stomatal aperture (Trejo *et al.*, 1993) and

associated signalling pathways (for example McAinsh *et al.*, 1990, 1991, 1992; Gilroy *et al.*, 1991; Staxén *et al.*, 1996, 1997). In some instances, the activity shown by the racemate was assumed to be solely due to (+)-ABA (Trejo *et al.*, 1993). This assumption is contradicted by the present data which shows that (–)-ABA is also active.

The very low activity of (±)-*trans, trans*-ABA, a geometric isomer of ABA, in the guard cell system (Figure 2.5.B and C), mirrors previous research on the activity of this molecule in other plant systems [see Section 1.7.4(b)]. The activity attributed to the molecule at 10^{-5} M is assumed to be due to light-induced isomerization of (±)-*trans, trans*-ABA to (±)-*cis, trans*-ABA. Indeed, the biological activity of a solution of 10^{-6} M (±)-*trans, trans*-ABA exposed to the light for 3 hours was eliminated by transferring the epidermal pieces to 'fresh' (i.e. not exposed to the light) solutions of (±)-*trans, trans*-ABA every 30 minutes (Figures 2.5.A and B). In the future, this assumption could be tested by using a continuous perfusion system in which (±)-*trans, trans*-ABA would be only exposed to the light for ten minutes [see Section 4.2.5(b)]. It is interesting that the simple change to the orientation of the side chain apparent in *trans, trans*-ABA results in a molecule which is unable to inhibit stomatal opening or promote stomatal closure. This suggests a specific requirement of the ABA "receptor(s)" for both these processes.

The optical isomers of the ABA analogue, dihydroacetylenic abscisyl alcohol [the (+)-isomer designated PBI-63, and the (–)-isomer designated PBI-51] were markedly less active than the optical isomers of ABA at inducing changes in stomatal aperture in detached epidermis from *C. communis* and *V. faba*. However, the reduction in activity seen with PBI-63 and PBI-51 was much less extreme in detached epidermis from *N. tabacum*. Overall, these findings suggest the combination of changes to the structure of

the ABA molecule in PBI-63 and PBI-51 (see Figure 1.4) had a profound effect on the ability of the molecules to evoke a response. A similar ABA analogue, (acetylenic abscisyl alcohol) which only differs from PBI-63 by the presence of a double bond in the ring of the molecule, was a more effective antitranspirant than (±)-ABA in several coniferous species (Blake *et al.*, 1989, 1990a & b). The difference in activity between this analogue (Blake *et al.*, 1989, 1990a & b) and PBI-63 and PBI-51 could be due to the presence of the ring double bond or to the fact that two types of ABA analogue were tested in two very different systems.

The differences in biological activity seen between plant species in the relative effectiveness of PBI-63 and -51 [and to a lesser extent (-)-ABA], on stomatal opening, compared with (+)-ABA was an interesting finding, but plant species differences in the effectiveness of ABA analogues are by no means an unusual phenomenon (see below). Indeed, the sensitivity of different plant species to naturally-occurring (+)-ABA varies (see Quarrie, 1983; Schulte & Hinckley, 1987; Blake *et al.*, 1990a). Therefore, it is not unexpected that the sensitivity of guard cells to ABA analogues will vary between plant species. Species differences have been seen in the requirement of the 4' carbonyl group of ABA for stomatal closure. Thus, the lack of the 4' carbonyl group or the replacement of this group with a hydrazone group, led to a loss of activity in closure of stomata of *C. communis* (Hite *et al.*, 1994; Yamamoto & Oritani, 1995), but not of *V. faba* (Hornberg & Weiler, 1984). In addition, it was shown that ABA analogues without a 4' carbonyl group still retained biological activity in a barley inhibition of transpiration assay, as long as the C-8' and C-9' groups were present (Schubert *et al.*, 1991). Species differences have also been reported in the properties of ABA uptake carriers (Astle & Rubery, 1987; Windsor *et al.*, 1994; Perras & Abrams, 1993). Methyl ABA inhibited the uptake of ABA by a carrier in *Phaseolus coccineus* suspension-

cultured cells (Astle & Rubery, 1987), but not in barley or carrot suspension-cultured cells (Perras & Abrams, 1993; Windsor *et al.*, 1994;).

Why these plant species differences exist is unclear, but they could be due to differences in: (i) the "physiological address" (see McAinsh *et al.*, 1997) of the guard cells and epidermal cells; (ii) in the structural requirements, density and location (intracellular or extracellular) (see Section 1.5.1) of the guard cell ABA "receptor(s)";or (iii) in the effect of the molecules on endogenous ABA concentration (see Wilen *et al.*, 1993).

The most striking difference between plant species was seen in the effect of PBI-63 and -51 on stomata in detached epidermis of *N. tabacum* and *C. communis* (Figure 2.7.A and C). It was shown that the higher biological activity of PBI-63 and -51 in detached epidermis from *N. tabacum* compared with *C. communis* was not due to differences in the conditions under which the epidermis was incubated (Figure 2.9). Recently, patch-clamp studies (see Section 1.2) have revealed that some aspects of the signalling pathway(s) which regulate guard cell ion channel activity in response to ABA differ between plant species (see Armstrong *et al.*, 1995; Schmidt *et al.*, 1995; Esser *et al.*, 1997; Grabov & Blatt, 1997; Pei *et al.*, 1997). Importantly for the present study, differences have emerged between ABA signalling pathways that control guard cell turgor in tobacco compared with *V. faba* and *C. communis* (Pei *et al.*, 1997; Armstrong *et al.*, 1995; Schmidt *et al.*, 1995; Esser *et al.*, 1997). The work of Schmidt *et al.* (1995) and Esser *et al.*, (1997) suggests that ABA-induced stomatal closure in *V. faba* and *C. communis* involves the activation of a protein kinase (see Section 1.5.3) and/or the inhibition of a protein phosphatase. In contrast, for ABA-induced stomatal closure in tobacco (*Nicotiana benthamiana*) activation of a protein phosphatase and inhibition of a protein kinase are reported to be important (Armstrong *et al.*, 1995).

Further investigations are, however, required to determine the precise underlying causes of the species differences reported in the present study.

One of the reasons for investigating the activity of PBI-63 and -51, was to determine whether PBI-51 would act as a ABA antagonist in the guard cell system as it does in other plant systems (Wilen *et al.*, 1993, 1996). The data described show clearly, that 10^{-5} M PBI-51 did not antagonize ABA-induced changes in stomatal aperture in detached epidermis from *C. communis* (Figure 2.9) or stomatal opening in detached epidermis from *N. tabacum* (Figure 2.10), whether or not a pre-incubation in 10^{-5} M PBI-51 was carried out.

N. tabacum was the most likely plant species in which PBI-51 may have displayed some degree of ABA-antagonism. This was due to the fact that the relative biological activities of PBI-51, -63 and (±)-ABA in the guard cell system of *N. tabacum* were the most similar out of the three plant species investigated in this study, to those seen for ABA-induced gene expression in *B. napus* microspore-derived embryos (Wilen *et al.*, 1993). However, PBI-51 was relatively more active in the guard cell system than in the gene expression system and this made any antagonistic effect of PBI-51 on (±)-ABA action difficult to determine because the effect could only be measured over a small concentration range. In addition, the changes in stomatal aperture were small (see Figure 2.10). The data suggest that a 1 hour pre-incubation in PBI-51 reduced slightly the biological activity of a solution containing (±)-ABA and 10^{-5} M PBI-51 (Figure 2.10.B). However, these data do not suggest that PBI-51 would act as a powerful antagonist to ABA-induced changes in guard cell turgor. It may be of interest to investigate the effect of a longer pre-incubation in PBI-51 on (±)-ABA-induced changes in *N. tabacum* guard cell turgor. A 2 to 3 h pre-incubation in PBI-51 was needed for PBI-51 to act as an antagonist of (±)-ABA-induced gene expression

(Wilen *et al.*, 1993). However, the present study showed that a pre-incubation in PBI-51 of this duration had no affect on (±)-ABA-induced promotion of stomatal closure in epidermal strips of *C. communis* (Figure 2.9.C).

It is interesting that PBI-51 does not antagonize ABA-induced changes in stomatal aperture width (guard cell turgor), but does antagonize ABA-induced changes in gene expression in *Brassica napus* microspore-derived embryos (Wilen *et al.*, 1993) and ABA-induced freezing tolerance (including the accumulation of heat stable and dehydrin-like proteins) in bromegrass cell culture (Wilen *et al.*, 1996). It may be that the stereochemistry of PBI-51 is not specific enough to interact with the guard cell ABA "receptor(s)" in ABA signalling pathways that lead to changes in guard cell turgor. However, it may be fruitful to investigate the possible antagonistic properties of lower concentrations of PBI-51 (see Appendix C for a preliminary study) because concentration-dependent antagonism by certain agents has been reported previously in biological systems (e.g. calcium channel blocker activity in guard cells - McAinsh *et al.*, 1991a).

In conclusion, (–)-ABA affected guard cell turgor to a greater extent than previously recorded; its degree of effectiveness, relative to (+)-ABA varied somewhat between plant species. For some plant species, this discovery may lead to a revision of the hypothesis that there are different ABA receptors for the "fast" and "slow" responses to ABA (Milborrow, 1980) which was based on (–)-ABA having very little effect on stomatal aperture. The biological inactivity of (±)-*trans, trans*-ABA in the present study concurs with previous research and suggests that the *cis* orientation at C-2 [see Section 1.7.4(b) and Figure 1.4] is essential in the perception of ABA in the signalling pathway which leads to a change in guard cell turgor. There were plant species differences in the biological activity of the ABA analogues, PBI-51 and PB1-

63. They were more biologically active in detached epidermis of *N. tabacum* than of *C. communis* or *V. faba.* The reason(s) for such a difference needs further investigation. PBI-51 did not antagonize ABA-induced changes in stomatal aperture (guard cell turgor). This suggests that the perception mechanisms involved in the signalling pathways leading to ABA-induced stomatal movements are stereochemically different from those terminating in changes in gene expression and freezing tolerance (Wilen *et al.*, 1993, 1996).

Chapter 3

The Effect of ABA on Turgor and Gene Promoter Activity in Tobacco Guard Cells

3.1 Introduction

3.1.1 The effects of ABA on guard cells

ABA regulates both turgor and gene expression in stomatal guard cells (see Sections 1.5, 1.6.2 and 1.8). However, neither the signalling pathway(s) by which ABA regulates gene expression in the guard cell (see Section 1.6.4) nor the relationship of this pathway with the ABA signalling cascade which terminates in a change in guard cell turgor (see Section 1.5) are fully understood. One way to investigate the relationship between these signal transduction pathways is to study simultaneously how ABA affects the turgor and gene expression of guard cells of a single species.

To date, there have been no direct reports of simultaneous measurements of the effects of ABA on turgor and gene expression in guard cells. However, two recent studies have simultaneously examined the effect of drought stress on guard cell turgor and gene expression in a single species; and it has long been recognized that ABA plays a major role in the response of plants to drought stress (Bray, 1991; Hartung & Davies, 1991; Hetherington & Quatrano, 1991; Willmer & Fricker, 1996; see Section 1.5). In summary, levels of ABA increase in leaves and the transpiration stream of plants in

response to drought and this correlates with a reduction in stomatal conductance which is believed to reduce leaf water loss (see Davies & Zhang, 1991). In addition, many genes which are drought-responsive are also regulated by ABA (see Section 1.6; Chandler & Robertson, 1994). Therefore, it will be of interest in the future to determine the role of ABA in the drought-induced effects of guard cell turgor and gene expression described below (Thompson & Corlett, 1995; Kopka *et al.*, 1997).

Thompson and Corlett (1995) attempted to link quantitatively gene expression in tomato leaves with plant water status during drought treatments. Specifically, they compared stomatal conductance (g_s) (amongst other parameters such as leaf water potential and relative water content) and the levels of mRNA for different genes in paired leaves. It was found that only one of the four genes studied, *le25*, appeared to respond in a simple way to plant water status. The induction of *le25* mRNA occurred when leaves began to show severe water stress (leaf water potential > - 0.9 MPa) and this correlated with a reduction in stomatal conductance. Interestingly, *le25* is a *lea* gene (see Section 1.6) identified on the basis of induction by elevated endogenous ABA levels in leaves under water deficit (Cohen *et al.*, 1991; Kahn *et al.*, 1993). *Le25* has been shown to increase in developing seeds prior to desiccation and in dehydrating shoots and roots (Cohen *et al.*, 1991; Kahn *et al.*, 1993) suggesting that it plays a role in stabilizing membranes in dehydrating cells (Dure *et al.*, 1989). In contrast, *le20*, another gene identified on the basis of induction by elevated endogenous ABA levels in leaves under water deficit, showed a very different expression pattern. *Le20* was very sensitive to slight water deficit, showing responsiveness to short-term diurnal fluctuations in water status, and had a low level of expression which suggested a regulatory role rather than a protective role (Thompson & Corlett, 1995). In conjunction with the regulatory patterns of the other two genes studied (*ni3212* and

lycp2), the results showed a complex relationship between gene expression and plant water status.

More recently, drought-induced changes in guard cell gene expression and turgor have been reported (Kopka *et al.*, 1997). The authors reported that drought induced a complex change in the expression of genes related to carbon metabolism and turgor regulation in guard cells of potato (Kopka *et al.*, 1997). Interestingly, two genes, *kst1* (an inwardly rectifying K^+ channel from guard cells) and *pha2* (a plasma membrane H^+-ATPase) were reported to be down-regulated by drought treatment. Kopka *et al.* (1997) studied the time course of changes in guard cell gene expression relative to stomatal closure and loss of leaf water potential in potato plants under drought treatment. They found that changes in steady-state transcript levels were complete by the time that a reduction in stomatal aperture width was reported (i.e. on the second day after withholding water) and before the onset of a decrease in leaf water potential. They suggest that drought not only induces short term stomatal movements but also has a long term effect on guard cell gene expression. The latter response may affect the sensitivity of guard cells to environmental change in the future, thereby playing a role in the adaptation of a plant to its habitat (Hirasawa *et al.*, 1995; Kopka *et al.*, 1997).

Similarly, ABA may affect the expression of genes whose products are components of the signalling pathways by which ABA brings about changes in guard cell turgor and/or further changes in guard cell gene expression [for example *kst1* and *pha2* genes (see Kopka *et al.*, 1997) and genes encoding *trans*-acting elements in the regulation of gene expression by ABA (see Section 1.7.2)]. This would alter the sensitivity of guard cells to ABA in the future (see Hetherington & Quatrano, 1991), as

described above for drought-induced gene expression in guard cells (Hirasawa *et al*, 1995; Kopka *et al*, 1997).

3.1.2 The *CDeT6-19* gene

Guard cells were first shown to be competent to relay the (±)-ABA signal from the site of perception to the nucleus by Taylor *et al.* (1995) (see Section 1.6.2). These authors reported that (±)-ABA and drought stress enhanced GUS activity driven by the *CDeT6-19* gene promoter in guard cells of both transgenic *N. tabacum* and *A. thaliana*. The *CDeT6-19* gene was isolated from the resurrection plant *C. plantagineum* as one of a number of genes which were shown to be responsive to (±)-ABA and desiccation (Bartels *et al.*, 1990; Piatkowski *et al.*, 1990).

Resurrection plants, such *C. plantagineum*, provide an excellent system in which to study the processes leading to desiccation tolerance. This is because the mature vegetative tissue of these plants is unique amongst higher plants in that it can be desiccated up to 1% relative water content and yet still be viable upon rehydration (Gaff, 1971). In contrast, the only tissue in the majority of higher plants which can withstand such extreme desiccation is the seed. Comparisons of air-dried and fully hydrated leaves of *C. plantagineum,* showed that many new transcripts accumulated rapidly after the onset of desiccation (Bartels *et al.*, 1990). For each of these genes, transcript accumulation is correlated with increased levels of ABA. Although there is plenty of evidence that the expression of these genes is modulated by ABA, the precise molecular mechanism is still unknown.

The cDNA clone pcC6-19, encoded by the gene *CDeT6-19* (Piatkowski *et al.*, 1990), represents a prominent group of the transcripts induced by desiccation in *C. plantagineum* (Bartels *et al.*, 1990; Michel *et al.*, 1994). The *CDeT6-19* gene shares

characteristic sequence motifs with a ubiquitously occurring class of desiccation-related and ABA-inducible genes in higher plants. This class of genes codes for hydrophilic proteins (which contain a stretch of serines and conserved lysine rich repeat motifs) and are either constitutively expressed in mature embryos or the expression is induced by osmotic stress (Bray, 1991; Skriver & Mundy, 1990). The *CDeT6-19* polypeptide, designated dsp 16 (dsp - desiccation stress protein) has substantial homologies to *lea* proteins in many higher plants, for example RAB 16 from rice (Mundy & Chua., 1988) and lea D11 from cotton (Dure *et al.*, 1989). In *Craterostigma,* dsp 16 proteins (Mw 18-21 KDa) accumulate rapidly in the leaves in response to dehydration; the maximal signal occurring 0.5 h after the onset of dehydration treatment, at which point there has only been about a 4% loss of fresh weight (Schneider *et al.*, 1993). The same proteins are present in both dry roots and mature seeds, but in the latter tissue an additional protein of 28 KDa is found. A unique feature of the dsp 16 protein family in *C. plantagineum* is that a low level of constitutive expression, at the protein level, is found in both fully hydrated leaves and roots, indicating that some members of this family may play a role in normal cellular processes (Schneider *et al.*, 1993). At both a transcript and protein level expression was found in the cytoplasm and was particularly abundant in cytoplasmically rich cells such as the phloem sieve tube elements in leaves (Schneider *et al.*, 1993). In callus tissue, ABA induced dsp 16 transcripts and proteins at high levels, and this level could be enhanced by additional drying of the callus (Schneider *et al.*, 1993). The transcripts and proteins of maize genes homologous to the *CDeT6-19* gene were induced to high levels by ABA in embryos and seedlings (Pla *et al.*, 1989). In addition, the rice RAB 16 gene (another *CDeT6-19* gene homologue) is activated by osmotic stress (salt and cold) and ABA (Mundy & Chua, 1988; Hahn & Walbot, 1989).

As already indicated (see Section 1.6.2), the mechanism of *CDeT6-19* gene regulation has been studied by fusing the promoter of this gene to the β-glucuronidase (GUS) reporter gene [construct used in the work by Taylor *et al.* (1995)]. Initially, this construct was introduced into protoplasts or used to transform plants of tobacco and *Craterostigma* (Michel *et al.*, 1994). In transient expression assays, 889 bp of the *CDeT6-19* promoter were sufficient to confer ABA-inducibility of GUS activity. Comparisons with the promoters of related genes revealed the conservation of potential ABRE elements (see Section 1.6.2). In transgenic tobacco, GUS activity driven by the *CDeT6-19* promoter was tissue-specific and showed developmental regulation. The maximum activity was found in the mature seeds (embryos) and dehydrated pollen. In addition, *CDeT6-19* driven GUS activity showed a constitutive level of expression in the leaves of transgenic tobacco which could be enhanced by an ABA or drying treatment. The pattern of GUS activity driven by the *CDeT6-19* promoter in transgenic *Craterostigma* plants was similar to that seen in tobacco and coincided with the expression of *CDeT6-19* gene encoded proteins. However, the levels of GUS activity were lower in transgenic *Craterostigma* than in transgenic tobacco.

More recently, GUS activity driven by the *CDeT6-19* promoter has been examined in whole plant tissue of transgenic *Arabidopsis* (Furini *et al.*, 1996). It was found that (±)-ABA enhanced GUS activity driven by the *CDeT6-19* promoter in vegetative tissue of transgenic *Arabidopsis* plants and again, as seen in transgenic tobacco plants, there was a constitutive level of *CDeT6-19* driven GUS activity. This finding fits in with the fact that *CDeT6-19* gene products are found in fully hydrated leaves of *Craterostigma* and suggests that in developed plants the expression of this gene is not strictly ABA dependent.

3.1.3 Aims

It was envisaged that guard cells of *N. tabacum* (tobacco) could be used as a model system to study the ABA signalling pathways which terminate in alterations in guard cell turgor and in gene expression. It was intended that these guard cells could play a role in determining whether the signalling pathways linking ABA to alterations in turgor and gene expression are similar or totally separate. In this way, tobacco guard cells may provide a unique opportunity to investigate the signalling pathways in plants by which ABA regulates two completely different activities.

Tobacco provided a most attractive system in which to study ABA-regulated changes in turgor and gene expression in guard cells. Firstly, it has been reported that (±)-ABA enhances GUS activity driven by the *CDeT6-19* gene promoter in guard cells of transgenic tobacco (Taylor *et al.*, 1995; see Section 3.1.2). Secondly, it was easy to obtain viable, detached epidermis from tobacco leaves (see Section 2.2.3). And thirdly, the effects of (±)-, (+)-ABA and a number of ABA analogues on stomatal aperture (guard cell turgor) in detached epidermis from tobacco leaves have already been investigated in Chapter 2.

The first priority was to examine the effect of (±)-ABA on GUS activity driven by the *CDeT6-19* gene promoter in guard cells (see Section 3.1.2) in detached epidermis from leaves of transgenic tobacco (see Section 3.1.2). These studies would be followed by simultaneous measurements of both guard cell *CDeT6-19* gene promoter activity and guard cell turgor in response to (±)-ABA. Previously, the effect of (±)-ABA on *CDeT6-19* driven GUS activity in guard cells of tobacco leaves was studied by a foliar application *in planta* followed by a histochemical localization of GUS activity in detached epidermis 1 to 2 day(s) later (Taylor *et al.*, 1995). In the present study, it was intended to treat detached epidermis with (±)-ABA so that direct

and rapid measurements of the effect of (±)-ABA on both *CDeT6-19* driven GUS activity in guard cells and stomatal aperture (see Chapter 2) could be made.

Having simultaneously studied the effect of (±)-ABA on both *CDeT6-19* driven GUS activity in guard cells and stomatal aperture it was envisaged that the ABA analogues described in Section 1.7.4 (also see Section 1.8) could be used as tools to probe the perception mechanisms of the two responses to ABA in tobacco guard cells. It is possible that different ABA-induced responses have different ABA perception mechanisms/receptors (see Sections 1.5 and 1.7.2). ABA analogues could be used to uncover differences in the stereochemical requirements of the perception mechanism/receptors with which ABA interacts to bring about different types of response (see Section 1.7.2). This would be apparent, for example, if one ABA analogue was more effective at inducing one type of ABA response than another (see Section 1.7.2). In addition, multiple sites of action of ABA are believed to exist for the effect of ABA on guard cell turgor (Anderson *et al.*, 1994; Allan *et al.*, 1994; Schwartz *et al.*, 1995; MacRobbie, 1995a & b; MacRobbie, 1997; see Section 1.5.1).

Uncovering the mechanisms by which guard cells respond to ABA, in terms of turgor regulation and the activity of an ABA and desiccation-responsive gene promoter, would contribute to an understanding of how plants adapt to drought. Such information would complement that recently gained on drought-induced alterations in gene expression and turgor in guard cells (Kopka *et al.*, 1997). These data could be used in the future to modify plants (either genetically or chemically) so that they have guard cells that function in a manner advantageous to producing high crop yields under mild to moderate drought stress (Tan *et al.*, 1992).

3.2 Materials and Methods

3.2.1 Chemicals

All chemicals described were from Sigma Chemical Co. (UK) unless otherwise stated.

3.2.2 Plants and growth conditions

Wild-type (WT) *Nicotiana tabacum* (cv. Petit Havana SR1) (tobacco) plants and those transformed with the *CDeT6-19* gene promoter fused to the *β*-glucuronidase (GUS) reporter gene (construct 604 - see Michel *et al.*, 1994; Taylor *et al.*, 1995) were used in the investigations. A single line (line 4) representing an individual transformant of the *CDeT6-19*-GUS plants was used, because it had been reported by Taylor *et al.* (1995) that *CDeT6-19* driven GUS activity in guard cells in both of the two individual tobacco transformants available was equally responsive to (±)-ABA .

Seeds were placed on agar plates [0.6% agar (Tissue culture grade) and half strength Murashige-Skoog (MS) salts]. Transgenic tobacco plants were selected by including 50 µg/ml kanamycin in the medium. Seeds were allowed to germinate under greenhouse conditions (described in Section 2.2.2). When seedlings were of a suitable size (after about 7 to 10 days) they were transferred to pots containing Fisons M3 general purpose compost and watered regularly. After 2-3 weeks, plants were transferred to larger pots. Plants which were 8-weeks-old were transferred to a controlled environment room maintained under the same conditions as the greenhouse, except that all the light was supplied by "daylight" fluorescent tubes (Thorn EMI, UK). They were kept in this room, on self-watering gravel trays or damp capillary matting, for 3 or 4 days before use and for the duration of the experiments in which (±)-ABA was applied to whole plants.

3.2.3 (±)-ABA-induced *CDeT6-19* driven GUS activity in guard cells of tobacco leaves

3.2.3(a) Treatment of plant tissue with (±)-ABA

(i) Detached, abaxial epidermis

The abaxial epidermis was removed from leaves 3 and 4 (counted down from the shoot apex, leaf 1 being the youngest leaf over 2 cm in length) of six, 8-week-old WT and transgenic tobacco plants for each experiment, according to the methods described in Section 2.2.3. (Leaf 4 was chosen in this study because it was used in investigations of (±)-ABA-induced changes in guard cell turgor in Chapter 2). The epidermal pieces were then incubated in 5 cm Petri-dishes on MES/KOH (see Section 2.2.3), 25±1°C in the presence or absence of 10^{-5} M (±)-ABA (made by diluting a stock solution of 10^{-2} M (±)-ABA [made in 100% (v/v) ethanol] in MES/KOH) under the conditions that promote stomatal opening described in Section 2.2.4 except that no KCl was present in the incubation medium. 10^{-5} M (±)-ABA was used because it was envisaged that in the future the effects of the ABA analogues described in Chapter 2 on *CDeT6-19* driven GUS activity in guard cells would be investigated. Furthermore, 10^{-5} M is the highest concentration at which the effects of these analogues can be studied without resulting in a high ethanol concentration which itself will influence stomtal behaviour (see Section 3.4, for a more detailed discussion).

(ii) Leaves of tobacco plants

WT and transgenic tobacco plants (8-weeks-old) were sprayed twice daily (at 6 and 10 h into the 16 h photoperiod) for 2 days with MES/KOH, 0.1% (v/v) Tween, 25±1°C, with or without 10^{-4} M (±)-ABA (made by stirring the solid in MES/KOH at 4°C overnight) until run off, or left untreated. At the end of the experimental period

epidermal peels were made from the abaxial surface of a designated area of numbered leaves from each plant (see Section 2.2.3). Leaves were numbered so that leaf 1 was the oldest leaf on the plant and were grouped into categories according to age: "old" - leaves being those numbered from 2-4, "medium" - leaves numbered from 5-7, and "young" - leaves numbered from 8-10. In some experiments the leaves of the treated tobacco plants were subjected to daily stomatal conductance measurements (see Section 3.2.4 for details).

3.2.3(b) The histochemical GUS assay

The activity of the *CDeT6-19* gene promoter was studied in plants transformed with a construct consisting of this promoter fused to the β-glucuronidase (GUS) reporter gene (Jefferson *et al.*, 1986, 1987). The histochemical localization of GUS activity was determined using the substrate 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (X-Gluc) (Stomp, 1992).

(i) Background

The GUS gene, encoded by the *uid* A locus, was isolated from *E. coli* and is a hydrolase that catalyses a wide variety of β-glucuronides (see Jefferson *et al.*, 1986, 1987). Many of the glucuronides are available commercially as spectrophotometric, fluorometric and histochemical substrates (Naleway, 1992). The GUS gene has been cloned, sequenced and encodes a stable enzyme that is particularly suitable for the construction and analysis of gene fusions (Jefferson *et al.*, 1986, 1987). The GUS reporter gene is a superior plant reporter gene over others, such as the bacterial enzymes chloroamphenicol acetyl transferase (CAT) or neomycin phosphotransferase (NPT11) because it is easily, sensitively and cheaply assayed in plants *in vitro* (see Jefferson *et al.*, 1987) and more recently an *in vivo* substrate has become available

(ImaGene Green, Molecular Probes, USA; used by Herd *et al.*, 1997). In addition, the majority of plant species show no detectable endogenous GUS activity thus providing a null background in which to assay for the activity of GUS driven by the promoter being studied. X-Gluc (a colourless compound) is the preferred substrate for histochemical staining of GUS activity (Pearson *et al.*, 1961). This is due to the fact that it is readily detectable at low concentrations (high extinction coefficient) and the of the final cleavage product, dichlorodibromo-indigo (ClBr-indigo) is insoluble in water. The ClBr-indigo immediately precipitates upon formation thus allowing precise cellular location of enzymatic activity and little loss of ClBr-indigo in solvents (Stomp, 1992) (see Figure 3.1).

(ii) Methodology used in the present study

Histochemical localization of GUS activity in the epidermal pieces was carried out by placing the epidermis on the surface of a GUS assay solution [0.1 M phosphate buffer pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1.0 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide; Gold Biotech., USA), 0.1% (v/v) Triton X-100] (Stomp, 1992; Taylor *et al.*, 1995), followed by an incubation at 37°C for 2-26 h. The tissue was examined microscopically and photographs taken on Agfachrome slide film 200 RS (Agfa) using a Wild Leitz 35 mm camera. A semi-quantitative determination of GUS activity in the guard cells was made by either counting the number of stomata stained blue out of the total number of stomata in 40 random fields of view per treatment (for the treatments to the epidermal peels) and per experiment; or alternatively, an estimation was made of the percentage of guard cells stained light, medium or dark blue over the whole epidermis for each treatment (for the treatments to the whole plants). For the second type of measurement a colour-coded chart (see Figure 3.2) was used as a scale by which to measure the

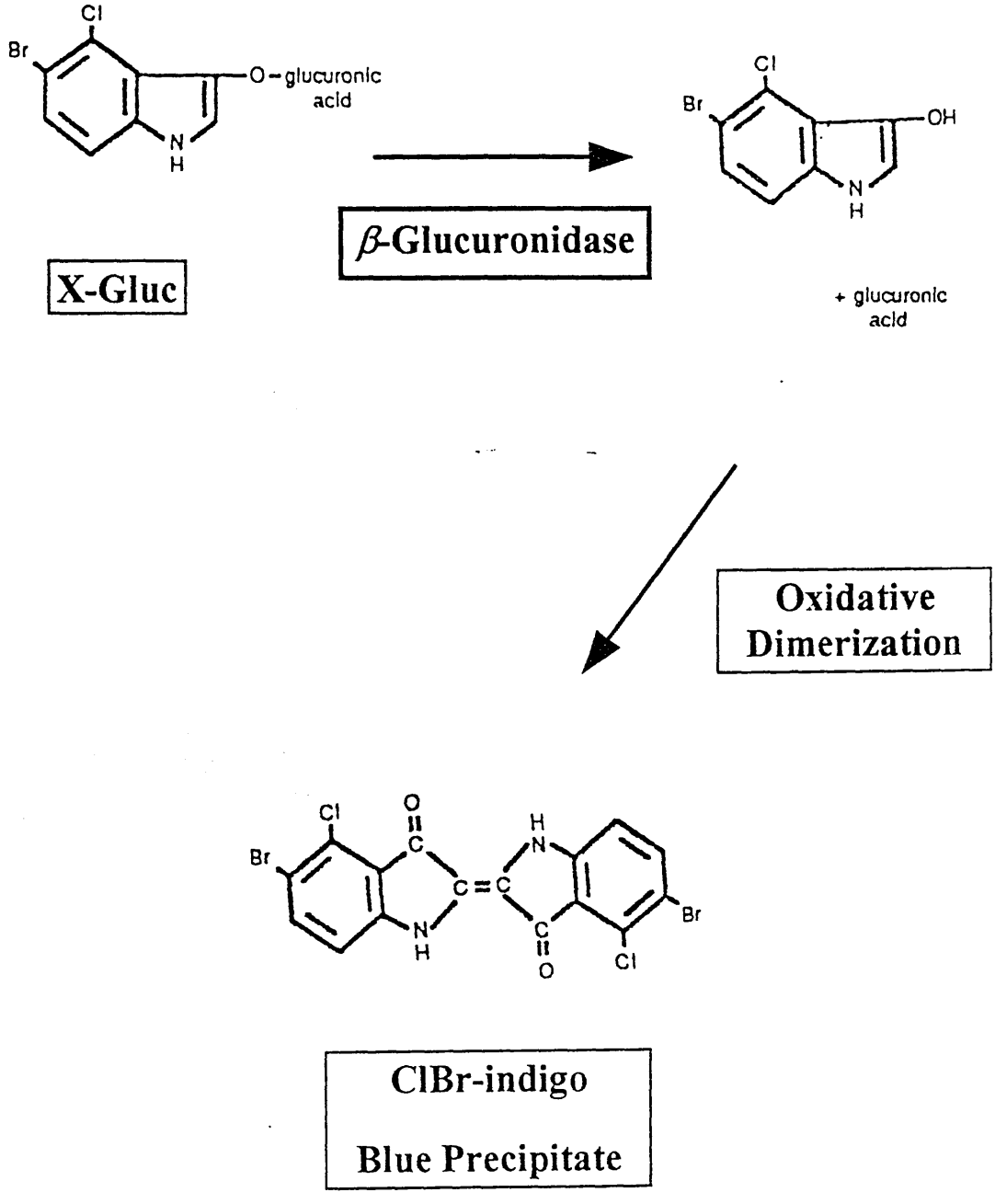


Figure 3.1 The reactions involved in the histochemical localisation of β-glucuronidase (GUS) activity using the substrate X-Gluc (Stomp, 1992).

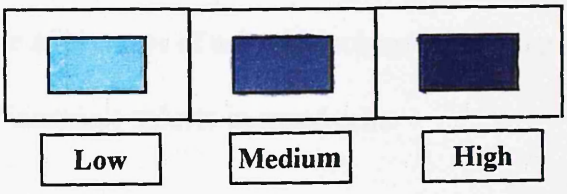


Figure 3.2 Colour-coded scale used to measure the relative intensity of the blue precipitate formed in the histochemical localization of *CDeT6-19* driven GUS activity in guard cells of transgenic tobacco leaves.

relative degree of GUS activity. The method of estimating the percentage of guard cells stained varying shades of blue was validated by a detailed count of guard cells in one experiment, see Section 4.2.3(d).

It is important to note that using histochemical methods to study *CDeT6-19* driven GUS activity provides only semi-quantitative information. It does, however, allow GUS activity in the guard cells to be visualized and assessed without the need for the isolation of guard cells. This would be necessary if a fluorometric substrate was used to determine GUS activity in guard cells. Recently, Talbott and Zeiger (1996) have described the advantages of using histochemical staining of intact leaf epidermis in determinationsof inorganic solutes in guard cells.

3.2.4 Stomatal conductance of leaves of tobacco

For the WT and *CDeT6-19*-GUS tobacco plants the stomatal conductance of a number of leaves (ranging from leaf 1 to leaf 12; leaf 1 being the oldest) of (±)-ABA -, buffer-treated or untreated plants was measured using an Delta-T AP4 porometer (Delta-T Devices Ltd., Cambridge, England).

(i) Background

The Delta-T model AP4 is a cycling, diffusion porometer used for measuring the stomatal resistance or conductance of plant leaves (see Weyers & Meidner, 1990). It works by measuring the time it takes for a leaf to release sufficient water vapour to change the relative humidity in a small chamber by a fixed amount (for diagrams of the head unit of the AP4 promoter see Figure 3.3). This is compared to measurements made with a calibration plate of known resistance in order to derive the stomatal resistance or conductance of the leaf. This is a well established method supported by a good theoretical understanding of a cycling porometer (see Bragg *et al.*, 1991). With

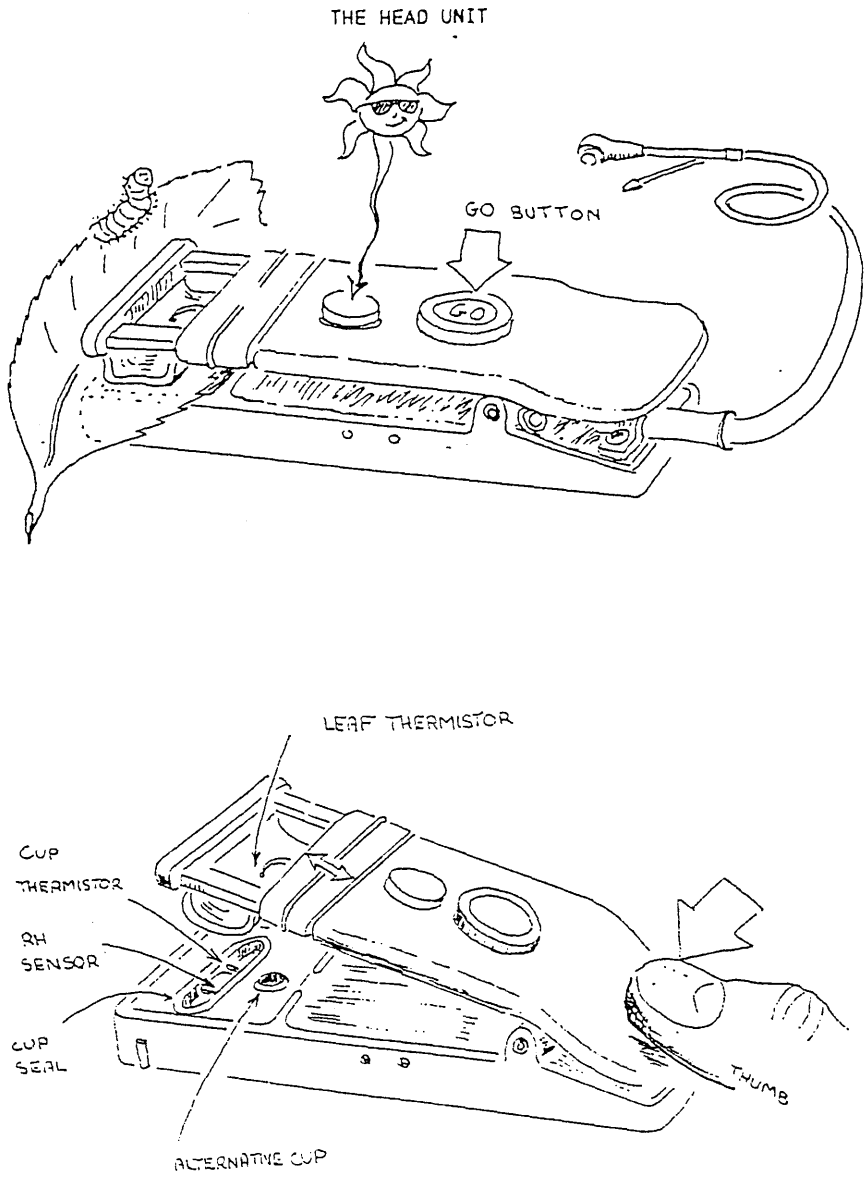


Figure 3.3 Diagrams of the head unit of the AP4 porometer (Taken from the AP4 Users Manual, Bragg *et al.*, 1991 [with permission]).

the AP4, readings are automatically converted into units of resistance or conductance which can be displayed, saved and transferred to a computer or printer (Bragg *et al.*, 1991). It is important to note that all instruments that measure the diffusive flow of water out of leaves give an estimate of leaf conductance as opposed to stomatal conductance. This is because they include cuticular and trichome (leaf hair) vapour loss and cannot take into account the resistance to vapour flow or changes in the vapour concentration within the leaf (see Weyers & Meidner, 1990, for a full discussion). For the purposes of this thesis (and often done elsewhere) the measurements made by the AP4 porometer will be referred to as "stomatal conductance" although the considerations described above should always be borne in mind.

(ii) Methodology used in the present study

The calibration plate supplied with the AP4 porometer was made up on the day before measurements were to be taken. It was then used to calibrate the machine just before readings were taken. The left side of a leaf on the tobacco plant was gently placed in the head unit at a point 1/3 of the way up the leaf from the base and between two major, radial veins. A reading was accepted after 4 to 5 cycles or when the machine indicated that there was a difference of less than a 2% change between consecutive cycles. All measurements were carried out in a climate controlled growth room between 3 and 4 h into the 16 h photoperiod on each day of the experiment. All the plants on which stomatal conductance measurements were made were also assayed for GUS activity [described in Section 3.2.3(b)]. The GUS activity measurements were made on a different area of the same leaf, or a leaf adjacent to that used for the stomatal conductance measurements.

3.2.5 Statistics

A generalized linear model with binomial error structure and logit function (see Aitkin *et al.*, 1990) was used to determine differences between treatments in the % of guard cells stained blue on treated, detached epidermis. The test can be thought of as comparing the mean number of "successes" (a "success" being a blue guard cell) in each treatment with a model in which the mean number of successes is the same for each treatment. Wald tests (see Aitkin *et al.*, 1990) were used on pairs of treatments to determine where the differences between treatments lay. One way analyses of variance and least significant difference (LSD) tests were used to determine differences between treatments in leaf stomatal conductance and the % of guard cells stained blue on detached epidermis from treated plants.

3.3 Results

3.3.1 (±)-ABA (10^{-5} M) has a variable effect on GUS activity driven by the *CDeT6-19* gene promoter in guard cells of detached epidermis from tobacco leaves

The underlying aim of these experiments was to measure simultaneously the effect of (±)-ABA on GUS activity driven by the *CDeT6-19* gene promoter in guard cells and guard cell turgor (see Section 3.1.3). However, it was first important to determine the effect of (±)-ABA on each of these guard cell activities (i.e. gene promoter activity or turgor changes) separately prior to the simultaneous measurements. The effect of (±)-ABA [and (+)-ABA and ABA analogues] on guard cell turgor in detached epidermis was established in Chapter 2. Therefore, in the present chapter it was necessary to determine the effect of (±)-ABA on GUS activity driven by the *CDeT6-19* gene promoter in guard cells of detached epidermis of tobacco leaves. A preliminary experiment revealed that 10^{-5} M (±)-ABA significantly enhanced *CDeT6-19* driven GUS activity in guard cells of the ABA-treated detached epidermis from leaf 4 of transgenic tobacco plants (Figure 3.4 and 3.5). There was a low level of GUS activity in the guard cells of epidermal pieces incubated on MES/KOH buffer with or without 0.1% (v/v) ethanol (Figure 3.5.B). Similar results were found for detached epidermis from leaf 3 of transgenic tobacco plants (data not shown). However, the overall data set (which consisted of five replicate experiments, including the one described above) revealed that 10^{-5} M (±)-ABA did not significantly enhance *CDeT6-19* driven GUS activity in the guard cells of detached epidermis from leaf 4 of transgenic tobacco plants (Figure 3.6). The level of GUS activity in detached epidermis

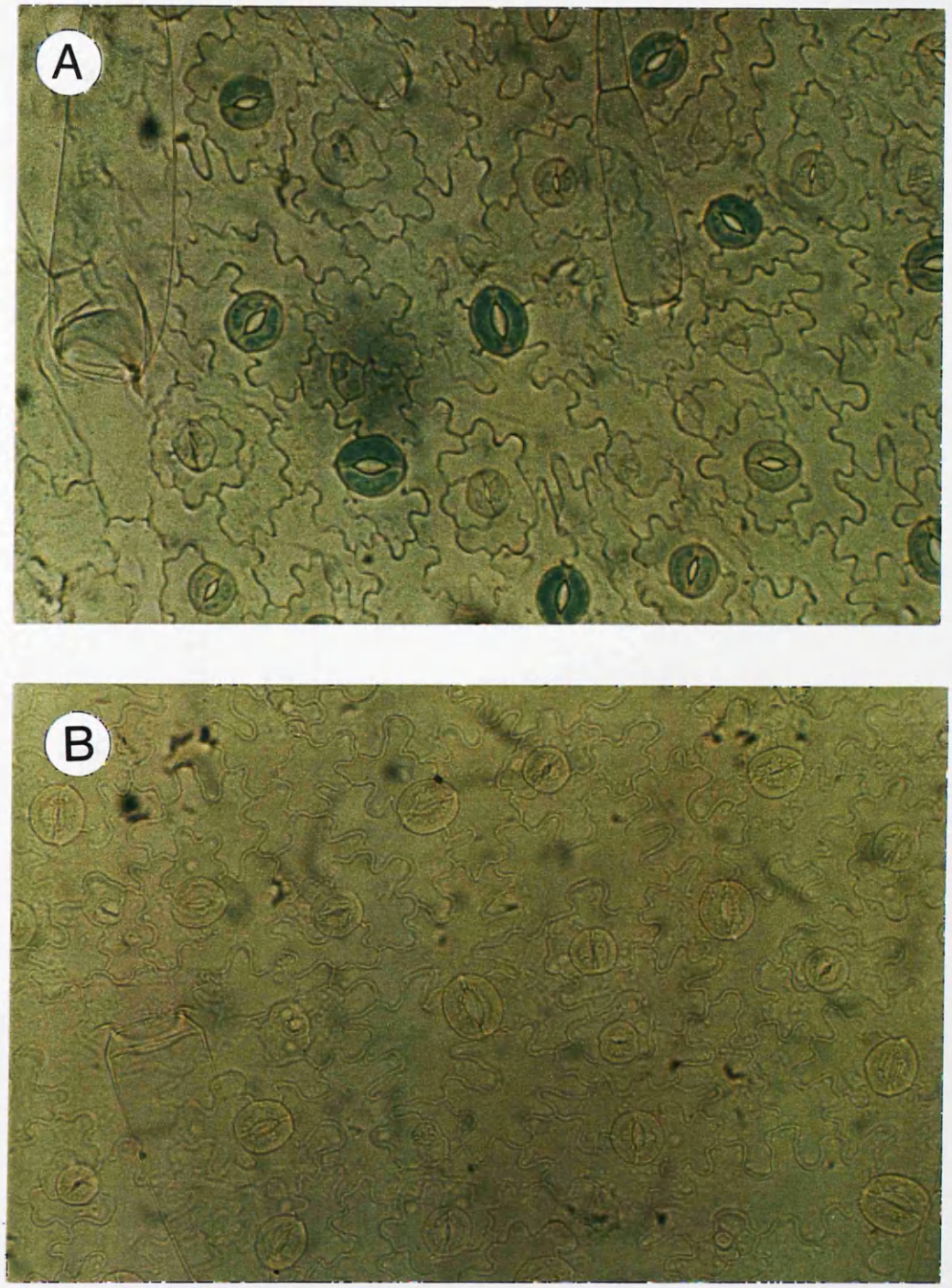


Figure 3.4 Histochemical localisation of *CDeT6-19* driven GUS activity in guard cells in detached, abaxial epidermis from leaf 4 of transgenic tobacco plants. The photographs show detached epidermis which has been incubated on MES/KOH containing 10^{-5} M (±)-ABA (**A**), 0.1% (v/v) ethanol or MES/KOH only (**B**). These data are from one experiment. (Magnification x 200).

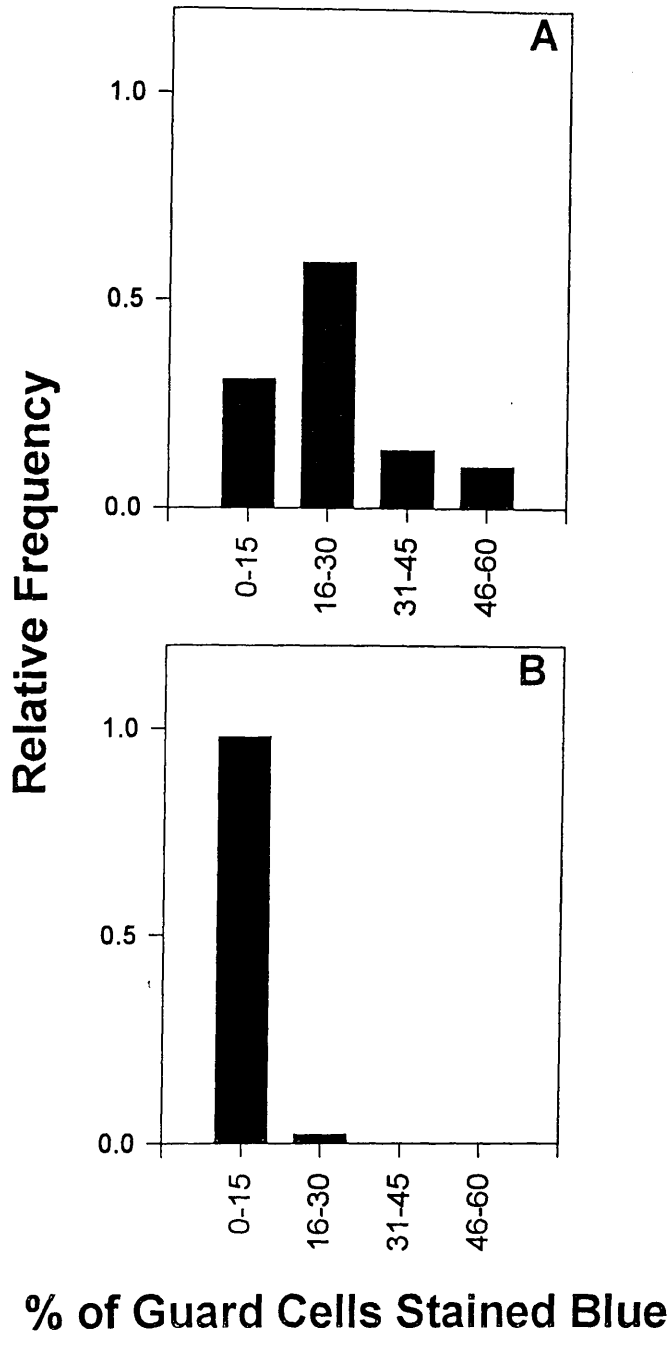


Figure 3.5 The relative frequency of the percentage of guard cells stained blue (indicative of *CDeT6-19* driven GUS activity as determined by histochemical localization) in detached epidermis from leaf 4 of transgenic tobacco plants incubated on MES/KOH containing 10^{-5} M (±)-ABA (A), 0.1% (v/v) ethanol or MES/KOH only (B). The data are from one experiment.

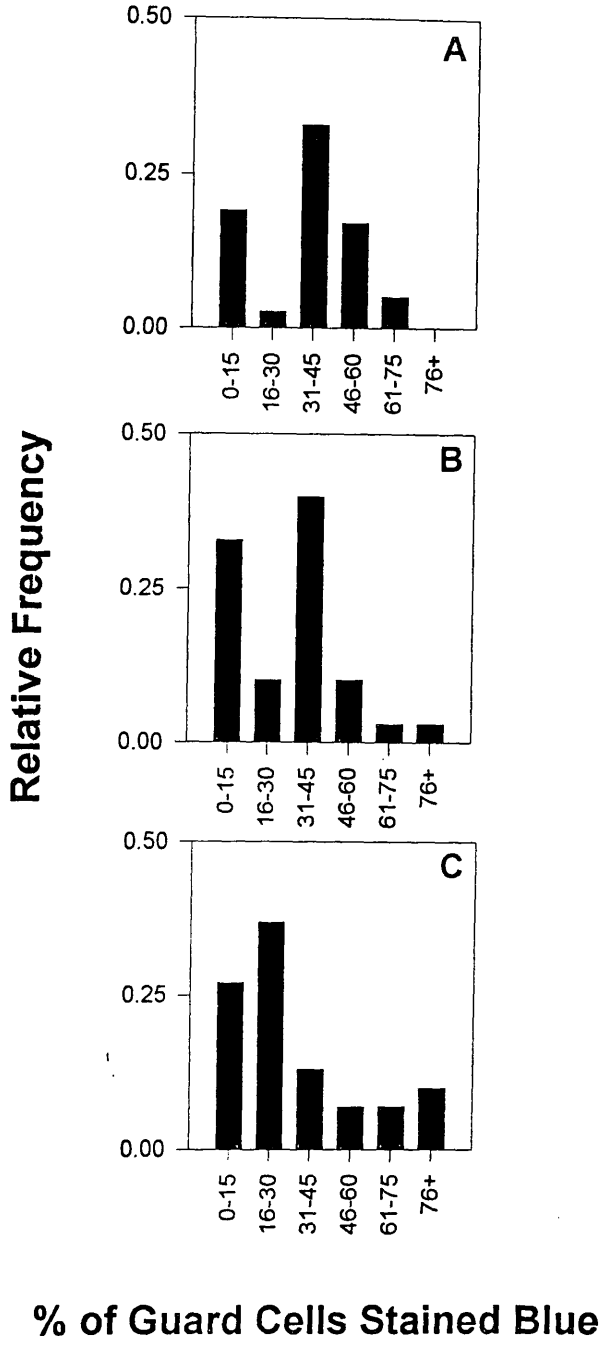


Figure 3.6 The relative frequency of the percentage of guard cells stained blue (indicative of *CDeT6-19* driven GUS activity as determined by histochemical localization) in detached epidermis from leaf 4 of transgenic tobacco plants incubated on MES/KOH containing 10^{-5} M (±)-ABA (**A**), 0.1% (v/v) ethanol (**B**) or MES/KOH only (**C**). The data are from five replicate experiments.

incubated on MES/KOH only or on MES/KOH containing 0.1% (v/v) ethanol was relatively high (Figure 3.6.B and C). The probability of the appearance of guard cells displaying GUS activity driven by the *CDeT6-19* promoter in detached epidermis from tobacco leaf 4 was equally likely in all treatments (see Section 3.2.5 for the statistics used). However, Wald tests (see Section 3.2.5) revealed that the probability of there being a lower number of guard cells displaying GUS activity driven by the *CDeT6-19* promoter in the MES/KOH + 0.1% (v/v) ethanol-treated tissue (Figure 3.6.B) compared with tissue in the other two treatments (Figure 3.6.A and C) was approaching significance at the 5% level. A similar result was found for leaf 3 (data not shown). In all these experiments no GUS activity was seen in the (±)-ABA-treated abaxial epidermis from WT tobacco leaves (data not shown).

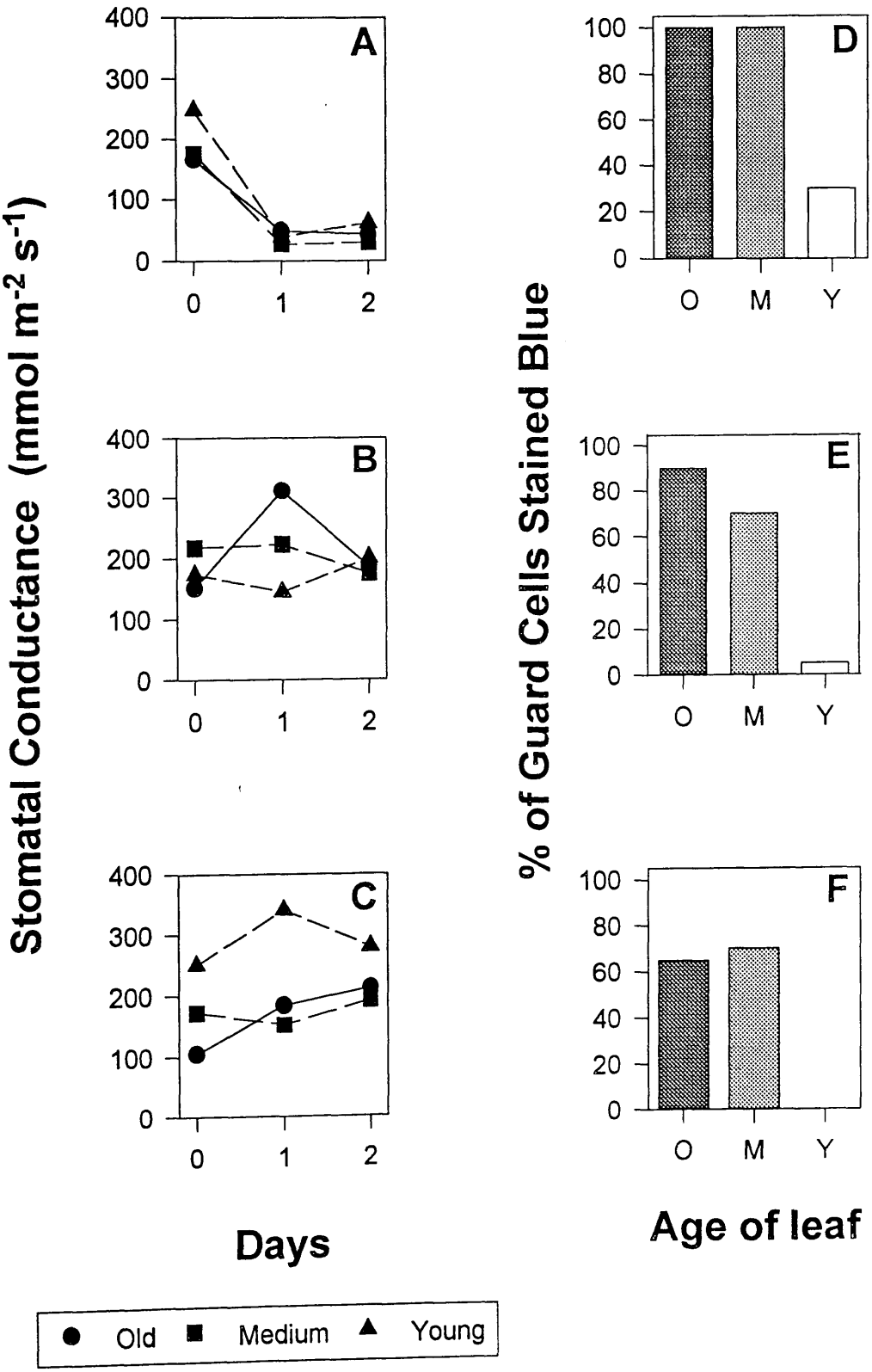
3.3.2 (±)-ABA-induced changes in guard cells of leaves of transgenic tobacco plants

As described in Section 3.3.1, on the whole 10^{-5} M (±)-ABA did not enhance *CDeT6-19* driven GUS activity when applied to detached abaxial epidermis from tobacco leaves. Therefore, attention turned to studying the effects of (±)-ABA on guard cells in attached epidermis of treated tobacco leaves with the epidermis being removed at the end of the treatment in order to histochemically localize GUS activity (as described by Taylor *et al.*, 1995). The effect of (±)-ABA on the stomatal conductance of tobacco leaves and on *CDeT6-19* driven GUS activity in guard cells of abaxial epidermis of the same or adjacent leaves was investigated. 10^{-4} M (±)-ABA was used in these investigations, with a view to decreasing the concentration to 10^{-5} M (so that the effect of the ABA analogues could be investigated) once a significant treatment effect at this concentration was established for both responses investigated.

(i) *Preliminary investigations*

Figure 3.7 shows the results from a preliminary experiment in which the stomatal conductance of leaves was measured before and after foliar treatment applications of MES/KOH in the presence or absence of 10^{-4} M (±)-ABA.. At the end of the experimental period, *CDeT6-19* driven GUS activity was assessed in epidermis detached from the same leaf in which the stomatal conductance measurements were made (although not from the precise area of the leaf which was placed in the porometer). These preliminary data showed that stomatal conductance in all ages of leaf was reduced drastically by (±)-ABA (Figure 3.7.A). The stomatal conductance of MES/KOH-treated or untreated leaves did not show any dramatic decrease (Figure 3.7.B and C). In fact, in many cases no change or increases in stomatal conductance were observed (Figure 3.7.B and C). *CDeT6-19* driven GUS activity was higher in all ages of (±)-ABA-treated tobacco leaves compared with MES/KOH-treated or untreated leaves (Figure 3.7.D, E and F). The most dramatic difference in *CDeT6-19* driven GUS activity between treatments was seen in the youngest leaf; virtually no GUS activity was reported in the leaves of MES/KOH-treated or untreated plants (Figure 3.7.E and F) whereas approximately 30% of guard cells were stained blue in the leaves of (±)-ABA-treated tobacco plants (Figure 3.7.D). GUS activity (background or (±)-ABA-enhanced) was highest in the older leaves Figure 3.7.D, E and F). In conclusion, this preliminary experiment showed that (±)-ABA reduced stomatal conductance (and hence reduced guard cell turgor) and enhanced *CDeT6-19* driven GUS activity in the guard cells of transgenic tobacco leaves. It also suggested that there may be a developmental aspect, in terms of leaf age, to the responsiveness of *CDeT6-19* driven GUS activity in the guard cells. Replicate experiments were carried

Figure 3.7 The stomatal conductance of leaves of an old (**O**), medium (**M**) and young (**Y**) age (**A-C**) of transgenic tobacco plants treated with MES/KOH containing 10^{-4} M (±)-ABA (**A**), MES/KOH (**B**) or untreated (**C**). The percentage of guard cells stained blue (indicative of *CDeT6-19* driven GUS activity as determined by histochemical localization) in detached epidermis removed from the same leaves as described above (**D-F**). The epidermis was removed from 10^{-4} M (±)-ABA-treated (**D**), MES/KOH-treated (**E**) and untreated (**F**) leaves. "Days" refers to the number of days after the initial treatment application. Stomatal conductance was measured daily between 3 and 4 h into the 16 h photoperiod and *CDeT6-19* driven GUS activity was assayed on Day 2 (for more details, see Sections 3.2.3 & 3.2.4). The data are from one experiment.


Stomatal Conductance (mmol m-2 s-1)
A
B
C
D
E
F
400
300
200
100
0
0
1
2
100
80
60
40
20
O
M
Y
Days
% of Guard Cells Stained Blue
Age of leaf
Old
Medium
Young

out, the results of which are described in the two following sections [Sections 3.3.2 *(ii)* and *(iii)*].

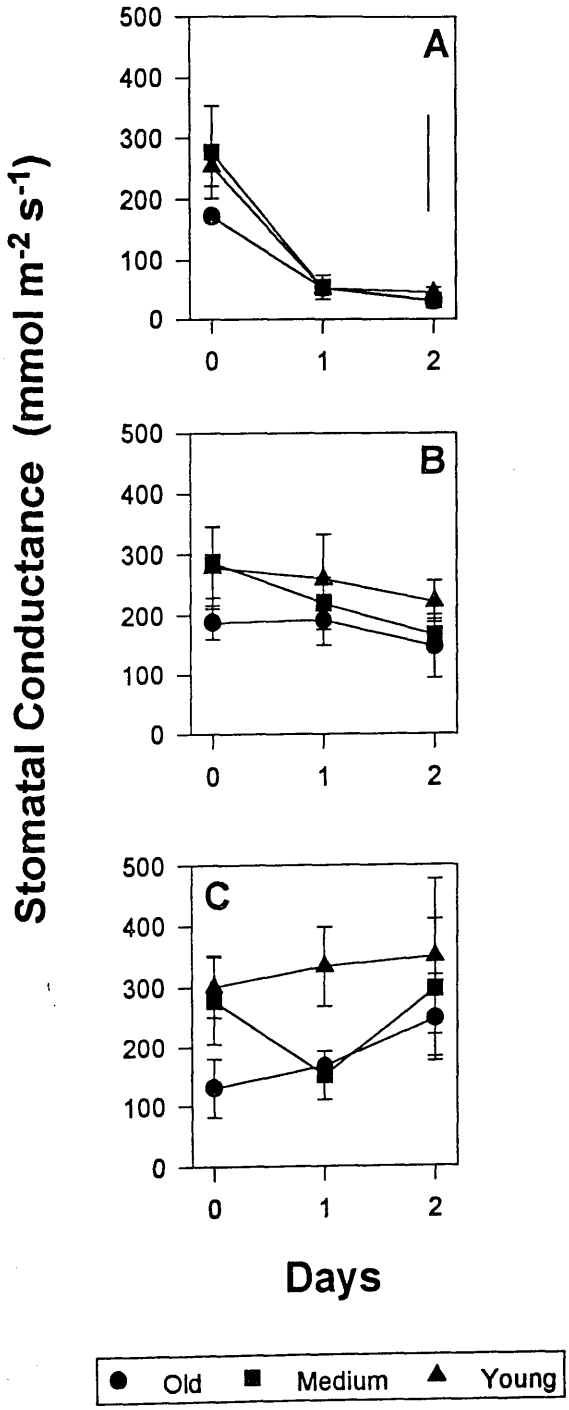
(ii) Stomatal conductance

The stomatal conductance of tobacco leaves of both young and medium age was significantly reduced (by about 80%) one day after of a foliar application of 10^{-4} M (±)-ABA (Figure 3.8.A). The stomatal conductance of these leaves remained low after a second day of (±)-ABA application. Although there was a decrease in the stomatal conductance of the oldest (±)-ABA-treated leaf on day 1, it was not statistically significant when the variation in the whole data set was taken into account (see LSD bar). By way of contrast, there were no significant differences in stomatal conductance after 1 or 2 day(s) of treatment for all ages of leaves sprayed with MES/KOH (Figure 3.8.B) or left untreated (Figure 3.8.C). A difference in the stomatal conductance of leaves of different ages (an "age effect") was only seen for the untreated plants. The younger leaves of these plants had a significantly higher stomatal conductance on "day 0" and "day 1" compared with the older leaves; and on "day 1" only compared with the medium aged leaves. The range of stomatal conductance values of tobacco leaves measured in this study correlate well with measurements made in other studies (Krapp *et al.*, 1994; Delgada *et al.*, 1994; Majeau *et al.*, 1994).

(iii) CDeT6-19 driven GUS activity in guard cells

CDeT6-19 driven GUS activity was measured in the guard cells in epidermis removed from the same leaves or ones adjacent to those on which the stomatal conductance measurements, described above, were made. The results of four individual

Figure 3.8. The stomatal conductance of leaves of an old, medium and young age from transgenic tobacco plants treated with MES/KOH containing 10^{-4} M (±)-ABA (**A**), MES/KOH (**B**) or untreated (**C**). The data represent the means of 4 individual experiments. The vertical bar shown in (**A**) represents the LSD for the whole data set. Leaves from WT tobacco plants responded in a similar manner (data not shown). "Days" refers to the number of days after the initial treatment application. Stomatal conductance was measured daily between 3 and 4 h into the 16 h photoperiod (for more details, see Sections 3.2.3 & 3.2.4).


Stomatal Conductance (mmol m-2 s-1)
A
B
C
500
400
300
200
100
0
0
1
2
Days
Old
Medium
Young

experiments revealed a high degree of variability in the percentage of guard cells showing GUS activity (as determined by the appearance of a blue precipitate in the guard cells) within treatments (Table 3.1). This will contribute to the fact that there were no statistically significant differences between treatments (as determined by one-way analyses of variance). Table 3.2 shows the results of an experiment [unpublished data of L.T. Montgomery, J.E. Taylor and Lancaster University undergraduates (1994)] using a larger sample size (8 plants per treatment). The results represent the total number of epidermal peels with guard cells displaying *CDeT6-19* driven GUS activity for each treatment. Leaves of all ages were used for each treatment. These results showed that (±)-ABA enhanced *CDeT6-19* driven GUS activity in the guard cells of abaxial epidermis from leaves of transgenic tobacco plants (see Table 3.2). A low level of GUS activity was seen in guard cells in just under half the epidermal peels taken from the leaves of MES/KOH-treated plants. No GUS activity was reported in the epidermis of leaves from WT tobacco plants treated with (±)-ABA.

3.3.3 Leaf age affects the (±)-ABA -responsiveness of *CDeT6-19* driven GUS activity in guard cells of transgenic tobacco leaves

A detailed study of the effect of (±)-ABA on *CDeT6-19* driven GUS activity in guard cells in epidermis from leaves of different ages (the oldest being leaf 5 and the youngest leaf 8) was carried out (Figure 3.9). It revealed that there was a high level of constitutive GUS activity in all ages of leaf (untreated plant) except for the youngest (leaf 8). There was no significant difference between the % of guard cells stained light blue in epidermis from all ages of leaf and for all treatments (Figure 3.9.A). The only statistically significant difference between treatments (when one age of leaf was

Table 3.1 The estimated percentage of guard cells in four replicate experiments (**1-4**) stained with either a low or medium intensity blue precipitate (indicative of the relative level of *CDeT6-19* driven GUS activity) in detached, abaxial epidermis from leaves of an old (**O**), medium (**M**) and young (**Y**) age from transgenic tobacco plants treated with MES/KOH in the presence or absence of 10^{-4} M (±)-ABA, or untreated (n.d. = not determined). No blue precipitate was seen in guard cells in epidermis from the leaves of WT plants (data not shown).

		Intensity of Precipitate							
		Low				Medium			
Treatment	**Age of Leaf**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
MES + 10^{-4} M ABA	**O**	100	40	0	60	0	60	100	0
	M	100	20	20	60	0	0	80	0
	Y	30	1	n.d.	80	0	0	0	0
MES/KOH	**O**	90	20	80	100	0	80	20	0
	M	70	20	40	100	0	65	10	0
	Y	5	0	n.d.	80	0	0	0	0
Untreated	**O**	65	20	0	n.d.	0	0	0	0
	M	70	5	0	n.d.	0	0	0	0
	Y	0	0	n.d.	n.d.	0	0	0	0

	INTENSITY OF PRECIPITATE		
TREATMENT	**Low**	**Medium**	**High**
(±)-ABA	14	23	4
MES/KOH	18	0	0

Table 3.2 The number of epidermal peels with guard cells containing either a low, medium or high intensity blue precipitate (indicating the relative level of *CDeT6-19* driven GUS activity) from leaves of transgenic tobacco plants treated with MES/KOH in the presence and absence of 10^{-4} M (±)-ABA. Forty one epidermal pieces were removed from leaves (numbered 3 to 8) of eight transgenic tobacco plants for each treatment. All forty one of the epidermal pieces taken from leaves of WT tobacco plants showed no GUS activity.

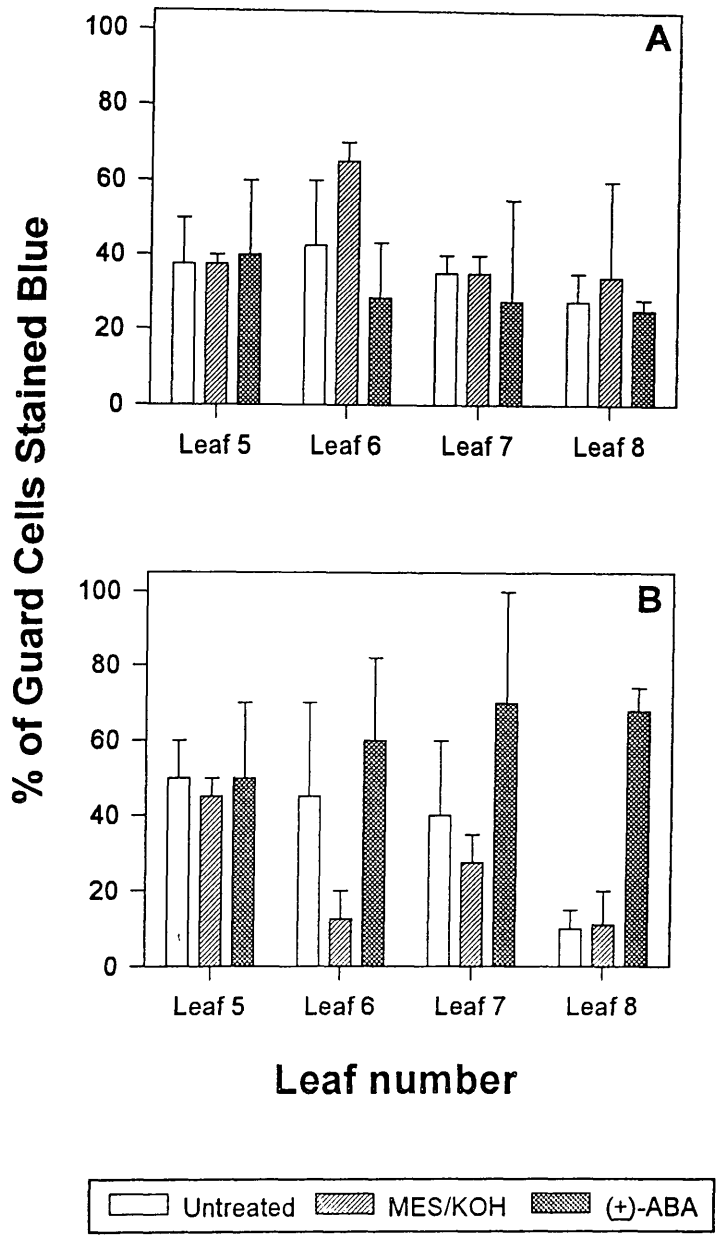


Figure 3.9 The estimated percentage of guard cells containing either a low (**A**) or medium (**B**) intensity blue precipitate (indicating the relative level of *CDeT6-19* driven GUS activity determined by histochemical localization) in abaxial epidermal pieces from tobacco leaves of different ages (Leaf 5 being the eldest) from plants treated with MES/KOH containing 10^{-4} M (±)-ABA, MES/KOH only, or from untreated plants. Each bar represents the mean of 3 plants; the standard error bars are shown.

considered on it own using a one-way analysis of variance) was found for the percentage of guard cells stained dark blue on epidermis from the youngest leaf (leaf 8) (Figure 3.9.B). The (±)-ABA treatment resulted in a significantly higher percentage of guard cells stained dark blue in epidermis from this age of leaf than the MES/KOH-treatment or no treatment (Figure 3.9.B). In order to further investigate this developmental effect, it would be advisable to increase the number of replicate experiments so that the within treatment variation could be reduced.

3.4 Discussion

It was envisaged that the tobacco guard cell could be used as a model system for studying ABA signalling pathways associated with changes in gene promoter activity and guard cell turgor. The investigation of these two types of ABA response in guard cells of treated, detached epidermis from the tobacco leaves aimed to provide more direct and rapid guard cell measurements than application to the whole plant. In Chapter 2 the effect of (±)-ABA on guard cell turgor in detached epidermis from tobacco leaves was investigated. Therefore, the present chapter was concerned with studying the effect of ABA on *CDeT6-19* driven GUS activity in guard cells of abaxial epidermis from leaves of transgenic tobacco plants. A preliminary result showed that 10^{-5} M (±)-ABA increased significantly *CDeT6-19* driven GUS activity in the guard cells of detached epidermis (Figures 3.4 and 3.5). However, a more thorough investigation revealed that 10^{-5} M (±)-ABA had no apparent effect on *CDeT6-19* driven GUS activity in these guard cells (Figure 3.6). This could be due to: (i) the concentration of (±)-ABA not being high enough; (ii) the period of exposure to (±)-ABA not being long enough and/or; (iii) a lack of certain "factors" (see Sections 1.6.3 and 1.6.4) in the epidermal tissue for 10^{-5} M (±)-ABA-regulated *CDeT6-19* gene promoter activity in the guard cells. Indeed, a relatively low level of responsiveness of this gene construct (604) to (±)-ABA was found by Michel *et al.*, (1994). However, a significant level of responsiveness was found in transgenic tobacco leaves treated with 5×10^{-4} M (±)-ABA (Michel *et al.*, 1994) and in guard cells of such leaves treated with 10^{-4} M (±)-ABA (Taylor *et al.*, 1995). Some studies have shown that stomatal guard cells in detached epidermis behave differently, both quantitatively and qualitatively, from those in the intact leaf (Trejo *et al.*, 1993; Lee & Bowling, 1995). These studies

were concerned with the control of guard cell turgor, although there is no reason to suppose that the same phenomenon could not hold true for ABA-regulated gene promoter activity in the guard cell.

The absence of a consistent effect of 10^{-5} M (±)-ABA on *CDeT6-19* driven GUS activity in guard cells of detached epidermis from tobacco leaves, suggests that it would not be advisable to study the effect of the ABA analogues investigated in Chapter 2 on this response. The effect of concentrations of (±)-ABA higher than 10^{-5} M (±)-ABA on *CDeT6-19* driven GUS activity in guard cells of detached epidermis was not investigated for the following reasons. It was envisaged that the effect of the ABA analogues, (–)-ABA, PBI-63 and PBI-51 (see Section 1.7.4 and Chapter 2) on *CDeT6-19* driven GUS activity in guard cells of detached epidermis from tobacco leaves would be investigated once a significant (±)-ABA effect had been established. The results from these studies would be comparable with the results gained in Chapter 2 on the effects of these molecules on guard cell turgor. Such comparisons would provide information on the stereochemical requirements of the perception processes for the two distinct types of ABA response. The highest concentration of either PBI-63 or PBI-51 which could be made in a stock solution was 10^{-2} M in ethanol due to the insolubility of these ABA analogues at higher concentrations and in other solvents. This meant that at a concentration of 10^{-5} M PBI-63 or PBI-51 there was 0.1% (v/v) ethanol in the incubation medium. The present study showed that the presence of 0.1% (v/v) ethanol in the incubation medium (MES/KOH) resulted in a decrease, which was approaching statistical significance, in the number of guard cells displaying *CDeT6-19* driven GUS activity compared with the MES/KOH treatment (see Section 3.3.1) This suggests that it would not be advisable to have an even higher concentration of ethanol present. The effect of concentrations of (±)-ABA higher than 10^{-5} M on *CDeT6-19*

driven GUS activity in guard cells were therefore not investigated in the detached epidermis system because of the concomitant increase in ethanol concentration in the incubation medium which would be needed to act as a control for the ABA analogue work. Admittedly, 10^{-4} M concentrations of the analogues [1 % (v/v) ethanol] were used in Chapter 2 when studying the effect of the analogues on stomatal aperture. However, 1% ethanol had no effect on stomatal aperture in any of the species in which it was investigated (see Appendix A) and the results gained at this concentration were not fundamental to the overall investigations in Chapter 2 as they would be in the present chapter.

There was a relatively high level of GUS activity in the guard cells of MES/KOH + 0.1% (v/v) ethanol- and MES/KOH-treated detached epidermis from transgenic tobacco leaves (Figure 3.6). Taylor *et al.* (1995) reported that there were variable levels of constitutive *CDeT6-19* driven GUS expression in the guard cells of epidermis from MES/KOH-treated leaves of transgenic tobacco plants. However, the levels that they reported varied from zero to very low. In addition, a significant level of constitutive GUS activity was reported in the leaves of transgenic tobacco plants and *Craterostigma* plants transformed with the *CDeT6-19-GUS* construct (Michel *et al.*, 1994). In the present study, there was a possibility that the removal of epidermis from the tobacco leaves prior to incubation on the treatment solutions may have led to the high level GUS activity seen in the guard cells due to a wounding response. However, the *CDeT6-19* gene promoter has been shown to be unresponsive to wounding (Piatkowski *et al.*, 1990). In addition, a high level of *CDeT6-19* driven GUS activity was evident in guard cells in epidermal tissue which was removed from untreated plants immediately prior to incubation on the GUS assay solution (see Figure 3.9). This is a situation in which there is presumably insufficient time for wound-induced activation of

CDeT6-19 driven GUS activity. These facts suggest that the level of *CDeT6-19* driven GUS activity in guard cells of MES/KOH-treated, detached epidermis was not related to the removal of epidermis prior to the treatment application.

In the light of the above results obtained using (±)-ABA-treated, detached epidermis, the effects of ABA on guard cells on tobacco leaves were investigated by a foliar application of 10^{-4} M (±)-ABA. Interest focused on comparing the effects of 10^{-4} M (±)-ABA on stomatal conductance (an indirect measurement of guard cell turgor) and *CDeT6-19* driven GUS activity in the guard cells of the same or adjacent leaves of the transgenic tobacco plants. Similar studies have revealed a complex relationship between guard cell turgor, plant water status and transcript levels in leaves (Thompson & Corlett, 1995) and guard cells (Kopka *et al.*, 1997) of plants under drought treatment. A preliminary experiment, in the present study, revealed that stomatal conductance was reduced and *CDeT6-19* driven GUS activity enhanced in the leaves of (±)-ABA-treated transgenic tobacco plants (Figures 3.7 and 3.8). However, a comparison between the overall results for (±)-ABA-regulated *CDeT6-19* driven GUS activity and stomatal conductance was not feasible. This was due to the unexpected result that in this study (±)-ABA did not have a significant effect on *CDeT6-19* driven GUS activity in guard cells (Table 3.1) when both ABA responses were measured on the same or adjacent leaves. This could be due to the relatively small sample size on which both types of measurements (GUS activity and stomatal conductance) were made. Indeed, increasing the sample size resulted in a significant ABA effect on *CDeT6-19* driven GUS activity in guard cells of transgenic tobacco leaves.(Table 3.2). The results in Table 3.2 are in agreement with the work by Taylor *et al.* (1995). They suggest that a larger scale study of both types of ABA response may be profitable. The differences between the results in Tables 3.1 and 3.2 may also be due to the fact that

the plants used to provide the data in Table 3.2 were not subjected to any porometric analysis and were not moved to a controlled environment room for the application of treatments. Thus the data in Table 3.1 may have come from plants which were slightly stressed. This could have resulted in an increase in endogenous ABA levels and hence *CDeT6-19* driven GUS activity in all plants, thus reducing or eliminating the responsiveness of the *CDeT6-19* gene promoter to (±)-ABA. In this respect, it could be of interest to determine the endogenous concentrations of ABA in the leaves (using an enzyme-linked immunoabsorbant assay [ELISA] [see Walker-Simmons & Abrams, 1991] or by radio-immunoassay [RIA] [Quarrie *et al.*, 1988; Vernieri *et al.*, 1989]) both pre- and post-treatment application.

Leaf age affected the responsiveness of *CDeT6-19* driven GUS activity to (±)-ABA in guard cells. (±)-ABA induced a significant level of *CDeT6-19* driven GUS activity in guard cells in abaxial epidermis from only the youngest leaves (Figure 3.9.B). Similarly, it was reported that *CDeT6-19* driven GUS activity was the most responsive to (±)-ABA in vegetative tissue of the youngest *A. thaliana* plants (Furini *et al.*, 1996) and the responsiveness of osmotin promoter driven GUS activity decreased with leaf age in transgenic tobacco plants (Nelson *et al.*, 1992). The results from the present study showed that in the older leaves there was no statistically significant difference between treatments in the level of *CDeT6-19* driven GUS activity in guard cells (Figure 3.9). Following on from this, the results also suggested that the highest level of constitutive *CDeT6-19* driven GUS activity was found in the guard cells in the oldest leaves (see Figure 3.7 and 3.9.B), although a larger sample size would be needed to establish statistical significance due to the within treatment variation in the present study. In agreement with this, it was found that *CDeT6-19* driven GUS activity in the vegetative tissue of *A. thaliana* plants (Furini *et al.*, 1996) was highest in the older

plant tissue. In addition, GUS activity driven by the promoters of the cold- and ABA-regulated genes *kin1* and *cor6.6* in leaf tissue increased with leaf and plant age (Wang & Cutler, 1995). Taken together, the results for the *CDeT6-19* gene promoter suggest that its regulation may not be strictly ABA-dependent in mature plants (Furini *et al.*, 1996). It could be argued that older leaves may have higher endogenous ABA concentrations and this would result in a higher level of *CDeT6-19* driven GUS activity in the guard cells. However, there is no evidence for high levels of endogenous ABA in older leaves. In fact, all the available data point to the reverse scenario (Zeevaart & Kinilaan, 1972; Lorah, 1974; Raschke & Zeevaart, 1976). It is worth mentioning that it is possible that the differential level of GUS activity in guard cells in different ages of leaf tissue may be due to some property of the GUS reporter gene. The idea that GUS activity (the presence of the GUS enzyme) may not always accurately report the activity of the gene promoter to which the GUS gene is fused was highlighted in investigations using the *kin1* or *cor6.6* gene promoter-GUS fusions (Wang *et al.*, 1995); for a more detailed discussion of this phenomenon see Taylor (1997). As an example, *kin1* or *cor6.6* driven GUS activity (measured by an assay dependent upon the presence of the GUS protein) was not induced by cold treatment in transgenic tobacco and *A. thaliana* even though these promoters are from cold-responsive genes of *A. thaliana* (Kurkela & Franck, 1990). Northern analysis revealed that low temperature did significantly induce GUS mRNA under the control of these promoters, but GUS protein synthesis was inhibited under low temperature thus resulting in the reported lack of GUS activity (Wang *et al.*, 1995).

The stomatal conductance of all leaves, except the oldest, was significantly reduced by (±)-ABA (Figure 3.8), which correlates with the (±)-ABA-induced inhibition of stomatal opening (determined by measurements of stomatal aperture) in

detached, abaxial epidermis from tobacco leaves described in Chapter 2. It was interesting to note that for the untreated plants the stomatal conductance of the youngest leaves was significantly higher on the first 2 days of the experiment than that of the oldest leaves (Figure 3.8.C). It was suggested by Blackman (1984) that stomata of older leaves are generally less responsive and dynamic, which would result in their lower stomatal conductance and a reduced responsiveness to ABA. Similarly other studies have shown that once a leaf passes a certain age, stomatal responses become more sluggish and the maximum apertures reached are smaller (for example Ludlow & Wilson, 1971; Vaclavik, 1973; Davis & McCree, 1978; Willmer *et al.*, 1988).

In conclusion, the first report of an ABA-regulated gene promoter in the stomatal guard cell brought with it many hopes for research into nuclear and ABA signalling pathways in plants (Taylor *et al.*, 1995). The data from the present study suggest that stomatal guard cells of tobacco plants transformed with the *CDeT6-19*/GUS construct do not provide an ideal system in which to pursue these investigations. The lack of a well-defined, consistent ABA effect on *CDeT6-19* driven GUS activity in guard cells of tobacco leaves means that this species is unsuitable for simultaneously investigating ABA-induced changes in guard cell gene promoter activity and turgor. In addition, it would be unwise to use tobacco guard cells for single cell, nuclear ABA signalling studies due to the within treatment variation and the lack of a consistent ABA effect on *CDeT6-19* driven GUS activity when using a small sample size. The results of the study of *CDeT6-19* driven GUS activity in guard cells of leaves of different ages suggests that, in the future, it may be worthwhile to study GUS activity driven by the *CDeT6-19* promoter in ABA-treated detached epidermis from leaves of a younger age than those used in the present study. It may be more profitable, however, to turn our attention to studying ABA signalling pathways in the stomatal

guard cells of transgenic plants of *Arabidopsis thaliana* (transformed with the same construct as the tobacco plants) (see Chapter 4). Briefly, the reasons for this include (also see Section 4.1.1): (i) there is a lower level of constitutive *CDeT6-19* driven GUS activity in guard cells of abaxial epidermis from leaves of *A. thaliana* (J.E. Taylor, personal communication, and results of preliminary experiments carried out by the author of this thesis); (ii) there have been recent reports of viable *A. thaliana* guard cells that regulate their turgor in response to ABA (preliminary experiments by the author; Roelfsema & Prins, 1995; Webb & Hetherington, 1997); and (iii) it is extremely advantageous to investigate ABA signalling pathways in guard cells of *A. thaliana,* due to the high number of signal transduction mutants and transgenic plants of *A. thaliana* available for such studies (see Section 4.1.1).

Chapter 4

The Effects of ABA Analogues on Gene Promoter Activity and Turgor in Guard Cells of *Arabidopsis thaliana*

4.1 Introduction

4.1.1 Studying guard cells in the abaxial epidermis from leaves of transgenic *A. thaliana*

4.1.1(a) The present study

Studying two responses to ABA in guard cells has the potential of revealing important information concerning ABA signalling pathways in these cells (see Sections 1.8 and 3.1.1). As described previously, ABA analogues can be used as tools to investigate ABA signalling pathways in plant cells (see Sections 1.7 and 1.8). The effect of ABA on *CDeT6-19* gene promoter driven GUS activity and turgor in guard cells of *Nicotiana tabacum* was investigated in the previous chapter. However, it was found that this species was unsuitable for studying the effect of ABA analogues on these two ABA-induced responses because of the lack of a well-defined, consistent effect of ABA on *CDeT6-19* driven GUS activity (see Chapter 3). To overcome these difficulties, experiments were carried out in the present chapter in *Arabidopsis thaliana* transformed with the construct consisting of the *CDeT6-19* gene promoter fused to the GUS reporter gene (see Taylor *et al.*, 1995; see Sections 1.6.2 and 3.1.3). Information

from two sources (preliminary experiments by the author and J.E. Taylor, personal communication) suggested that the guard cells of transgenic *A. thaliana* have a lower level of constitutive *CDeT6-19* driven GUS activity than those of transgenic *N. tabacum*.

4.1.1(b) Overall perspectives

The use of guard cells of *A. thaliana* for investigating ABA signalling pathways brings with it another advantage. *A. thaliana* is ideally suited for genetic analysis and there is a wealth of genetically modified plants of *A. thaliana* which are proving to be invaluable in investigations of ABA signalling pathways in plants. (for reviews see Giraudat *et al.*, 1994; Giraudat, 1995; Merlot & Giraudat, 1997; also see McAinsh *et al.*, 1997). Such plants include the ABA biosynthesis mutants, *aba*1 (*ABA absent* 1) (Koornneef *et al.*, 1982; Duckham *et al.*, 1991; Rock & Zeevaart, 1991) and the more recently discovered *aba*2 and *aba*3 (Léon-Kloosterziel *et al.*, 1996; Schwartz *et al.*, 1997). In addition, several *abi* (*ABA-Insensitive*) mutants have been discovered (for reviews see Giraudat, 1995; Merlot & Giraudat, 1997) including the mutants *abi*-1, *abi*-2 and *abi*-3 (Koornneef *et al.*, 1984). Studies of these mutants are proving to be invaluable in uncovering the complexity of ABA signalling pathways in plants [for example: Armstrong *et al.*, 1995; Parcy & Giraudat, 1995; Furini *et al.*, 1996; Parcy *et al.*, 1997; Pei *et al.*, 1997; Roelfsema & Prins, 1995; Webb & Hetherington, 1997; see Section 4.1.1(c) below for a detailed discussion of the *abi*-1, *abi*-2 mutants of *A. thaliana*]. In addition to mutational analysis, the ease with which *A. thaliana* can be genetically manipulated has led to the production of transgenic plants of *A. thaliana* useful for ABA signalling studies. These transgenic plants include those containing ABA-regulated gene promoter-reporter gene constructs. (Terryn *et al.*, 1993; Taylor *et*

al., 1995; Nakamura *et al.*, 1995; Wang *et al.*, 1995) and ectopically expressed genes such as the *ABI3* gene (Furini *et al.*, 1996; Parcy & Giraudat, 1995).

The significant advances that have been made in understanding ABA signalling pathways by genetically manipulating *A. thaliana* are now being complemented by the discovery that the guard cell physiology of *A. thaliana* can be studied in isolated epidermis (Roelfsema & Prins, 1995; Pei *et al.*, 1997; Webb & Hetherington, 1997). This has meant that the regulation of guard cell turgor by ABA can be studied in mutant *A. thaliana* [for e.g. the *abi* mutants, see Sections 1.5.3, 1.6.3(d) and 4.1.1(c)] using techniques such as patch-clamping (Pei *et al.*, 1997) which until recently were restricted to plants unsuited to genetical manipulations. The marrying of these two approaches (genetical and physiological) makes it an exciting time to be studying ABA signalling pathways in guard cells of *A. thaliana.*

4.1.1(c) The *abi1* and *abi2* mutants of *A. thaliana*

The *abi1* and *abi2* mutants of *A. thaliana*, isolated in 1984, display reduced seed dormancy and defects in the multiple actions of ABA in vegetative tissues (Koornneef *et al.*, 1984; Finkelstein & Sommerville, 1990; Finkelstein, 1994; Giraudat *et al.*, 1994). One of the phenotypic characteristics of these mutants is an increased loss of water and a tendency to wilt (Koornneef *et al.*, 1984) which was traced to improper stomatal regulation (Koornneef *et al.*, 1984; Finkelstein, 1994; Leung *et al.*, 1994). More recently, it has been confirmed that guard cells in detached epidermis of both the *abi1* and *abi2* mutants of *A. thaliana* are insensitive to ABA (i.e. they do not reduce their turgor in response to ABA) (Roelfsema & Prins, 1995; Pei *et al.*, 1997). These data suggest that the *ABI1* and *ABI2* gene loci encode important components of the ABA signalling pathway which terminates in a reduction in guard cell turgor. Indeed, it

is known that *ABI1* and *ABI2* encode proteins with homology to the 2C class of serine/threonine protein phosphatases (PP2C) (Leung *et al.*, 1994, 1997; Meyer *et al.*, 1994); and it has been confirmed that the *ABI1* and *ABI2* proteins exhibit PP2C activity (Bertauche *et al.*, 1996; Leung *et al.*, 1997).

There have been several attempts to assign roles for the *ABI1* and *ABI2* proteins in ABA signalling pathways in guard cells (Armstrong *et al.*, 1995; Grabov *et al.*, 1997; Pei *et al.*, 1997). In *Nicotiana benthamiana* expressing the *Arabidopsis abi1* transgene, the sensitivity of outward and inward K^+ channels in guard cells [see Section 1.5.3(a)] to ABA was abolished (Armstrong *et al.*, 1995). Armstrong *et al.* (1995) also reported that two broad range protein kinase inhibitors could recover normal sensitivity of these channels to ABA. From these studies the authors concluded that the product of the *ABI1* gene can be implicated as part of a phosphatase/kinase pathway that modulates the sensitivity of guard cell K^+ channels to ABA. In addition, they reported that ABA-induced cytosolic alkalinization [see Section 1.5.4(b)] was observed in the *abi1*-mutant. From these data the authors suggest that the product of the *ABI-1* gene acts downstream of the pH signal. The presence of the *abi1* transgene in *N. benthamiana* has been reported to have no effect on guard cell anion channels (Armstrong *et al.*, 1995; Grabov *et al.*, 1997). By way of contrast, the presence of the *abi1* and *abi2* mutant genes in *A. thaliana* abolished the ABA-induced activation of guard cell anion channels (S-type) [see Section 1.5.3(a); Pei *et al.*, 1997]. This effect could be partially rescued by kinase inhibitors in *abi1* but not *abi2* mutant guard cells. Based on these data, Pei *et al.* (1997) have put forward new models for ABA signalling pathways in guard cells. The reasons underlying the apparent difference between plant species described above remains to be investigated (also see Section 2.4).

As described in Section 1.6.3(d), the *abi1* mutation (and in some cases *abi2* mutation, for example; de Bruxelles *et al.*, 1996) has also been shown to interfere with ABA-responsive gene expression in *A. thaliana* (Finkelstein & Sommerville, 1990; Gilmour & Thomashow, 1991; Nordin *et al.*, 1991, 1993; Lang & Palva, 1992; Yamaguchi-Shinozaki & Shinozaki, 1993; Gosti *et al.*, 1995; de Bruxelles *et al.*, 1996; Söderman *et al.*, 1996). However, the effect of the mutant abi1 and abi2 proteins on ABA-responsive gene expression in guard cells of *A. thaliana* has not yet been published.

4.1.2 Structural features of the (+)-ABA molecule which are important for the regulation of gene expression

As stated earlier, this chapter is concerned with investigating the effect of ABA analogues on *CDeT6-19* gene promoter driven GUS activity and turgor in guard cells of *A. thaliana.* The structural features of the (+)-ABA molecule which have been reported to be important for the regulation of gene expression will be described below. The reader is referred to Section 2.1.2 for a description of the structural features of the ABA molecule which have been reported to be important for changes in guard cell turgor.

The structural features of the (+)-ABA molecule which are important for the regulation of gene expression in guard cells have not been studied previously. However, studies have been carried out to determine the structural features of the (+)-ABA molecule which are important for gene expression in other systems. These include barley aleurone protoplasts (Van der Meulen, 1993; Hill *et al.*, 1995), bromegrass cell cultures (Robertson *et al.*, 1994; Wilen *et al.*, 1996), white spruce protoplasts (Dong *et al.*, 1994), wheat embryos (Walker-Simmons *et al.*, 1992)

Brassica napus embryos (Wilen *et al.*, 1993) and callus tissue of *Craterostigma plantagineum* (Chandler *et al.*, 1997). The work of Chandler *et al.* (1997) is of special importance to the current study because it concerns the effect of several ABA analogues on transcripts which hybridize to the pcC6-19 cDNA probe in callus tissue of *C. plantagineum* [the cDNA clone pcC6-19 is encoded by the gene *CDeT6-19* (Piatkowski *et al.*, 1990; see Section 3.1.3)]; the promoter of the *CDeT6-19* gene is investigated in the present study. A summary of the important structural features of the (+)-ABA molecule required for the regulation of gene expression in the systems described above will now be presented (see Figure 4.1, Table 4.1 and also Section 1.7.4 for additional discussion).

The ABA analogues investigated in this chapter are described in Figure 1.4 and Section 1.7.4 and are the same as those investigated in Chapter 2. They include: (–)-ABA, (±)-*trans, trans*-ABA and (+)- and (–)-dihydroacetylenic abscisyl alcohol (PBI-63 and -51). The structural changes to the ABA molecule which are apparent in the ABA analogues used in the present study are highlighted in bold in the general summary below. The effects of (±)-ABA and (+)-ABA were also investigated in the present study and acted as positive controls to the ABA analogue work (see Section 1.8).

A detailed study has been carried out on the structural features of the ABA molecule important for the regulation of gene expression in barley aleurone protoplasts (Van der Meulen *et al.*, 1993). The data suggest an order of importance of the putative functional groups of the ABA molecule in this system: C-4' carbonyl- > C-1' hydroxyl- > **C-1 carboxyl** (Van der Meulen *et al.*, 1993). The 7' methyl group of ABA was reported to be important in the regulation of gene expression (Hill *et al.*, 1995). The requirement of a **(+)-orientation at C-1'** varied between the genes and systems

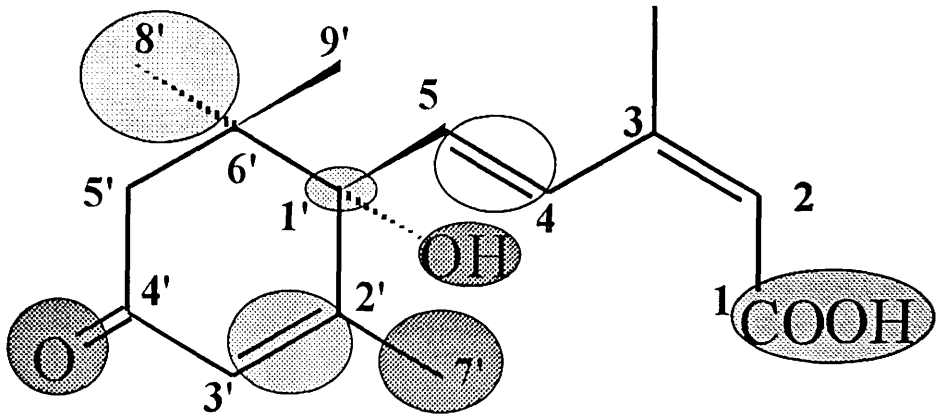


Figure 4.1 Structure of (+)-ABA. The structural features of the molecule shaded the darkest represent those which are important; the lighter shaded areas represent those which have a variable degree of importance; and the area circled with no shading represents the least important for (+)-ABA-responsive gene expression (see Table 4.1).

IMPORTANT	VARIABLE	LESS IMPORTANT
7' methyl group	(+)- orientation at C-1'	*trans* double bond at C-4 of side chain
C-4' carbonyl group	C-1 carboxylic group	
C-1' hydroxyl group	C-2', C-3' ring double bond	
	8' methyl group	

Table 4.1 A summary of the important structural features of the (+)-ABA molecule for (+)-ABA-responsive gene expression, based on the literature to date. The areas described are judged to be important, have a variable degree of importance (dependent on the gene or system under study) or to be less important (see text).

studied. For example, in some studies (–)-ABA was as active as (+)-ABA at affecting gene expression (Hill *et al.*, 1995; Robertson *et al.*, 1994; Chandler & Robertson, 1997), whereas in others it was much less active (Perras *et al.*, 1994; Dong *et al.*, 1994; Hill *et al.*, 1995; Walker-Simmons *et al.*, 1992; Wilen *et al.*, 1993). There was also variation in the biological activity of (–)-ABA between different genes investigated in individual systems (Walker-Simmons *et al.*, 1992; Hill *et al.*, 1995). The **C-1 carboxylic group** of ABA showed a variable level of importance in the regulation of gene expression (Robertson *et al.*, 1994; Van der Meulen *et al.*, 1993). The **lack of the ring double bond**, (dihydroABA) resulted generally in a molecule with biological activity similar to that of (+)-ABA (Robertson *et al.*, 1994; Hill *et al.*, 1995; Walker-Simmons *et al.*, 1992; Dong *et al.*, 1994; Chandler & Robertson, 1997). However, there were two exceptions, the wheat *Em* gene (Walker-Simmons *et al.*, 1992) and the barley WCS120 gene (Perras *et al.*, 1994) were both unresponsive to dihydroABA. The 8' methyl group of ABA has been reported to have a variable degree of importance. The presence of a hydroxyl group at C-8' in addition to the lack of the ring double bond resulted in an inactive ABA analogue (Hill *et al.*, 1995). This suggests that the 8' methyl group is important due to the fact that the lack of the ring double bond in this study did not markedly affect biological activity. However, (+)-8' 8'-difluroABA and (+)-8' 8' 8'-trifluroABA were as effective as (+)-ABA at inhibiting GA_3-induced alpha-amylase activity (Todoroki *et al.*, 1995). The presence of an **acetylenic group in the side chain of ABA** resulted in an ABA analogue which induced ABA-responsive gene expression (Perras *et al.*, 1994; Dong *et al.*, 1994).

Other ABA analogues with multiple changes to their structure will now be considered briefly. The presence of an **acetylenic group in the side chain of ABA in an addition to the lack of the ring double bond** resulted in an active ABA analogue

(Walker-Simmons *et al.*, 1992; Perras *et al.*, 1994; Dong *et al.*, 1994; Chandler & Robertson, 1997). It is interesting to note that for the induction of the *Em* gene in wheat embryos, dihydroacetylenic ABA was more active than dihydroABA (Walker-Simmons *et al.*, 1992). The (–)-enantiomers of many ABA analogues differ markedly in their effectiveness at inducing ABA-responsive gene expression compared with their respective (+)-enantiomers (Hays *et al.*, 1996; Perras *et al.*, 1994; Robertson *et al.*, 1994; Walker-Simmons *et al.*, 1992, Dong *et al.*, 1994). For example, (–)-dihydroABA induced a very low level of *CDeT6-19* gene expression in *Craterostigma* callus tissue and (–)-dihydroacetylenic ABA was inactive in this system, whereas both (+)-enantiomers were active (see above). **(+)-dihydroacetylenic abscisyl alcohol** [see Section 1.7.4(c)] induced ABA-responsive gene expression but was less active than ABA (Wilen *et al.*, 1993, 1996). By way of contrast, **(–)-dihydroacetylenic abscisyl alcohol** [see Section 1.7.4(c)] was inactive when applied alone but acted as an antagonist of (+)- or (±)-ABA-induced gene expression (Wilen *et al.*, 1993, 1996). The loss of the C-1 carboxylic group in conjunction with the C-1' hydroxyl group and the C-4' carbonyl group resulted in a completely inactive molecule (Van der Meulen *et al.*, 1993).

4.1.3 Aims

The first aim of this study was to investigate the effects of several ABA analogues (Figure 1.4) on *CDeT6-19* driven GUS activity in guard cells of *A. thaliana*. These investigations would be carried out in a manner similar to those in which (±)-ABA-responsive *CDeT6-19* driven GUS activity in guard cells of *A. thaliana* was determined (Taylor *et al.*, 1995). As stated earlier, the ABA analogues investigated in this chapter are the same as those studied in Chapter 2. Initially, it was envisaged that

the data gained in the present chapter could be compared with that reported in Chapter 2 for ABA analogue-induced alterations in guard cell turgor. Such a comparison may reveal whether the two guard cell ABA-induced responses (regulation of guard cell turgor and gene promoter activity) have similar perception mechanisms/ "receptors" (Montgomery *et al.*, 1996a & b; see Section 1.8). However, it was also envisaged that it would be of interest to measure both responses to ABA in guard cells of *A. thaliana*. This would allow a direct comparison to be made between the stereochemical requirements of the perception mechanisms of the two ABA-induced responses in guard cells of a single species.

4.2 Materials and Methods

4.2.1 Chemicals

All the chemicals described below were from Sigma Chemical Co. (UK) unless otherwise stated. (+)-ABA, (–)-ABA, PBI-51 and PBI-63 were kindly supplied by Dr. S. Abrams (Plant Biotechnology Institute, Saskatoon, Canada).

4.2.2 Plants and growth conditions

Wild-type (WT) *Arabidopsis thaliana* Columbia ecotype and those transformed with a chimeric construct consisting of the *CDeT6-19* gene translationally fused to the *Escherichia coli* β-glucuronidase (GUS) gene were used (Taylor *et al.*, 1995). Five individual lines, each of which originated from separate transformants carrying the construct described above, were available for study. One of the lines (line 4) was chosen for use in all experiments in this chapter. Preliminary experiments (not shown) established that this line was one of the two (lines 2 and 4) in which *CDeT6-19* driven GUS activity in the guard cells was particularly ABA-responsive. In addition, line four plants were easy to grow, displaying a vigorous and uniform growth habit.

Seeds were surface sterilized by a five-minute incubation in a solution containing no more than 0.5% (v/v) sodium hypochlorite (Household Bleach, Spa, UK) and then washed five times with sterile distilled water. Seeds were placed on agar plates (described in Section 3.2.2) and were germinated in a growth room, with a day/night temperature of 20±1°C/17±1°C and a 10 h photoperiod at a PFD of 150 $\mu mol\ m^{-2}\ s^{-1}$. The lighting comprised 400 W metal halide elliptical opal-coloured bulbs (Osram-Siemens, UK) and the 10 h light/14 h dark regime was chosen in order to encourage leaf growth and discourage flowering (Koncz *et al.*, 1992). When the

seedlings were of a suitable size to allow handling with fine ceramic coated forceps (Whatman International Ltd., Maidstone, UK) (after 7-10 days), they were carefully transferred to plug trays containing a 3:1 mix of sterilized compost (Levington M3 - Levington Horticulture, UK) and fine horticultural grit (William Sinclair Horticulture, Lincoln, UK). The plug trays were housed in propagators (B & Q, Lancaster, UK) containing damp capillary matting (LBS Polythene, Nelson, UK) and maintained at a 21-23°C day and a 17-19°C night and a RH of 70-85%. After 10-14 days the seedlings were carefully transferred to "Aracon baskets" (Arasystem, Betatech, Belgium) containing the same soil mix as described earlier, and held in "Aracon trays" in the propagators (under the conditions described previously). Plants were then allowed to grow for a further 10-14 days before use in experiments. In order to maintain seed stocks, plants were grown on for seed. The flowering stems were enclosed in perspex tubes supplied with the Aracon system and after seed pod formation the plants were allowed to dry out for 2-3 weeks. After this time, the seed was harvested and stored in vials at 4°C.

4.2.3 Measurement of the effect of ABA analogues on *CDeT6-19* driven GUS activity in guard cells of *A. thaliana*; foliar treatment application *in planta*

4.2.3(a) Treatment application

A. thaliana plants (6-weeks-old) were immersed in solutions of MES/KOH (see Section 2.2.3), 22±1°C in the presence or absence of 10^{-5} M (±)-ABA, (+)-ABA, (–)-ABA, PBI-63, PBI-51 or 0.1% (v/v) ethanol for five seconds twice a day (Taylor *et al.*, 1995). The solutions were made from stock solutions as described in Section 2.2.4. Each plant was placed in the base of a 10 cm Petri dish containing damp capillary matting to prevent cross contamination of solutions via the groundwater (see Figure

4.2). On the third day, the leaf tissue (detached abaxial epidermis or whole leaves) was subjected to a histochemical GUS assay [see Sections 4.2.3(b) and 4.2.3(c)]. For the detached epidermis experiments two plants were used for each treatment and the experiment was repeated five times. For the whole leaf experiments one plant was used for each treatment and the experiment was repeated three times.

The effect of (±)-*trans, trans*-ABA on *CDeT6-19* driven GUS activity in guard cells of the abaxial epidermis of leaves of *A. thaliana* was investigated by making foliar applications as described above, except that the applications were made in the dark and over an 18 h time period. Thus an initial application of MES/KOH, 22±1°C containing 10^{-5} M (±)-*trans, trans*-ABA, (±)-ABA or 0.1% (v/v) ethanol was made at the beginning of the dark phase of the normal light regime. The propagator was then placed in a darkened area of the growth room and two further applications of treatment solutions were made in the dark at the beginning of the next photoperiod and at 4 h into this photoperiod. In this manner the plants were exposed to an extra five hours of darkness. It was envisaged that this methodology would reduce light-induced isomerization of (±)-*trans* , *trans*-ABA to (±)-*cis, trans*-ABA (see Sections 1.7.4 and 2.2.4). A histochemical assay for *CDeT6-19* driven GUS activity in detached abaxial epidermis was carried out [see Sections 4.2.3(b) and 4.2.3(c)] 2 h after the final treatment application. Four plants were used for each treatment per experiment and the experiment was repeated three times.

4.2.3(b) Removal of epidermis

The abaxial epidermis was removed carefully, using fine forceps, from the youngest two leaves over 1.5 cm in length of each plant of *A. thaliana*. The leaves were numbered according to age, leaf 1 was the smallest leaf in the middle of the

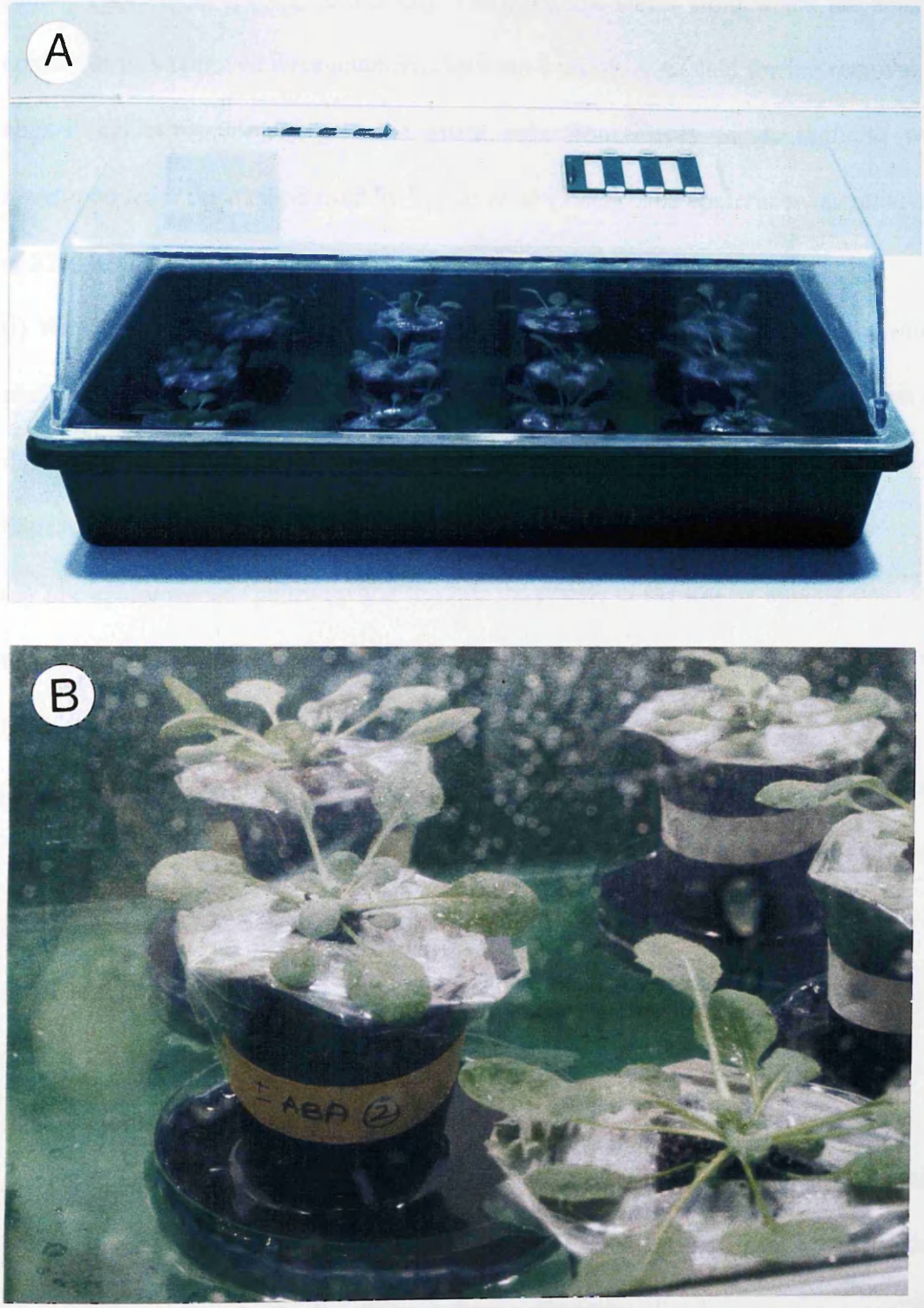


Figure 4.2 The experimental set-up for the treatment of transgenic *Arabidopsis thaliana* with ABA and ABA analogues [see Section 4.2.3(a)] (**A**). Note that each plant was placed in the base of a 10 cm Petri dish containing capillary matting to prevent cross-contamination of treatment solutions via the groundwater (**B**).

rosette (see Figure 4.10.A, further on). Therefore, the leaves from which the abaxial epidermis was removed were numbered between 4 and 6. A method for the removal of abaxial epidermis containing viable guard cells from leaves of *A. thaliana* was developed from the method used by Taylor *et al.* (1995). The epidermis was removed at 22±1°C in the manner described below:

(i) With the abaxial surface of the leaf uppermost, the epidermis (and initially a small piece of whole leaf tissue, hereafter known as the tab) on one side of the mid-vein at the leaf apex was pulled gently away from the rest of the leaf using fine forceps or fingers.

(ii) The epidermis was pulled up and towards the petiole of the leaf by holding onto the tab until epidermis was removed from approximately the upper quarter of one side of the leaf.

(iii) Holding the tab with forceps, the epidermis was placed cuticle side up on MES/KOH, 22±1°C with the leaf floating abaxial surface down (i.e. the leaf was flipped around the detached epidermis).

(iv) The remainder of the epidermis on that side of the leaf was then removed by gently pulling the tab away from the leaf, whilst holding the leaf with a pair of fine ceramic forceps (Whatman International Ltd., Maidstone, UK).

(v) The majority of the mesophyll tissue was removed from the detached epidermis using scissors, although it was necessary to leave a very small strip attached to the epidermis to prevent the fragile epidermis from curling up.

(vi) The whole process was repeated for the other side of the same leaf.

The epidermis was then transferred to the GUS assay solution [see Section 4.2.3(c)].

4.2.3(c) Histochemical localization of *CDeT6-19* driven GUS activity

A histochemical GUS assay was carried out on detached, abaxial epidermis from leaves of *A. thaliana* in the same manner as that described for detached, abaxial epidermis from transgenic tobacco plants in Section 3.2.3(b). In the majority of experiments, a photographic record was kept of each treatment (see Section 3.2.3). Alternatively, leaves of *A. thaliana* numbered 4-6 were placed in sealed 1.5 ml microfuge tubes (Sarstedt, Leicester, UK) containing 1 ml of the GUS assay solution described in Section 3.2.3(b). After incubation at 37°C for 10-14 h (see Figure 4.3) the chlorophyll was removed from the leaves by replacing the GUS assay solution in the microfuge tubes with 80% (v/v) ethanol and incubating at 60°C for 5 min (a hole was pierced in the lid of the tube with a mounted needle prior to incubation at 60°C). This procedure was repeated with fresh 80% (v/v) ethanol until a suitable degree of chlorophyll removal had been achieved. The leaves were examined under a dissecting microscope (Leica, Milton Keynes, UK) with illumination from a Schott KL1500 halogen light source (Orme Scientific, Manchester, UK) and also using the microscope described in Section 3.2.3. Photographs of the leaves were taken with a Wild Leitz camera (Leica, Milton Keynes, UK) connected to a Wild Leitz photoautomat (Leica, Milton Keynes, UK).

4.2.3(d) Variability in guard cell GUS activity in detached epidermis

The numbers of guard cells stained blue out of the total number of guard cells (approximately 800) in five fields of view on each of eight epidermal pieces per treatment were recorded. The colour-coded scale shown in Figure 4.4 was used to describe the intensity of the blue precipitate contained in the guard cells. The epidermal peels examined for each treatment were from two plants.

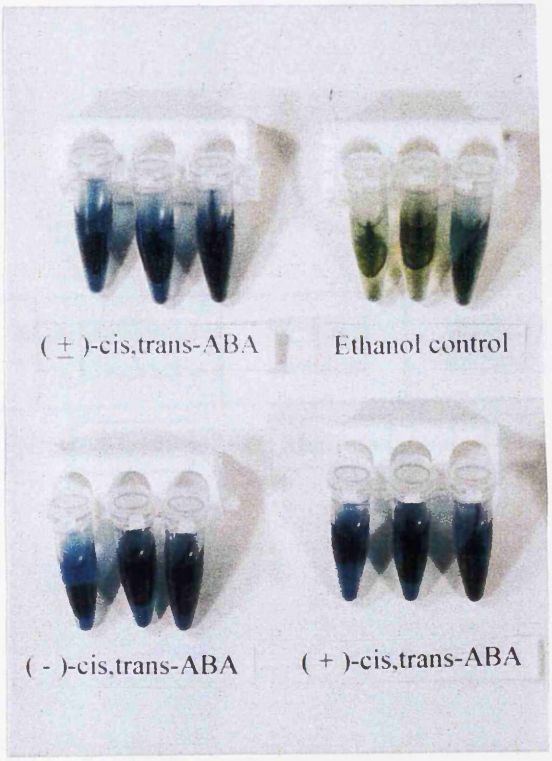


Figure 4.3 Microfuge tubes containing leaves of transgenic *Arabidopsis thaliana* following incubation at 37°C for 10-14 h in the GUS assay substrate used for the histochemical localisation of *CDeT6-19* driven GUS activity [see Section 4.2.3(c)]. The leaves were from plants treated with MES/KOH containing 10^{-5} M (±)-ABA, 0.1% (v/v) ethanol, 10^{-5} M (–)-ABA or 10^{-5} M (+)-ABA. The microfuge tubes shown for each treatment contain leaf 4, 5 and 6 (left to right) from one plant of *Arabidopsis thaliana*. See Figure 4.9. for results using this methodology.

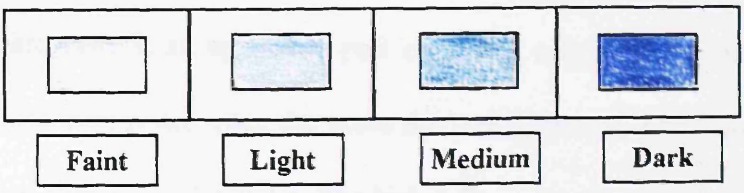


Figure 4.4 Colour-coded scale used to describe the shades of the blue precipitate formed in the histochemical localisation of *CDeT6-19* driven GUS activity in guard cells in abaxial epidermis from leaves of transgenic *Arabidopsis thaliana*. The corresponding GUS activity scores were as follows: Faint: 0.5; Light: 1; Medium: 3; Dark: 5 [see Section 4.2.3(e)].

4.2.3(e) Guard cell GUS activity scoring system

The estimated percentage of guard cells stained different shades of blue (see Figure 4.4) on an epidermal peel were used to calculate a guard cell *CDeT6-19* driven GUS activity score for each epidermal peel. The shade (or intensity) of the blue precipitate contained in the guard cells was described using a numerical scale (see Table 4.2). The guard cell GUS activity score for each peel was calculated by multiplying the (% of guard cells containing each shade of the blue precipitate / 100) by the numerical value given to that shade of blue in the table above. For example, if 30% of the guard cells in an epidermal peel contained a light blue precipitate and 70% a medium blue precipitate, then the score for that epidermal peel was [(30/100) x 1] + [(70/100) x 3] = 0.3 + 2.1 = 2.4. The higher the score the greater the level of *CDeT6-19* driven GUS activity. A mean guard cell GUS Activity Score per epidermal Peel (GASP) was calculated from the 30 peels examined for each treatment.

4.2.4 Measurement of the effect of ABA on *A. thaliana* leaf stomatal conductance; foliar treatment application *in planta*

4.2.4(a) Treatment application

A solution of MES/KOH, 22±1°C, 0.1% (v/v) ethanol (final concentration) in the presence or absence of 10^{-5} M (±)-ABA was applied to both surfaces of leaves of *A. thaliana in planta* by a fine spray from an aerosol spray bottle (fine mist nozzle, Nalgene Brand Products, Nalge Company, USA) at 2 h into the photoperiod until run off. In total, 3 ml of each treatment solution was applied to each plant. The plants were placed inside a large plastic bag in the controlled environment room during spraying, which lasted no more than 1 min, to avoid cross-contamination of the surrounding plants. The stomatal conductance of leaf 4 [see Section 4.2.3(b) for leaf numbering

Shade of the blue precipitate	**Guard cell GUS activity score**
No blue precipitate	0
Faint	0.5
Light	1
Light to Medium	2
Medium	3
Medium to Dark	4
Dark	5

Table 4.2 The guard cell GUS activity scoring system used to describe the shade (intensity) of the blue precipitate in the guard cells on detached epidermis from leaves of *Arabidopsis thaliana.*

system] was measured 6 h later (the leaves were totally free from surface moisture by this point). The experiment was repeated five times and the total number of plants measured was 14 per treatment.

4.2.4(b) Measurement of stomatal conductance

The stomatal conductance of leaf 4 of each *A. thaliana* plant was measured using a PMR-1 steady state porometer (PP Systems, Hitchin, Herts UK), which measures the net water vapour change between the air entering and leaving the cuvette in which a portion of the leaf is enclosed. This information is used along with other measurements such as temperature, air flow rate, PAR and leaf area exposed in the porometer to calculate stomatal conductance (for calculations, see PP systems, 1993). The porometer was used in conjunction with the EGM (Environmental Gas Monitor)-1 program (PP Systems, UK) and the data collected was downloaded to a PC in order to carry out the stomatal conductance calculations.

4.2.5 Measurement of the effect of ABA analogues on guard cell *CDeT6-19* driven GUS activity and turgor in detached, abaxial epidermis from *A. thaliana*

4.2.5(a) Removal of epidermis

Epidermis from the leaves of three plants of *A. thaliana* was removed [see Section 4.2.3(b)] and floated on MES/KOH, 22±1°C prior to the transfer of all the epidermal pieces to one of the treatment solutions (see below).

4.2.5(b) Treatment application

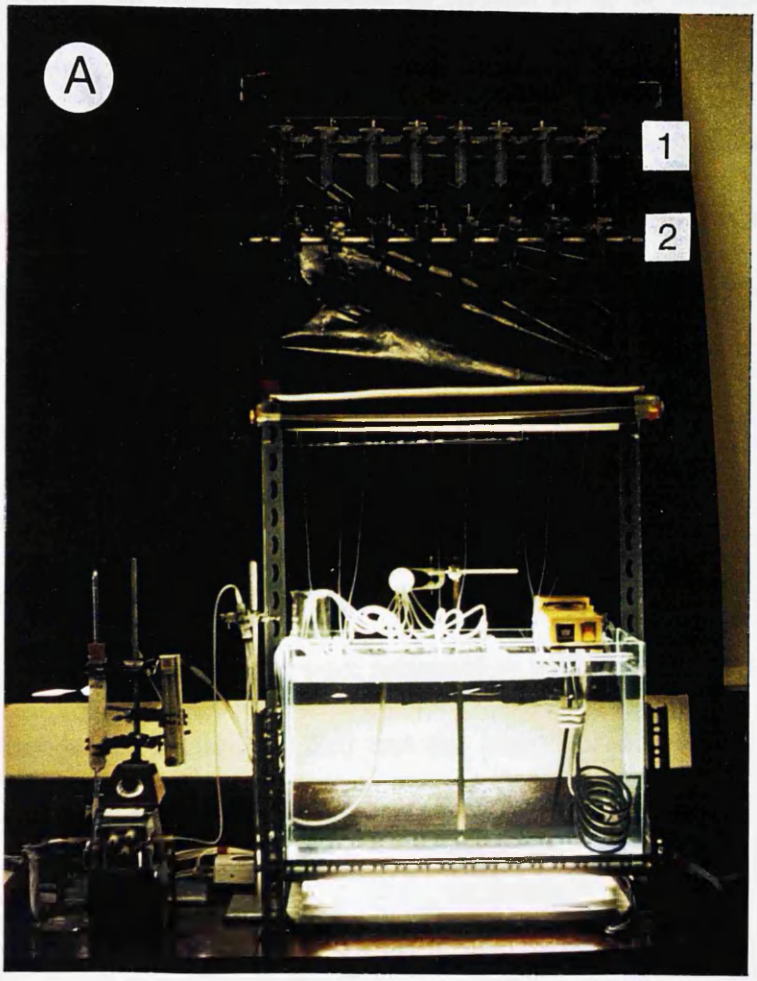
Detached abaxial epidermis from leaves of *A. thaliana* was incubated on MES/KOH, 50 mM KCl (preliminary experiments had confirmed that this

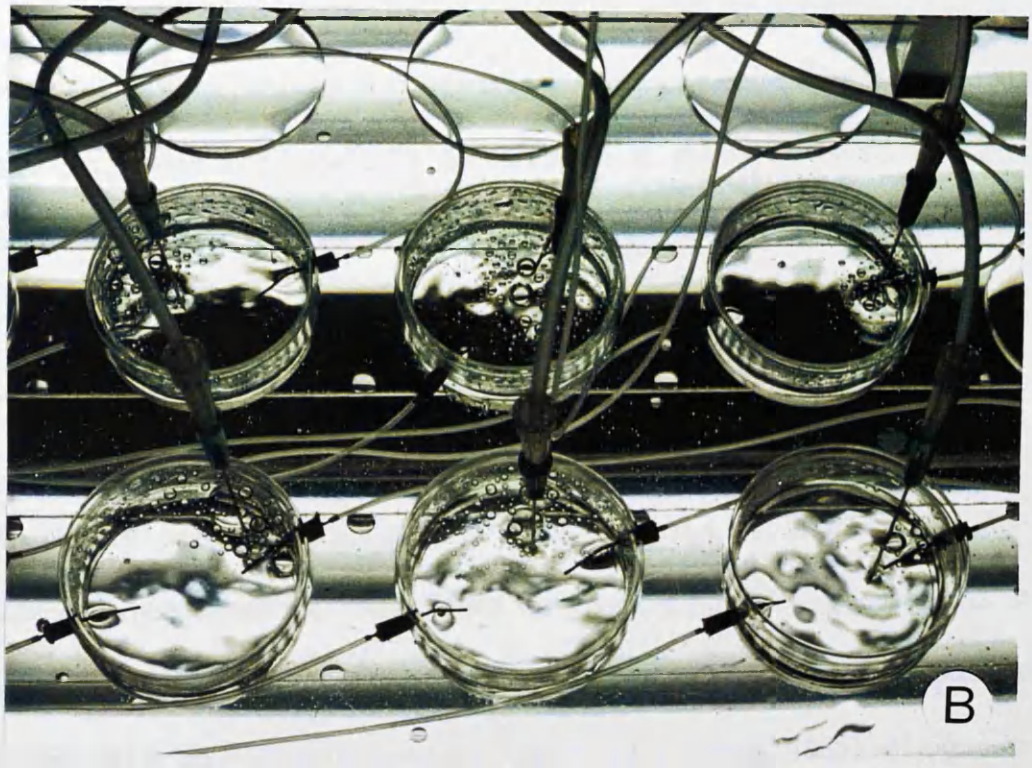
concentration of KCl resulted in maximal stomatal opening; Roelfsema & Prins, 1995), 22±1°C in the presence or absence of (±)-ABA, (+)-ABA, (–)-ABA, PBI-63, PBI-51, 0.01 or 0.1% (v/v) ethanol, under the conditions described in Section 2.2.4 that promote stomatal opening; the only differences being that the temperature was maintained at 22±1°C and the incubation period was for 2 h. The effect of the various compounds on stomatal opening was investigated by floating the epidermal pieces with "closed" (see below) stomata on the incubation medium. The removal of epidermis and subsequent flotation on MES/KOH resulted in "closed" stomata. These "closed" stomata had a stomatal aperture width of 3-4 μm.

A purpose built, continuous perfusion system (McAinsh *et al.*, 1991a) (see Figure 4.5) was used to investigate the effect of the light sensitive (±)-*trans, trans*-ABA molecule on stomatal opening. This system allowed the epidermal pieces to be maintained for 2 h in 10 cm^3 of continuously flowing (1 cm^3 min^{-1}) treatment medium so as to minimize the light induced isomerization of (±)-*trans, trans*-ABA to (±)-*cis, trans*-ABA.

Fresh solutions of ABA and its analogues were made up for each experiment. They were made as 10^{-2} M stock solution in 10% (v/v) ethanol and then diluted to the appropriate concentrations in MES/KOH, 50 mM KCl. In this way, the highest concentration of ethanol present in the incubation medium was 0.1% (v/v). Every attempt was made to prevent (±)-*trans, trans*-ABA from being exposed to light during the preparations of solutions. All solutions were allowed to come to equilibrium under the conditions described for stomatal opening for at least 1 h before use, except for those used in the 'flow-through' system. All the latter solutions were maintained as for the former except that they were kept free from light and were maintained at 22±1°C in an additional water bath prior to entry into the perfusion apparatus. The start time of

Figure 4.5 The continuous perfusion system (McAinsh *et al.*, 1991a) used to investigate the effect of (±)-*trans, trans*-ABA on guard cells in detached epidermis from leaves of transgenic *Arabidopsis thaliana*. The whole experimental set up is shown in (**A**); the reservoirs for all the treatment solutions at the positions shown as (**1**) and (**2**) were covered in foil (not shown) to minimize light-induced isomerization of (±)-*trans, trans*-ABA to (±)-*cis, trans*-ABA. A close-up of the apparatus used to supply and remove the treatment solutions to the 5 cm Petri-dishes in which the detached epidermal pieces were incubated is shown in (**B**). The vertical needle in the lid of each Petri dish supplied CO_2-free air to the treatment solutions.


A
1
2


B

incubation for each treatment was staggered by 15 min so as to allow time for the removal of epidermis for each treatment.

Experiments were conducted in which the effect of the treatment solutions described above were investigated on either *CDeT6-19* driven GUS activity [see Section 4.2.5(c)] or stomatal aperture [see Section 4.2.5(d)] in detached epidermis from leaves of *A. thaliana*. Each experiment was repeated three times.

4.2.5(c) Histochemical localization of *CDeT6-19* driven GUS activity

After incubation on the treatment solutions under the conditions described in Section 4.2.5(b) the epidermal pieces were subjected to a histochemical GUS assay in the manner described in Section 4.2.3(c) and the mean guard cell GASP for each treatment concentration calculated [see Section 4.2.3(e)].

4.2.5(d) Measurement of stomatal aperture

After incubation on the treatment solutions under the conditions described in Section 4.2.5(b) stomatal aperture widths in the epidermal peels were measured in the manner described in Section 2.2.5. However, for *A. thaliana*, stomata with a pore length of less than 7.5 μm were not measured (see Roelfsema & Prins, 1995).

4.2.5(e) Guard cell viability test

Guard cell viability in detached, abaxial epidermis from leaves of *A. thaliana* was assessed using fluorescein diacetate (FDA). This is a non-fluorescent, non-polar molecule which enters cells freely across the plasma membrane. Following this, it is hydrolyzed by the action of membrane bound esterases thus releasing the highly fluorescent, polar fluorescein molecule (Larkin, 1976; Dixon, 1985). Fluorescein is

much less permeant than FDA and accumulates in cells which possess an intact plasma membrane. Viable, intact cells exhibit a discrete distribution of fluorescence often associated with the cell membranes and organelles. However, accelerated leakage of the fluorescent product can occur following the hydrolysis of FDA if the plasma membrane of the cell has been damaged (Larkin, 1976). Detached, abaxial epidermis from leaves of *A. thaliana* was incubated on MES/KOH, 50 mM KCl, 22±1°C under the conditions described in Section 4.2.5(b). Subsequently, it was mounted in a 0.001% (w/v) solution of FDA in MES/KOH, 50 mM KCl, 22±1°C and examined microscopically using an epifluorescence microscope (Leitz, Milton Keynes, UK). The % of guard cells and other epidermal cells showing FDA staining in nine epidermal pieces (3 per experiment) were estimated [see Sections 4.2.3(e) and 4.3.1]. In addition, fluorescence (450-590 nm excitation filter; 515 nm emission filter [Leitz, Milton Keynes, UK]) and bright field photographs of detached epidermis were taken approximately 5 min after mounting. The viability of epidermal cells in detached, abaxial epidermis from leaves of *C. communis, V. faba* and *N. tabacum* was also assessed using FDA staining. The epidermis was mounted in the FDA solution after it had been incubated under the conditions that promoted stomatal opening for each species as described in Section 2.2.4.

4.2.6 Statistics

One way analysis of variance and LSD tests were used to determine differences between the mean guard cell GASPs for each treatment and the average stomatal aperture widths for each treatment. A t-test was used to compare the stomatal conductance of (±)-ABA and MES/KOH treated leaves (Section 4.2.4). T-tests were also used to compare the guard cell GASPs obtained from a detailed counting method

and the % estimation method. A generalized linear model with binomial error structure and logit function (Aitkin *et al.*, 1990; see Section 3.2.5) was used to determine statistically significant differences between treatments in the probability of obtaining blue guard cells of different shades of blue [see Section 4.2.3(c)]. Pairs of treatments were compared using a Wald test (Aitkin *et al.*, 1990). Significance was accepted at the 5% level in all tests unless otherwise stated.

4.3 Results

4.3.1 Variability in guard cell *CDeT6-19* driven GUS activity in epidermal peels

When assessing ABA- and ABA analogue-induced changes in *CDeT6-19* driven GUS activity in the guard cells of abaxial epidermis of leaves from transgenic *A. thaliana*, it was noticed that there was variability between guard cells in the intensity of the blue precipitate (indicative of *CDeT6-19* driven GUS activity) that they contained (for examples see Figures 4.7, 4.8, 4.11 and 4.12, further on). This variability was examined more closely by making detailed counts of the numbers of guard cells containing different intensities of the blue precipitate (zero, faint, light, medium or dark - see Figure 4.4) in epidermal peels from the leaves of *A. thaliana* which had been treated with ABA or ABA analogues. The results of these counts were expressed as percentages of the total number of guard cells examined for each treatment (Figure 4.6). From these data it can be seen that there was variation in the intensity of the blue precipitate contained in the guard cells in detached epidermis for all treatments. In addition to describing this variation, the data also show treatment effects in the distribution of guard cells displaying different intensities of the blue precipitate, such that, for example, treatment with (±)-ABA or PBI-63 resulted in a statistically significant higher number of guard cells stained with a medium or dark blue precipitate than for any other treatment.

A GUS activity scoring system was devised so that the variability could be incorporated into a description of the *CDeT6-19* driven GUS activity in the guard cells in abaxial epidermis for each treatment. A detailed description of this scoring system can be found in Section 4.2.3(e). The mean guard cell GUS activity score per

Figure 4.6 The percentage of guard cells stained dark, medium, light or faint blue or unstained (zero) out of the total number of guard cells counted for each treatment in detached, abaxial epidermis from leaves of transgenic *Arabidopsis thaliana.* The plants were treated with MES/KOH, 22±1°C containing 10^{-5} M (±)-ABA (**A**); PBI-63 (**B**); PBI-51 (**C**); 0.1% (v/v) ethanol (**D**); or were untreated (**E**).

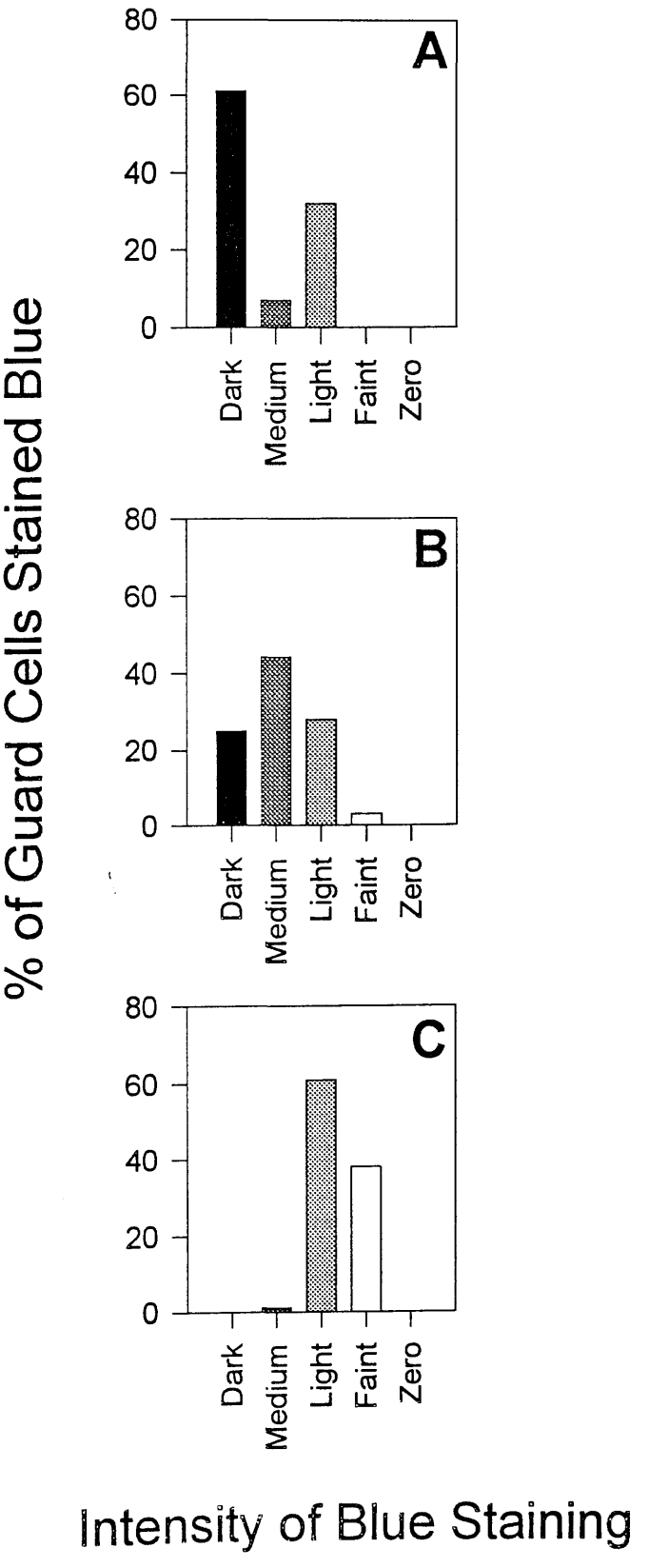
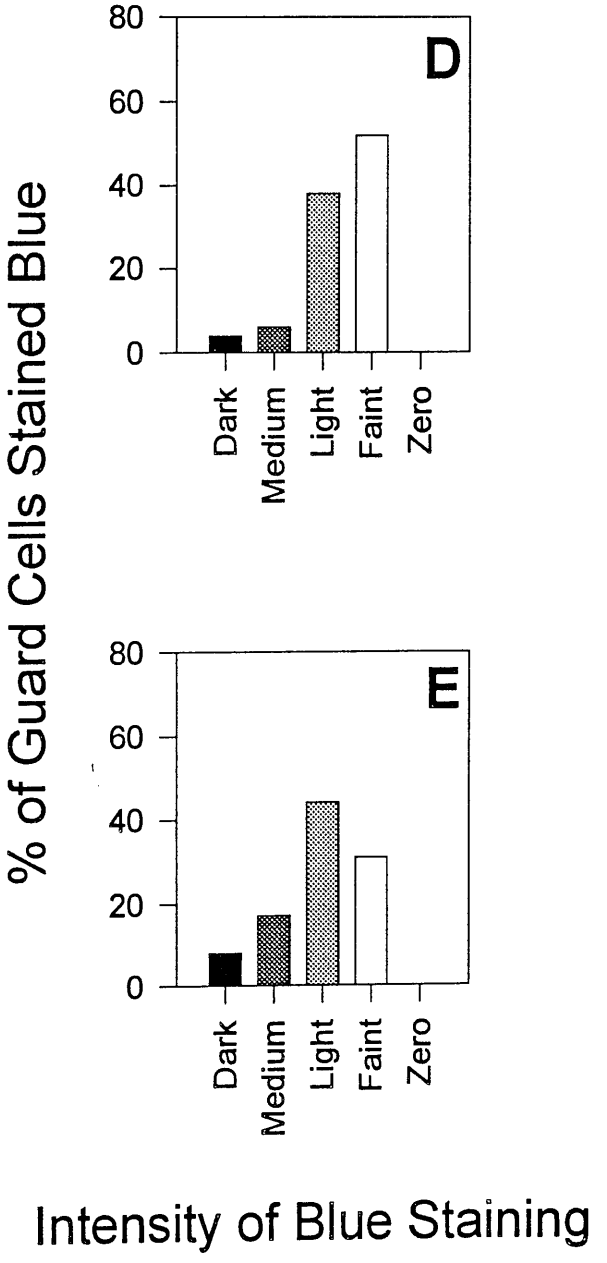

% of Guard Cells Stained Blue
80
60
40
20
0
A
Dark
Medium
Light
Faint
Zero
B
C
Intensity of Blue Staining

Figure 4.6 continued.

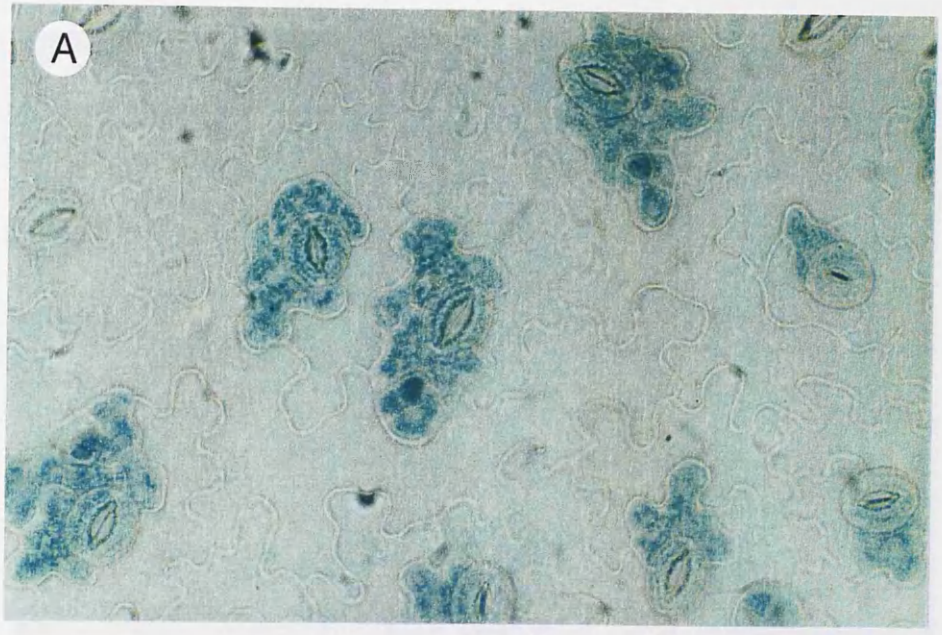


epidermal peel (GASP) was used to describe the *CDeT6-19* driven GUS activity of guard cells in different treatments. In order to calculate the mean guard cell GASP for each treatment the percentage of guard cells containing different intensities of the blue precipitate were estimated for the whole of each epidermal peel by microscopic observation. T-tests showed that the mean guard cell GASPs for each treatment obtained by using either percentages gained from detailed counts of guard cells [see Section 4.2.3(d)] or from estimating the percentage of guard cells containing different intensities of the blue precipitate by microscopic examination, were not statistically significantly different from one another. The latter method of obtaining the percentages (i.e. by estimation) was used in all experiments so that a large number of samples could be processed relatively quickly and also so that the whole population of guard cells on an epidermal peel could be assessed rather than taking samples as described for the detailed counting method [see Section 4.2.3(d)]. This latter consideration reduces the likelihood of stomatal patchiness (Martin & Rilling, 1993; Smith *et al.*, 1989) biasing the mean guard cell GASP.

4.3.2 The effect of ABA and ABA analogues on *CDeT6-19* driven GUS activity in guard cells in abaxial epidermis from leaves of treated, transgenic *A. thaliana*

Previously, it had been shown that a foliar application of 10^{-4} M (±)-ABA enhanced *CDeT6-19* driven GUS activity in guard cells in abaxial epidermis of transgenic *A. thaliana* leaves (Taylor *et al.*, 1995). The present study has investigated the effect of 10^{-5} M (±)-ABA and a range of ABA analogues on this gene promoter driven GUS activity. 10^{-5} M (±)-ABA significantly enhanced *CDeT6-19* driven GUS activity (see Figure 4.7.A and Table 4.3). This meant that it was possible to investigate

Figure 4.7 Histochemical localization of GUS activity driven by the *CDeT6-19* gene promoter in abaxial epidermis from leaves of transgenic *Arabidopsis thaliana* treated with MES/KOH, 22±1°C containing 10^{-5} M (±)-ABA (**A**); (+)-ABA (**B**); (–)-ABA (**C**); PBI-63 (**D**); PBI-51 or 0.1% (v/v) ethanol (**E**); or from untreated plants (**F**). (n = 10 plants per treatment). (Magnification x 400). Abaxial epidermis from leaves of WT *A. thaliana* treated with (±)-ABA showed no GUS activity (data not shown). See Table 4.3 for the mean guard cell GUS Activity Score per Peel (GASP) for each treatment.


A

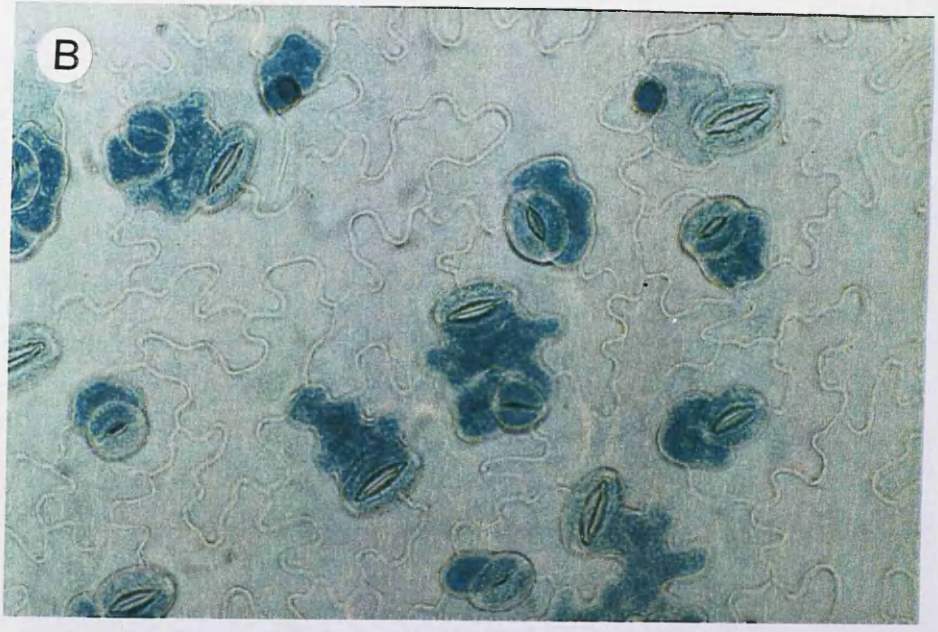

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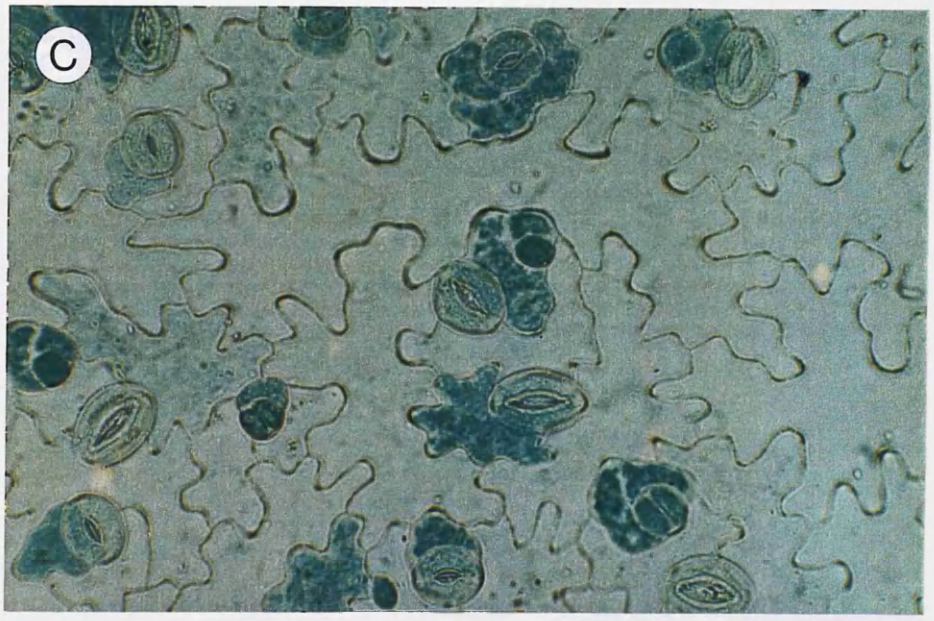
Figure 4.7 continued.



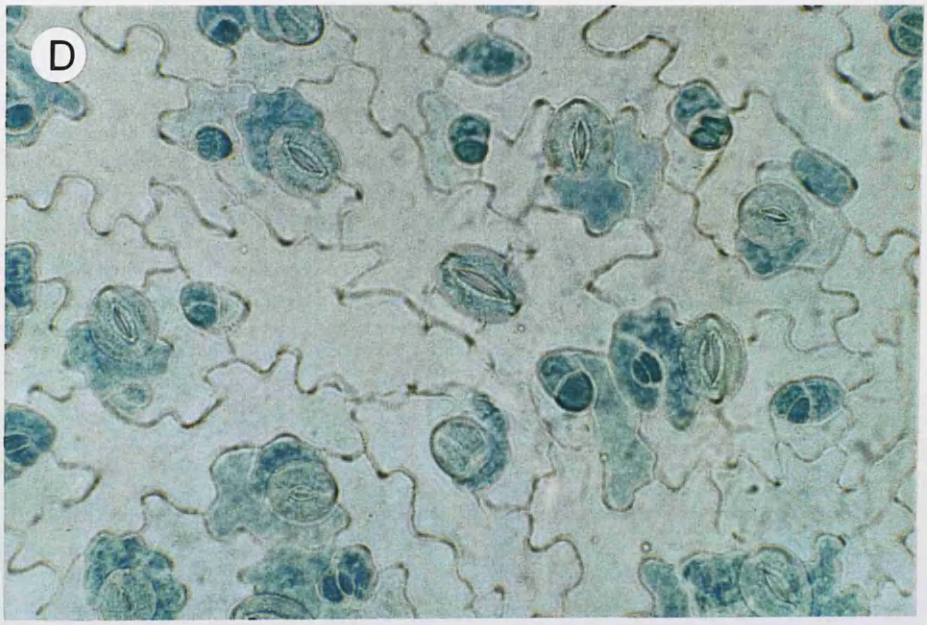
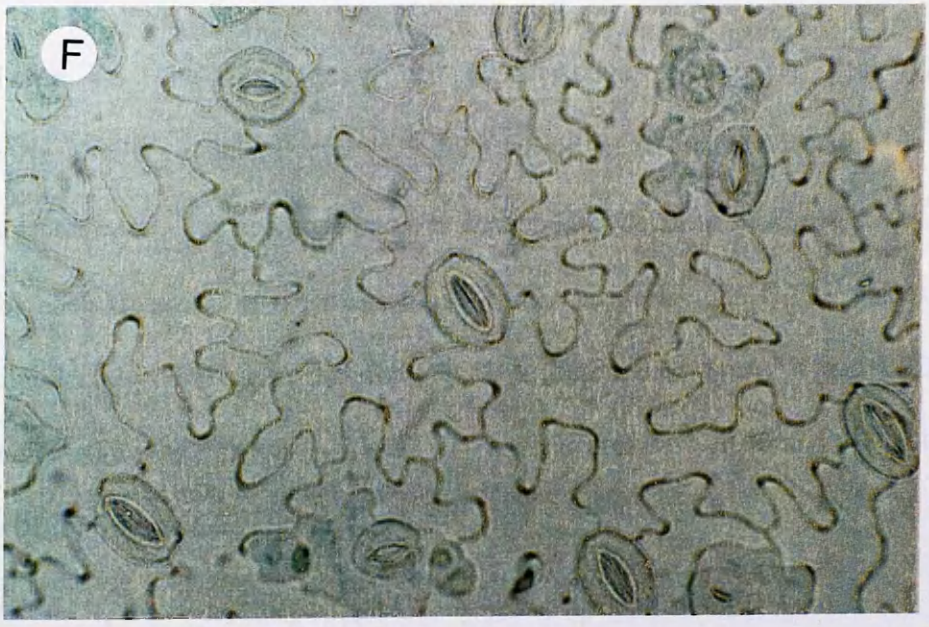


Figure 4.7 continued.



Treatment	Mean guard cell GASP	SE	Significantly different to (+)-ABA?
(±)-ABA	2.73	0.16	N.S.
(+)-ABA	3.40	0.20	-
(–)-ABA	2.40	0.20	*
PBI-63	2.50	0.14	*
PBI-51	0.88	0.15	***
0.1% (v/v) ethanol	0.88	0.40	***
Untreated	0.50	0.10	***

Table 4.3 The mean guard cell GUS Activity Score per Peel (GASP) and corresponding standard error (SE) for each of the treatments described in Figure 4.7 are presented. The scoring system is described in detail in section 4.2.3(e). The score for each treatment is the mean of 30 epidermal peels. The GASP values which are significantly different to that for (+)-ABA (determined by AOV and LSD tests) are indicated by the symbols " * " and " *** "; * indicates $p = < 0.05$ and *** indicates $p = < 0.01$; N.S. = Not significant.

the biological activity of the ABA analogues, PBI-63 and PBI-51 [(+)- and (–)-dihydroacetylenic abscisyl alcohol respectively] (see Figure 1.4). It was believed that these ABA analogues could not be investigated at concentrations higher than 10^{-5} M for the reasons described in Section 3.4. In addition, the biological activity of 10^{-5} M (+)- and (–)-ABA and (±)-*trans, trans*-ABA (see Figure 1.4) was investigated. (±)-*trans, trans*-ABA will be considered later in this Section because the experimental methodology for investigations with this molecule was different from that for other molecules [see Section 4.2.3(a)].

As detailed in Section 4.3.1, the effects of (±)-ABA and the ABA analogues described in Figure 1.4 on *CDeT6-16* driven GUS activity in guard cells of *A. thaliana* were quantified using a scoring system. The average guard cell GASP was calculated for each treatment and these were compared statistically in order to determine treatment effects. In addition to these scores, photographs of abaxial epidermis from the leaves of treated transgenic *A. thaliana* are presented (see Figures 4.7 and 4.8). These were selected from the photographic evidence collected for each treatment (see Section 4.2.3.3) and aim to be as representative of the mean guard cell GASP for each treatment as possible.

10^{-5} M (+)- and (±)-ABA induced the highest degree of *CDeT6-19* driven GUS activity in guard cells of abaxial epidermis from leaves of transgenic *A. thaliana* (Figure 4.7.A and B, and Table 4.3) and their respective mean guard cell GASPs were not significantly different from each other. Treatment with 10^{-5} M (–)-ABA or PBI-63 resulted in mean guard cell GASP which was significantly lower than that that for (+)-ABA but not (±)-ABA (Table 4.3, Figure 4.7.C and D). Treatment with 10^{-5} M PBI-51 resulted in a mean guard cell GASP which was not significantly different from those

resulting from an 0.1% (v/v) ethanol treatment or no treatment (Table 4.3, Figure 4.7.E and F).

The biological activity of (±)-*trans, trans*-ABA (*trans, trans*-ABA) was investigated in the dark due to the light-induced isomerization of this molecule to the biologically active molecule, (±)-*cis, trans*-ABA (*cis, trans*-ABA). Treatment with 10^{-5} M *trans, trans*-ABA resulted in a mean guard cell GASP which was not significantly different from that obtained with 0.1% (v/v) ethanol but was significantly lower than that for treatment of 10^{-5} M *cis, trans*-ABA (see Figure 4.8 and Table 4.4).

In all cases GUS activity was also apparent in epidermal cells other than mature guard cells. Thus, immature guard cells and other epidermal cells also contained the blue precipitate indicative of *CDeT6-19* driven GUS activity as can be seen in Figures 4.7 and 4.8. A detailed study of these phenomena was not carried out. No GUS activity was seen in leaves from WT plants treated with (±)-ABA (data not shown).

4.3.3 *CDeT6-19* driven GUS activity in leaves of transgenic *A. thaliana*

The effects of 10^{-5} M (±)-ABA and the ABA analogues described in the preceding Section on *CDeT6-19* driven GUS activity in leaves of transgenic *A. thaliana* were investigated. This was primarily to determine whether the histochemical localization of GUS activity in guard cells in abaxial epidermis attached to the plant, (i.e. *in planta*) resulted in a similar expression pattern to the histochemical localization of GUS activity in guard cells in detached abaxial epidermis from treated plants. A consequence of such studies was that the whole leaf response to ABA and the ABA analogues became apparent.

10^{-5} M (±)-ABA, (+)- and (−)-ABA resulted in an enhanced level of *CDeT6-19* driven GUS activity in guard cells in attached abaxial epidermis and whole leaf tissue

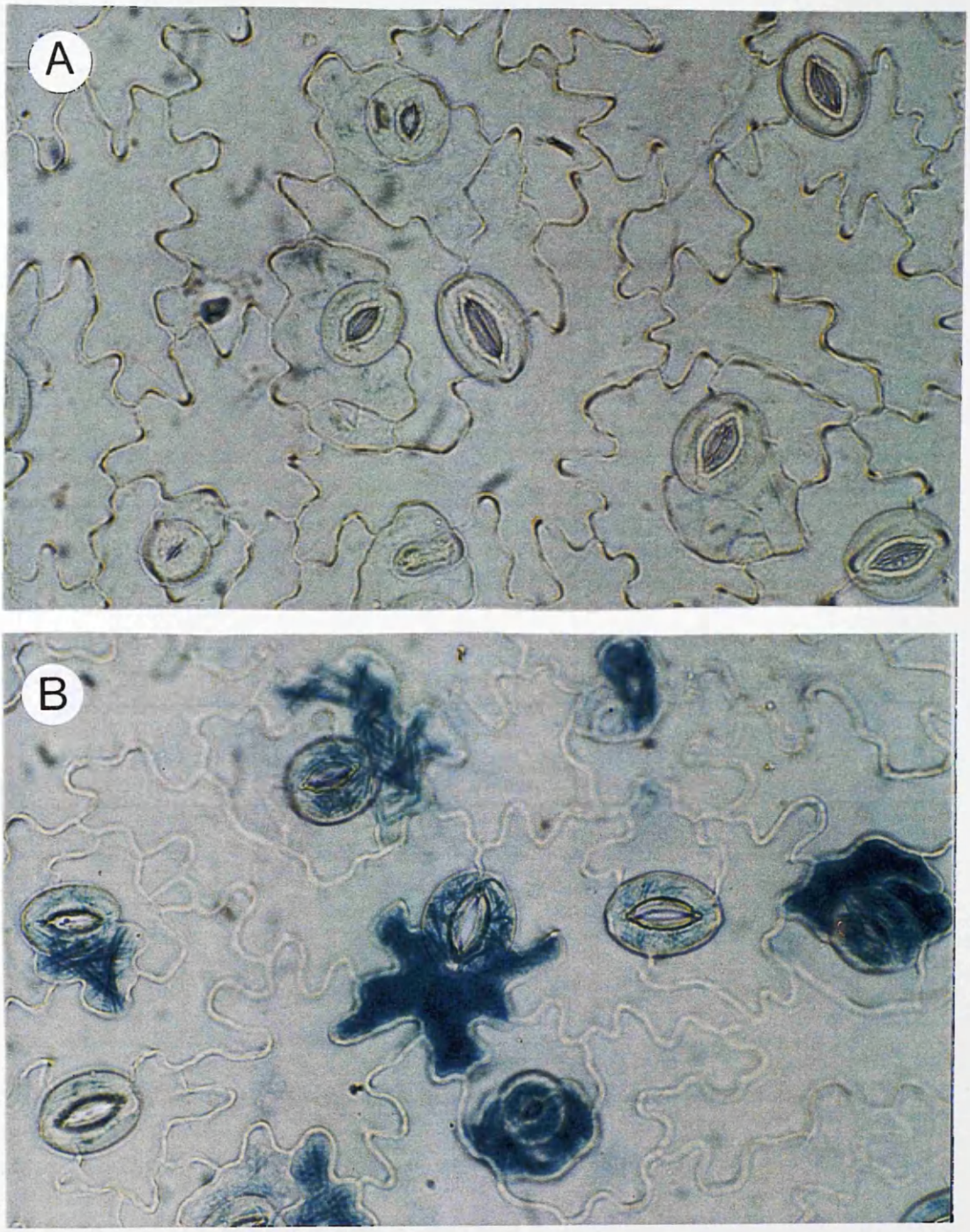


Figure 4.8 Histochemical localization of GUS activity driven by the *CDeT6-19* gene promoter in epidermis from leaves of transgenic *Arabidopsis thaliana* treated in the dark with MES/KOH, 22±1°C containing 10^{-5} M (±)-*trans, trans*-ABA or 0.1% (v/v) ethanol (**A**), or 10^{-5} M (±)-*cis, trans*-ABA (**B**). (n = 12 plants per treatment). (Magnification x 400). See Table 4.4 for the mean guard cell GUS Activity Score per Peel (GASP) for each treatment.

Treatment	Mean guard cell GASP	SE	Significantly different to (±)-*cis, trans*-ABA?
(±)-*trans, trans*-ABA	0.16	0.11	***
(±)-*cis, trans*-ABA	1.30	0.20	-
0.1% (v/v) ethanol	0.12	0.05	***

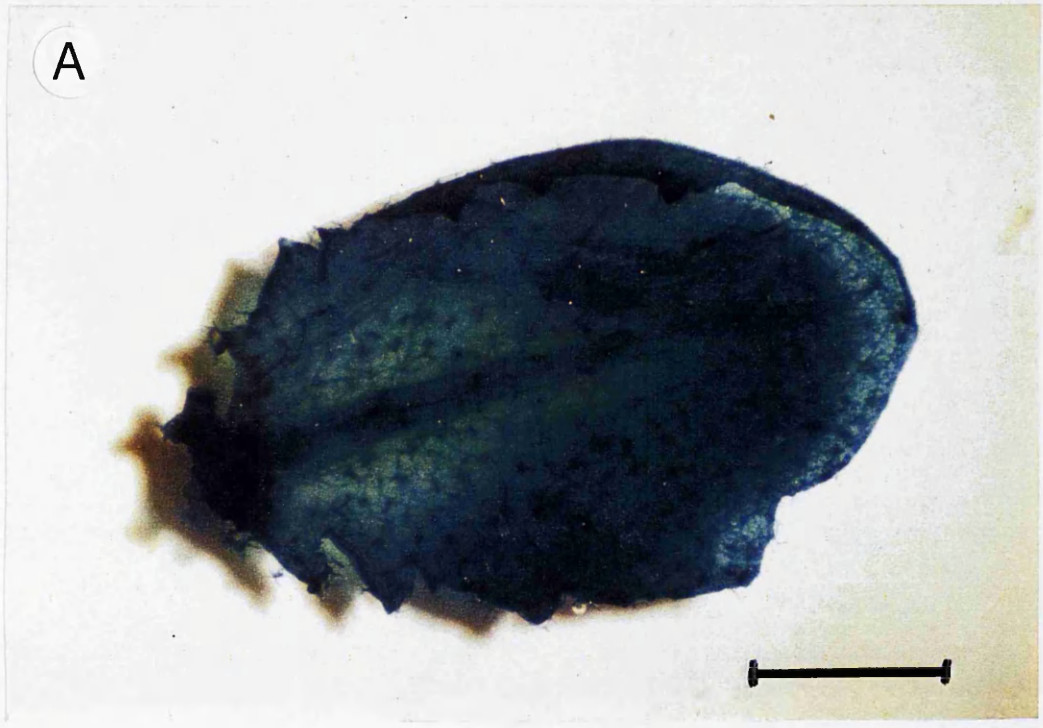
Table 4.4 The mean guard cell GUS Activity Score per Peel (GASP) and corresponding standard error (SE) for each of the treatments described in Figure 4.8 are presented. The scoring system is described in detail in section 4.2.3(e). The score for each treatment is the mean of 30 epidermal peels. The GASP values which are significantly different to that for (±)-*cis, trans*-ABA (determined by AOV and LSD tests) ($p = < 0.01$) are indicated by the symbol " *** ".

(mesophyll and vascular cells) as determined by a histochemical GUS assay (Figures 4.9.A and B). These results correlate with those in which only detached abaxial epidermis from the leaves of treated plants was subjected to a histochemical GUS assay (see Section 4.3.3). Detailed studies were not carried out on the whole leaf tissue, so it is not possible to determine the relative order of biological activity of these molecules in this tissue. The majority of guard cells in the attached abaxial epidermis of leaves treated with 10^{-5} M PBI-51, 0.1% (v/v) ethanol or untreated displayed no *CDeT6-19* driven GUS activity (Figure 4.9.C and D). However, a small number of guard cells at the leaf margins did display GUS activity under these treatments (Figure 4.9.E). Again, these results correlate with those described in Section 4.3.3 such that there was a low level of *CDeT6-19* driven GUS activity in epidermis from PBI-51, 0.1% (v/v) ethanol or untreated leaves (see Table 4.3). Figure 4.9 shows the histochemical localization of *CDeT6-19* driven GUS activity in guard cells in the abaxial epidermis of leaf 4 of *A. thaliana*. All leaves investigated (4, 5 and 6) showed a similar level of *CDeT6-19* driven GUS activity in guard cells. No GUS activity was seen in guard cells (or any other leaf tissue) in leaves from WT plants treated with (±)-ABA (data not shown). There was a higher level of *CDeT6-19* driven GUS activity in whole leaf tissue (excluding guard cells), both untreated and treated with PBI-51, 0.1% (v/v) ethanol than exclusively in the guard cells (Figure 4.9.C and E).

4.3.4 The viability of guard cells in detached abaxial epidermis of leaves of transgenic *A. thaliana*

The viability of cells in detached abaxial epidermis of transgenic *A. thaliana* leaves was assessed using fluoroscein diacetate (FDA) [see Section 4.2.5(e)]. The

Figure 4.9 Histochemical localization of *CDeT6-19* driven GUS activity in leaves of transgenic *Arabidopsis thaliana* (chlorophyll removed with 80% (v/v) ethanol). A representative leaf (number 4) from *A. thaliana* treated with MES/KOH, 22±1°C containing 10^{-5} M (±)-ABA, (+)-ABA, (–)-ABA or PBI-63 is shown (bar = 50 mm) (**A**). The abaxial surface near the centre of the leaf described in (A) (bar = 10 μm) is shown (**B**). A representative leaf (number 4) from *A. thaliana* plants treated with MES/KOH containing 10^{-5} M PBI-51, 0.1% (v/v) ethanol or untreated is shown (bar = 50 mm) (**C**). The abaxial surface near the centre of the leaf described in (C) is shown (bar = 10 μm) (**D**). The abaxial surface near the edge of the leaf described in (C) is shown (bar = 10 μm) (**E**). (n = 3 plants per treatment). WT *A. thaliana* plants treated with (±)-ABA displayed no GUS activity (data not shown).


A

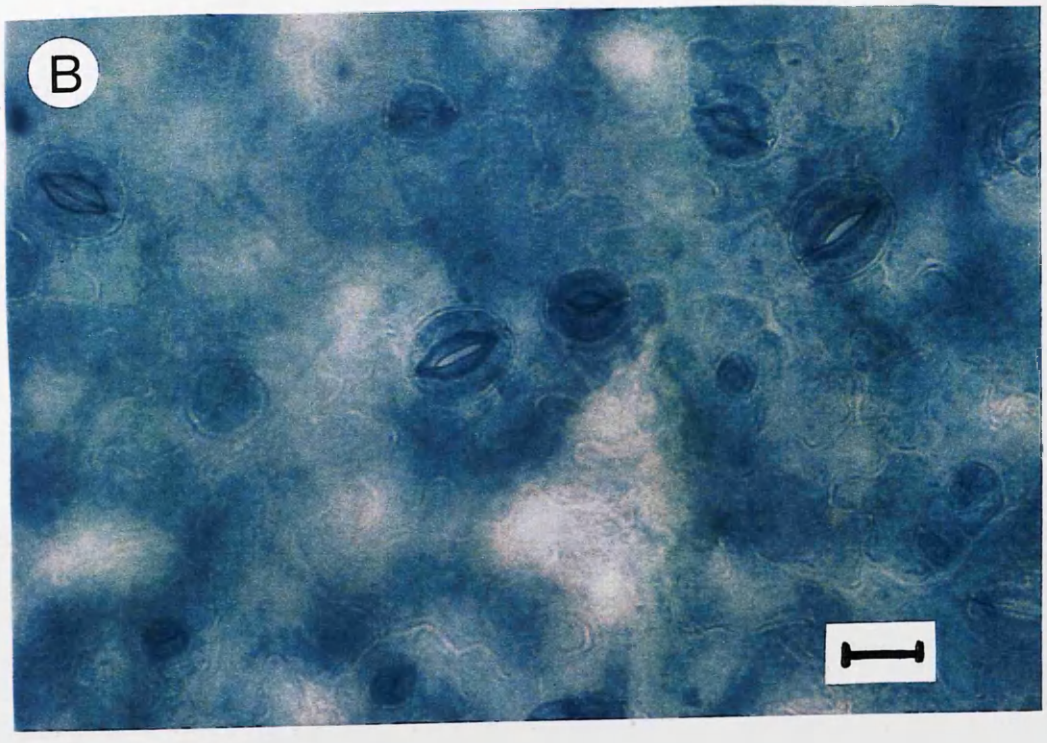
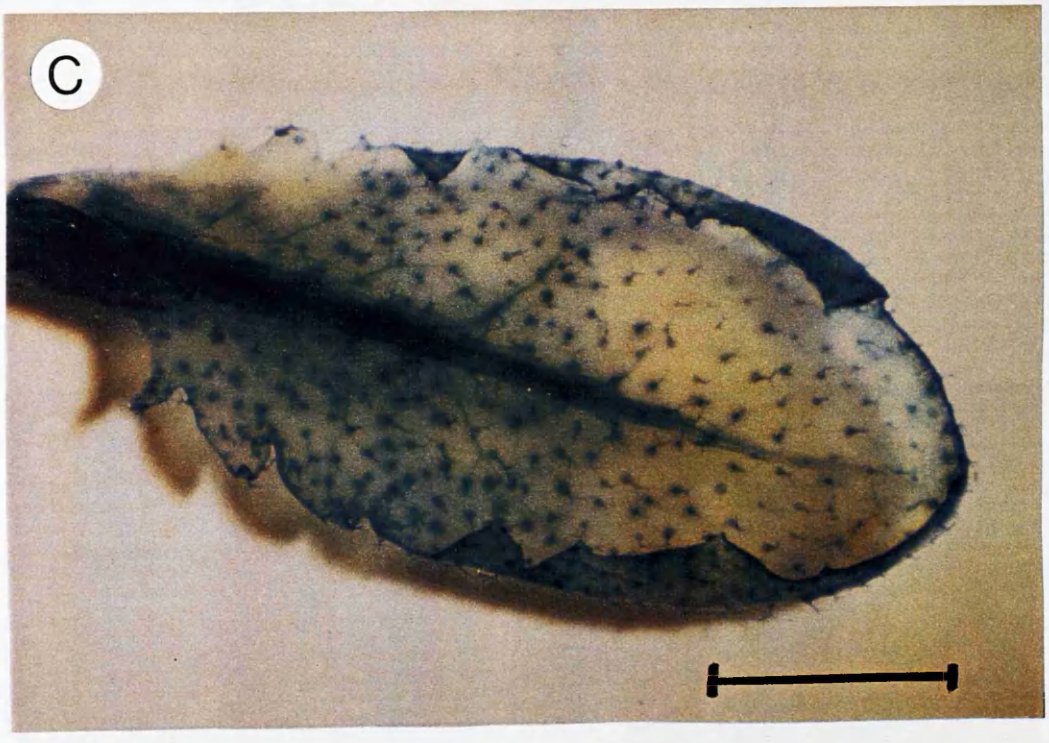

B

Figure 4.9 continued.



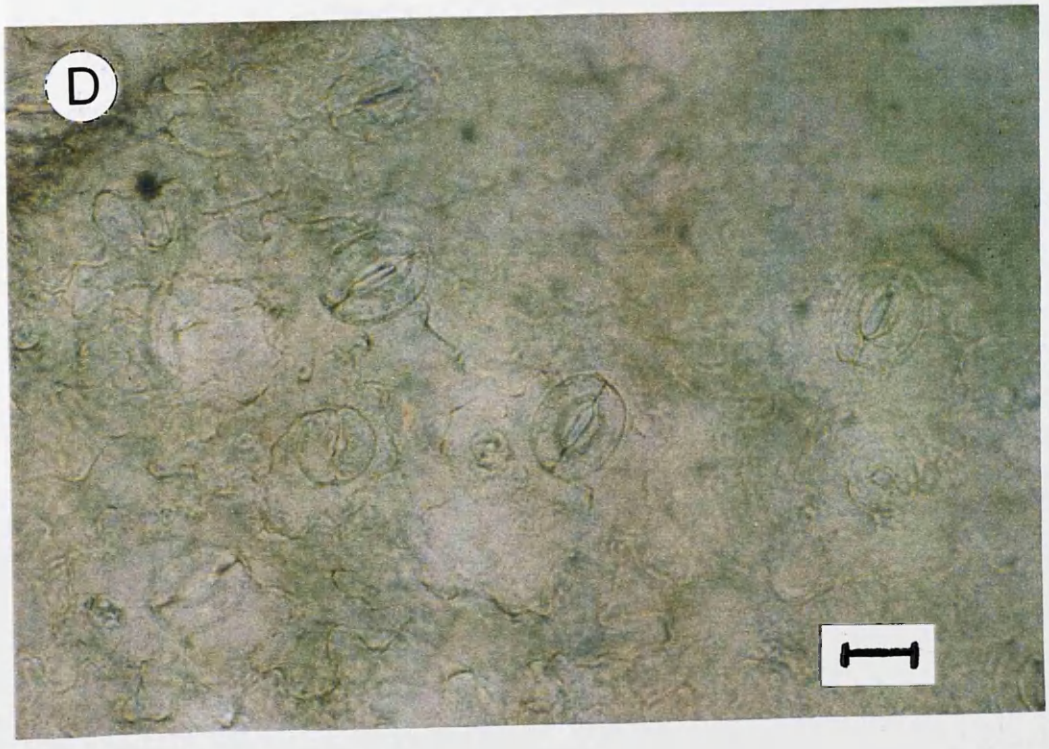
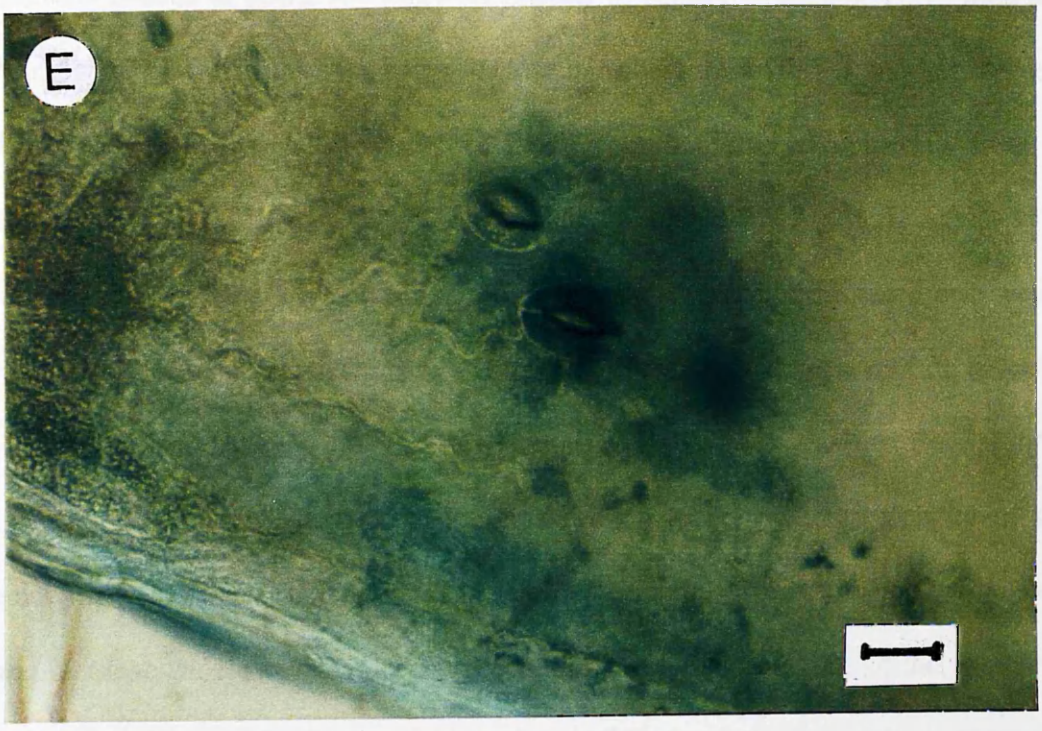


Figure 4.9 continued.



percentage of guard cells in abaxial epidermis detached from *A. thaliana* leaves which were viable as shown by FDA staining was 96% (SE = 1.4%; n = 9 epidermal strips) (Figure 4.10.C); only a very low percentage of the other epidermal cells [4% (SE = 2%; n = 9 epidermal strips)] were viable as shown by FDA staining. Detached abaxial epidermis from WT *A. thaliana* showed the same cell viability patterns (data not shown). These findings mirror those reported by Roelfsema and Prins (1995). The viability of cells in detached abaxial epidermis from the leaves of the plant species used in investigations of guard cell turgor in Chapter 2, namely *C. communis, V. faba and N. tabacum* was also assessed using FDA staining (Figure 4.10. I, J and M). As seen for *A. thaliana*, the vast majority of guard cells in detached epidermis from *C. communis, V. faba and N. tabacum* were viable (*C. communis* – 100%; *V. faba* – 96%; *N. tabacum* – 95%). The percentage of epidermal cells other than guard cells which were viable cells in detached epidermis from *C. communis, V. faba and N. tabacum* was higher than that seen in detached epidermis of leaves of *A. thaliana* (Figure 4.10. I, J and M). A detailed investigation was not carried out on the viability of the epidermal cells other than guard cells in detached epidermis from *C. communis, V. faba and N. tabacum*. However, it has been previously reported that 36% of the epidermal cells (other than guard cells) are viable in detached epidermis from *V. faba*, whilst in *C. communis* 54% of the epidermal cells (other than guard cells) and 99.6% of the subsidiary cells were viable (Weyers & Travis, 1981).

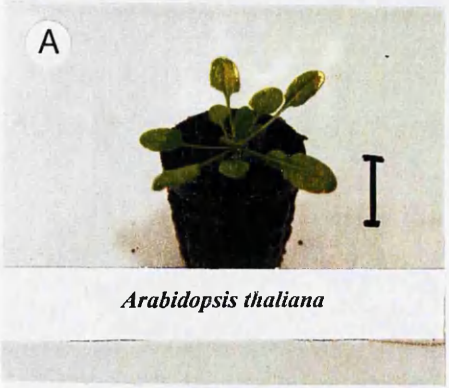
Figure 4.10 The viability of epidermal cells in detached, abaxial epidermis from leaves of *Arabidopsis thaliana*, *Commelina communis*, *Nicotiana tabacum*, and *Vicia faba* determined using fluoroscein diacetate (FDA) staining [see Section 4.2.4(e)].

A. thaliana: (**A**) plant (bar = 2 cm); (**B**) bright-field image of detached, abaxial epidermis from an experimental leaf (bar = 10 μm); (**C**) fluorescence image of that shown in (B).

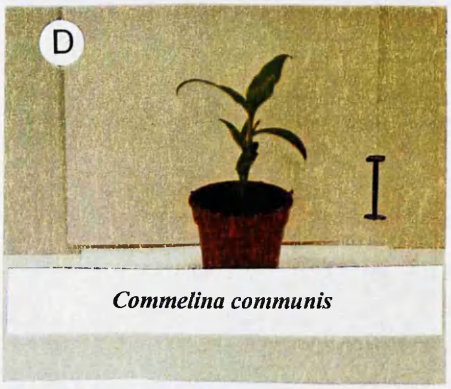
C. communis: (**D**) plant (bar = 2 cm); (**E**) bright-field image of detached, abaxial epidermis from an experimental leaf (bar = 10 μm); (**F**) fluorescence image of that shown in (E).

N. tabacum: (**G**) plant (bar = 10 cm); (**H**) bright-field image of detached, abaxial epidermis from an experimental leaf (bar = 10 μm); (**I**) and (**J**) fluorescence images of that shown in (H) in two focal planes - (**I**) focuses on the FDA staining of guard cells and (**J**) shows the FDA staining of the surrounding epidermal cells.

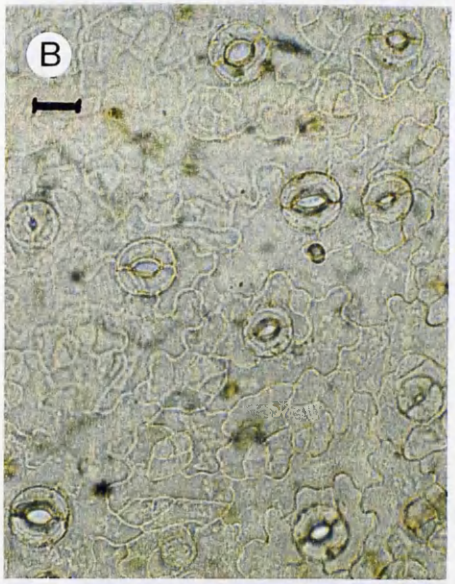
Vicia faba: (**K**) plant (bar = 10 cm); (**L**) bright-field image of detached, abaxial epidermis from an experimental leaf (bar = 10 μm); (**M**) fluorescence image of that shown in (L).

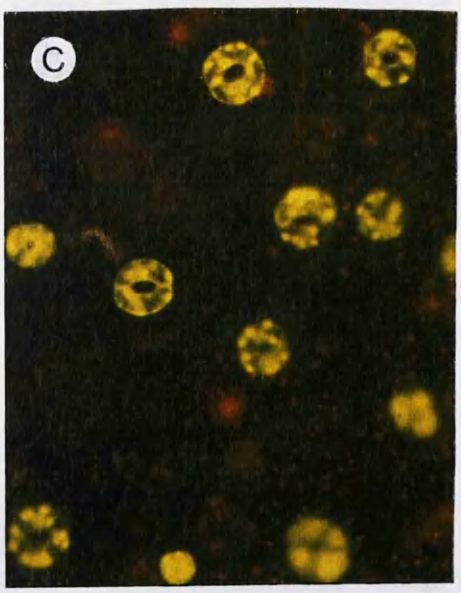


Arabidopsis thaliana



Commelina communis





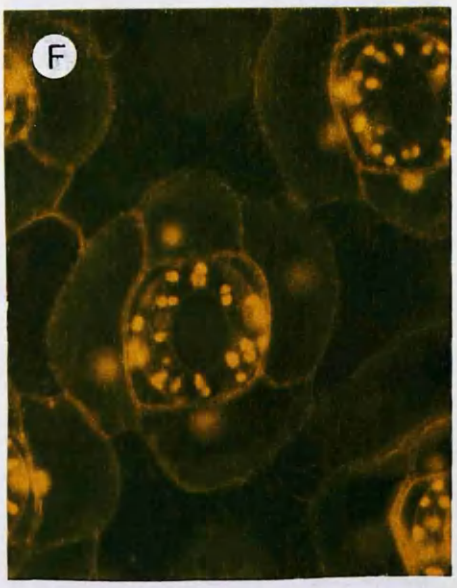
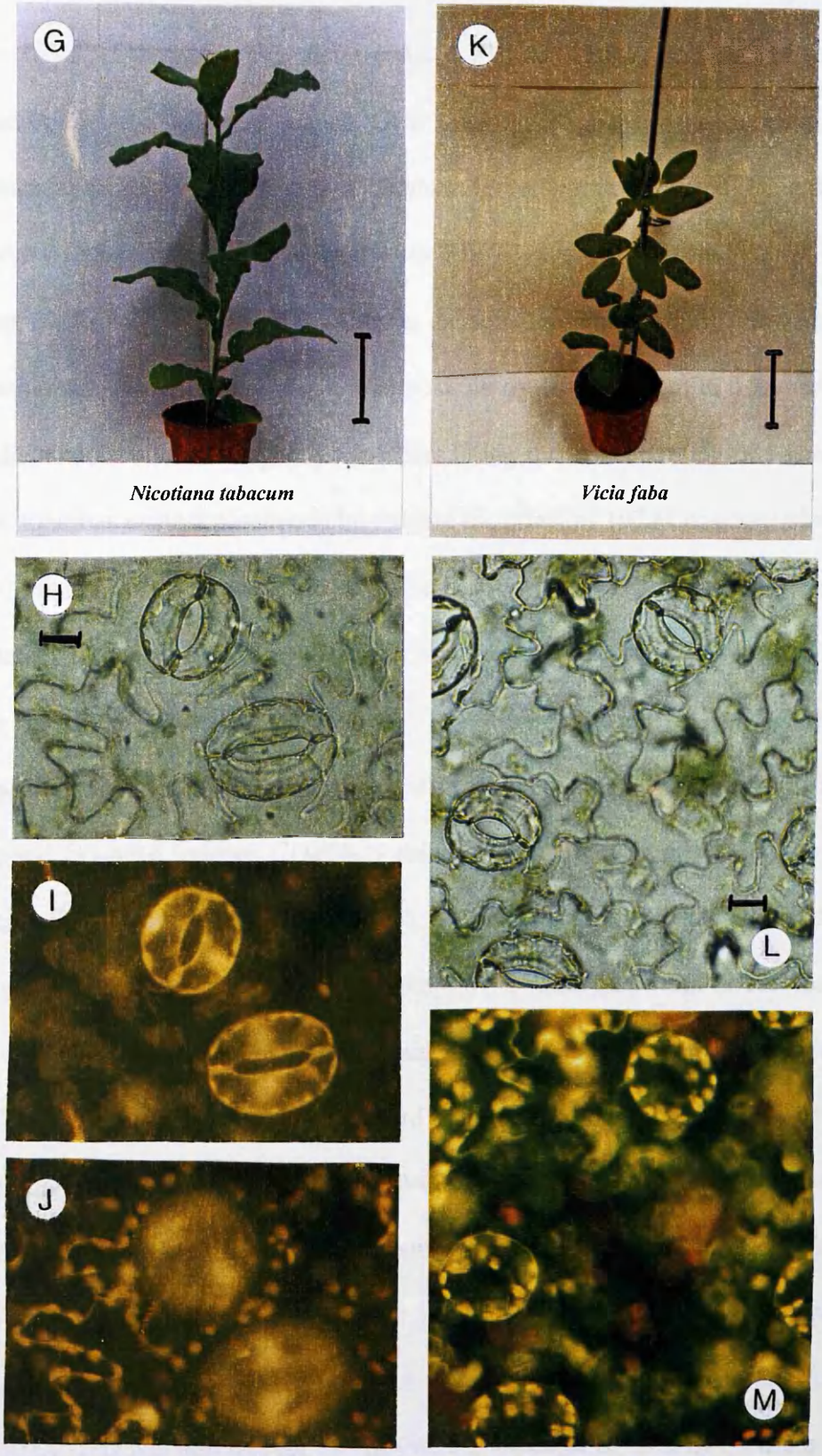


Figure 4.10 continued.



4.3.5 The effect of ABA and ABA analogues on *CDeT6-19* driven GUS activity in guard cells in detached, treated abaxial epidermis of transgenic leaves of *A. thaliana*

The effect of (±)-ABA, (+)- and (–)-ABA, PBI-63, PBI-51 and 10^{-4} M (±)-*trans, trans*-ABA on *CDeT6-19* driven GUS activity in guard cells in treated, detached, abaxial epidermis of transgenic *A. thaliana* leaves was investigated. Until this point, it was believed that the ABA analogues PBI-63 and PBI-51 could only be dissolved in 100% (v/v) ethanol (S.R. Abrams, personal communication). However, upon re-examination it was found that it was possible to dissolve them in 10% (v/v) ethanol if the mixture was stirred gently for 5 min. Using this concentration of ethanol in the stock solutions made it possible to investigate the effect of 10^{-4} M concentrations (0.1% (v/v) ethanol) of these molecules on *CDeT6-19* driven GUS activity in guard cells in treated, detached, abaxial epidermis of transgenic *A. thaliana* leaves. It was found that the GASP score for a 0.01% or a 0.1% (v/v) ethanol (in MES/KOH) treatment was not significantly different from a MES/KOH treatment (data not shown). 10^{-5} M (±)-and (+)-ABA induced *CDeT6-19* driven GUS activity in guard cells to a similar degree (see Figure 4.11 and Table 4.5). 10^{-5} M (–)-ABA also induced GUS activity in guard cells but resulted in a significantly lower mean guard cell GASP than 10^{-5} M (±)- or (+)-ABA (see Figure 4.11 and Table 4.5). 10^{-5} M PBI-63 and -51 had no effect on *CDeT6-19* driven GUS activity in guard cells (see Figure 4.11 and Table 4.5). 10^{-4} M (+)-ABA resulted in a mean guard cell GASP which was significantly higher than that for 10^{-5} M (+)-ABA or any other treatment. The mean guard cell GASP for 10^{-4} M (±)-ABA was no different from that for 10^{-5} M (±)-ABA. By way of contrast, the mean guard cell GASP for 10^{-4} M (–)-ABA was significantly higher than that for 10^{-5} M (–)-ABA. This was also true for 10^{-4} M PBI-63, although 10^{-4} M PBI-63 had a

Figure 4.11 Histochemical localization of GUS activity driven by the *CDeT6-19* gene promoter in abaxial epidermis of leaves of transgenic *Arabidopsis thaliana* treated with MES/KOH, 22±1°C containing 10^{-5} M or 10^{-4} M (+)-ABA, (**A**) and (**B**) respectively; 10^{-5} M or 10^{-4} M (–)-ABA, (**C**) and (**D**) respectively; 10^{-5} M or 10^{-4} M PBI-63, (**E**) and (**F**) respectively; 10^{-5} M or 10^{-4} M PBI-51, (**G**) and (**H**) respectively; 0.01 % (v/v) ethanol (**I**) or 0.1 % (v/v) ethanol (**J**). (n = 20 peels per treatment). (Magnification x 200). Abaxial epidermis from leaves of WT *A. thaliana* treated with (±)-ABA showed no GUS activity (data not shown). See Table 4.5 for the mean guard cell GUS Activity Score per Peel (GASP) for each treatment.

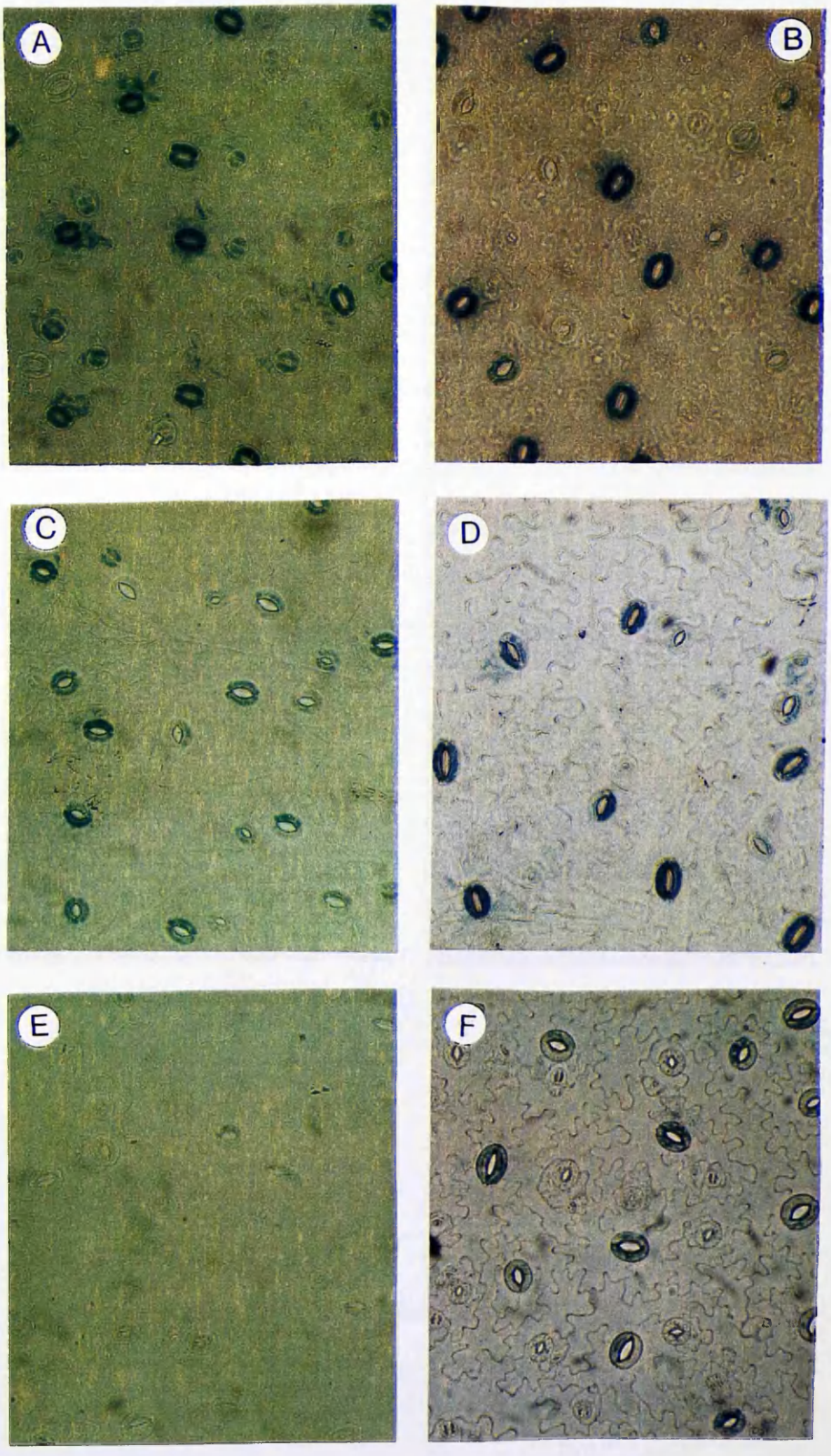

A
B
C
D
E
F

Figure 4.11 continued.

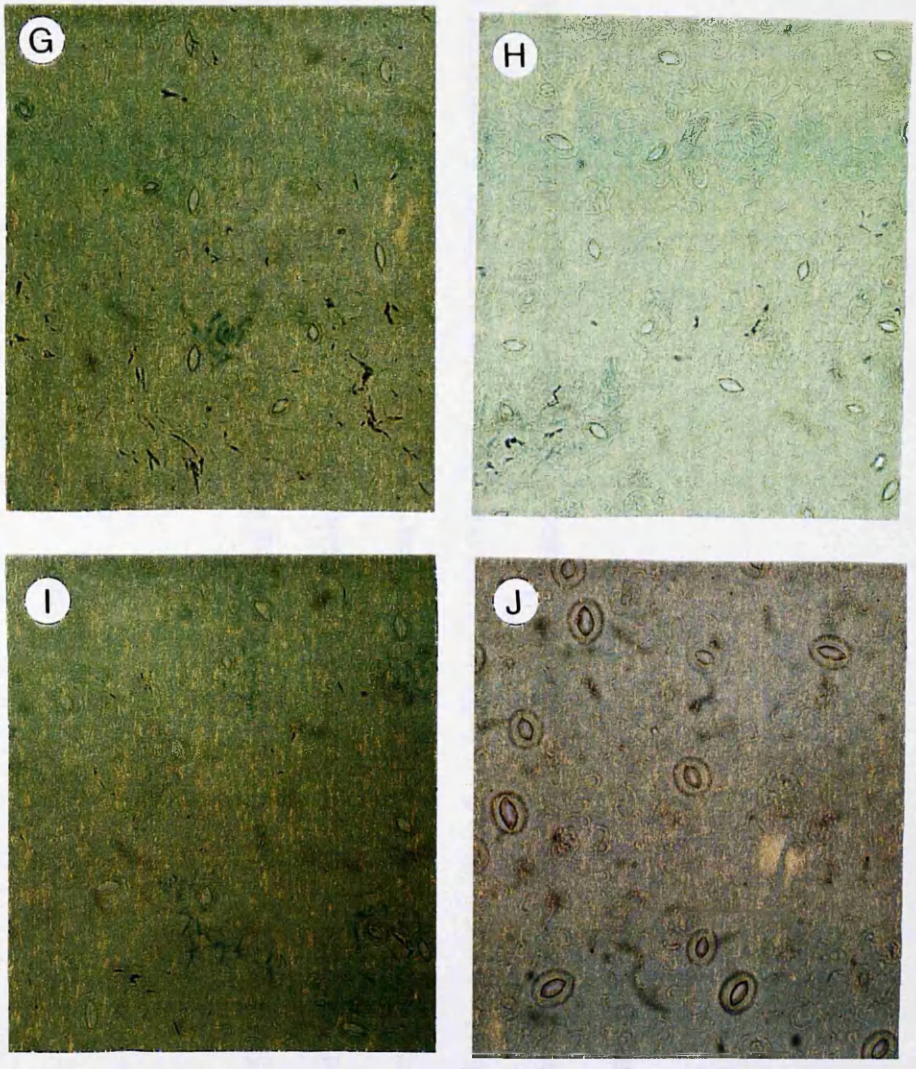


Table 4.5 The mean guard cell GUS Activity Score per Peel (GASP) and corresponding standard error (SE) for each of the treatments described in Figure 4.11 and also (±)-ABA are presented. The scoring system is described in detail in section 4.2.3(e). The score for each treatment is the mean of 30 epidermal peels. The GASP values which are significantly different to that for (+)-ABA (determined by AOV and LSD tests) are indicated by the symbols " * " and " *** "; * indicates $p = < 0.05$ and *** indicates $p = < 0.01$; N.S. = Not significant. A significant within treatment difference between the 10^{-5} and 10^{-4} M concentration is indicated by the symbols " * " and " *** " after the mean guard cell GASP score for each treatment; * indicates $p = < 0.05$ and *** indicates $p = < 0.01$.

	10-5 M			*10-4 M*		
Treatment	**Mean guard cell GASP**	**SE**	**Significantly different to 10-5 M (+)-ABA?**	**Mean guard cell GASP**	**SE**	**Significantly different to 10-4 M (+)-ABA?**
(±)-ABA	2.0	0.19	N.S	2.10	0.14	*
(+)-ABA	2.10*	0.20	-	2.53*	0.14	-
(–)-ABA	1.28***	0.11	***	2.11***	0.16	*
PBI-63	0.36***	0.08	***	1.36***	0.10	***
PBI-51	0.41	0.11	***	0.30	0.10	***
Ethanol control	[0.01% (v/v)] 0.32	0.08	***	[0.1% (v/v)] 0.21	0.08	***

significantly lower mean guard cell GASP than 10^{-4} M (–)-ABA. 10^{-4} M PBI-51 had no effect on guard cell *CDeT6-19* driven GUS activity in guard cells (see Figure 4.11 and Table 4.5).

A separate experiment involving the use of a continuous perfusion system [see Section 4.2.5(b) and Figure 4.5] was carried out to investigate the effect of (±)-*trans, trans*-ABA on *CDeT6-19* driven GUS activity in the guard cells. In this experiment, the mean guard cell GASP for 10^{-4} M (±)-*trans, trans*-ABA was not significantly different from that for 0.1% (v/v) ethanol (Figure 4.12.A and Table 4.6), whereas, the mean guard cell GASP for 10^{-4} M (±)-*cis, trans*-ABA was significantly higher than for any other treatment (Figure 4.12.B and Table 4.6).

4.3.6 The effect of ABA on guard cell turgor in leaves of transgenic *A. thaliana*

Concentrations of (±)-ABA of 10^{-6} M and above significantly inhibited stomatal opening in detached, abaxial epidermis of leaves of transgenic *A. thaliana* (Figure 4.13.A). (±)-ABA also reduced guard cell turgor *in planta* as determined by measuring the stomatal conductance of *A. thaliana* leaves treated with 10^{-5} M (±)-ABA (Figure 4.13.B).

4.3.7 The effect of ABA analogues on guard cell turgor in detached, treated abaxial epidermis of leaves of transgenic *A. thaliana*

The effect of (±)-ABA, (+)- and (–)-ABA, PBI-63, PBI-51 and (±)-*trans, trans*-ABA on stomatal opening in detached, abaxial epidermis from leaves of transgenic *A. thaliana* was investigated. By dissolving (±)-ABA, (+)- and (–)-ABA, PBI-63, PBI-51 and (±)-*trans, trans*-ABA in 10% (v/v) ethanol (see Section 4.3.5) it was possible to investigate the effect of 10^{-4} M [0.1% (v/v) ethanol] concentrations of

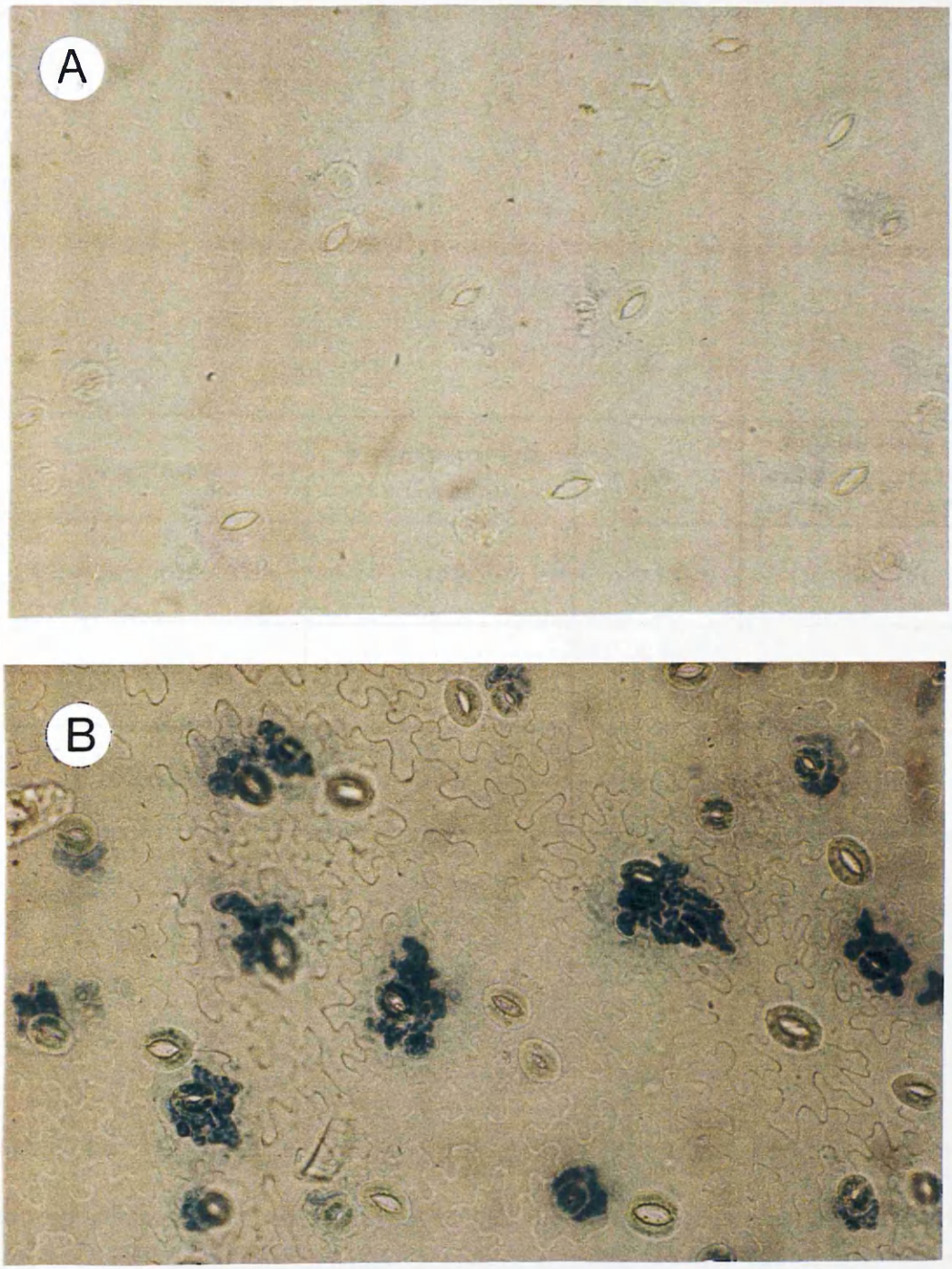


Figure 4.12 Histochemical localisation of GUS activity driven by the *CDeT6-19* gene promoter in abaxial epidermis of leaves of transgenic *Arabidopsis thaliana* treated with MES/KOH, 22±1°C containing 10^{-4} M (±)-*trans, trans*-ABA or 0.1% (v/v) ethanol (**A**); or 10^{-4} M (±)-*cis, trans*-ABA (**B**). (n = 20 peels per treatment). (Magnification x 200). See Table 4.6 for the mean guard cell GUS Activity Score per Peel (GASP) for each treatment.

Treatment	Mean guard cell GASP	SE	Significantly different to (±)-*cis, trans*-ABA?
(±)-*trans, trans*-ABA	0.58	0.13	*
(±)-*cis, trans*-ABA	1.54	0.45	-
0.1% ethanol	0.37	0.15	*

Table 4.6 The mean guard cell GUS Activity Score per Peel (GASP) and corresponding standard error (SE) for each of the treatments described in Figure 4.12 are presented. The scoring system is described in detail in section 4.2.3(e). The score for each treatment is the mean of 20 epidermal peels. The GASP values which are significantly different to that for (±)-*cis, trans*-ABA (determined by AOV and LSD tests) ($p = < 0.05$) are indicated by the symbol " * ".

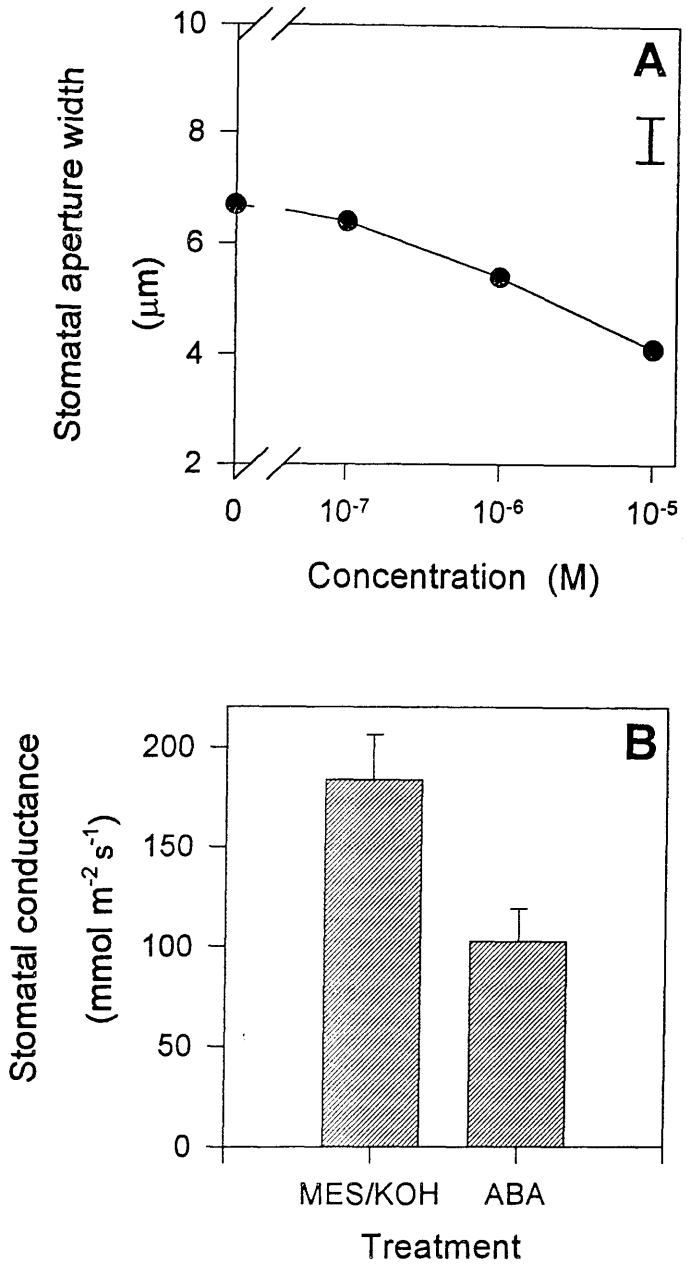
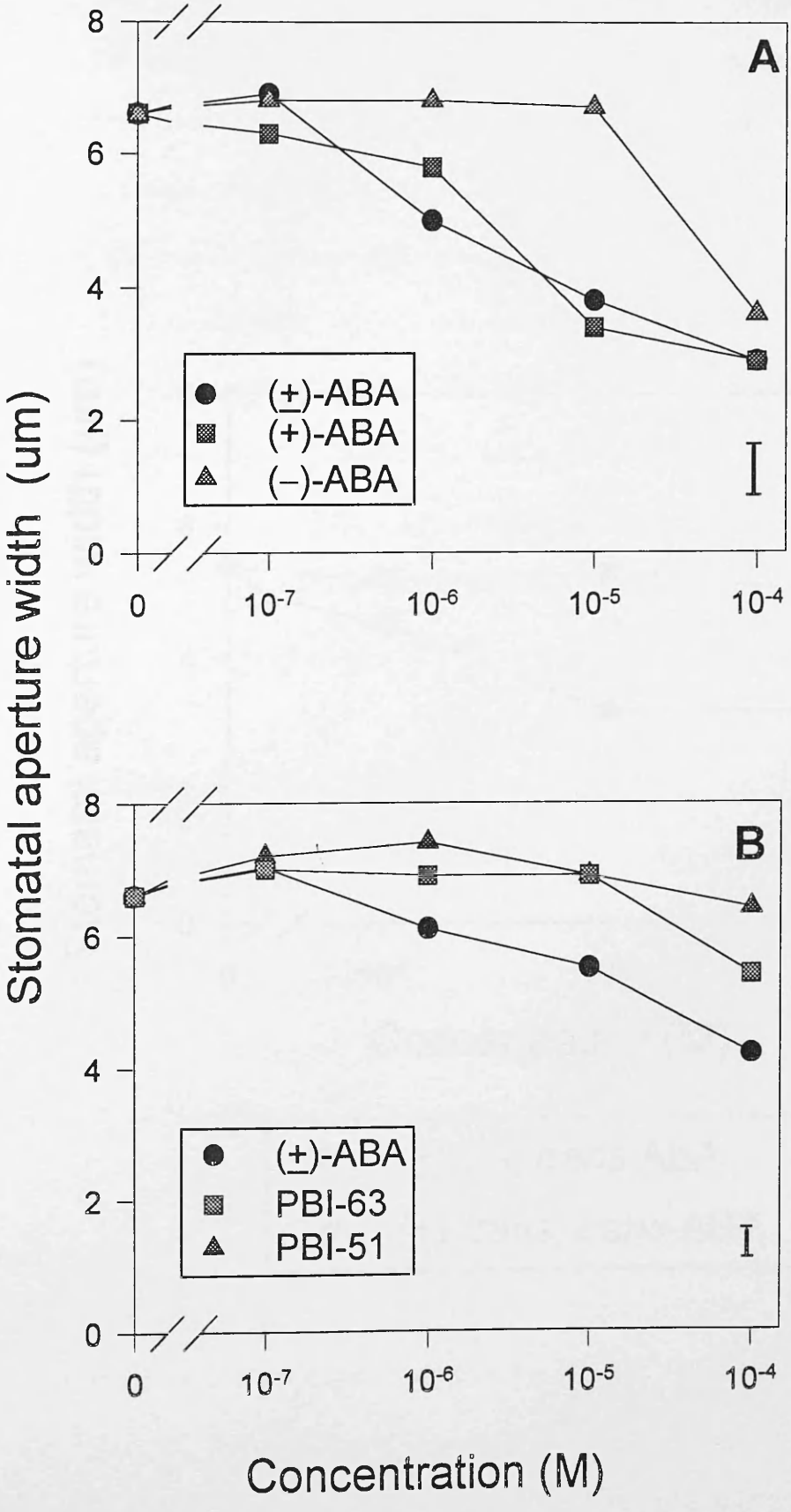


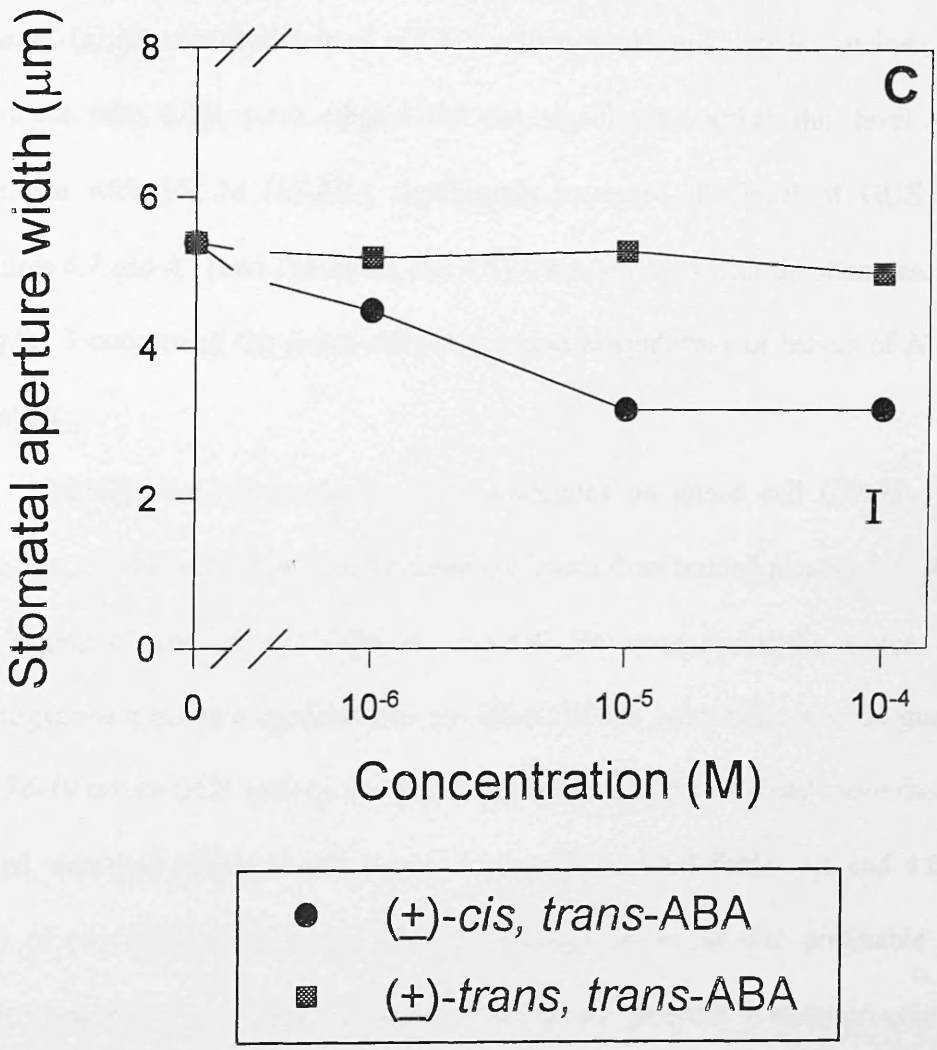
Figure 4.13 The effect of (±)-ABA on stomata of leaves of transgenic *Arabidopsis thaliana*. The effect of (±)-ABA on stomatal opening in detached epidermis (**A**). Each point represents the average of 80 aperture measurements. The vertical bar represents the LSD. The effect of MES/KOH, 22±1°C in the presence and absence of 10^{-5} M (±)-ABA on leaf stomatal conductance at 6 h post treatment application (**B**). Each bar represents an average ± standard error of 14 plants ($t = -2.95$; $p = < 0.01$).

these molecules on guard cell turgor in detached abaxial epidermis from leaves of *A. thaliana*. It was shown that 0.1% (v/v) ethanol has no effect on stomatal aperture in this epidermis (see Appendix D).

(+)-ABA inhibited stomatal opening to the same extent as (±)-ABA at all concentrations investigated (Figure 4.14.A), whereas (–)-ABA had no significant effect on stomatal opening until applied at a concentration of 10^{-4} M (Figure 4.14.A). PBI-63 was inactive until applied at a concentration of 10^{-4} M (Figure 4.14.B). At this concentration it slightly, but significantly, inhibited stomatal opening (Figure 4.14.B). PBI-51 and (±)-*trans, trans*-ABA had no significant effect on stomatal opening (Figure 4.14.B and C).

Figure 4.14 The effect of ABA analogues on stomatal aperture width in detached epidermis from leaves of transgenic *Arabidopsis thaliana.* The effect of (+)- and (−)-ABA (**A**), PBI-63 and PBI-51 (**B**),and (±)-*trans, trans*-ABA (**C**) on stomatal opening. Each point represents an average of 120 aperture measurements; the LSD bars are shown. [Positive and negative controls are (±)-ABA and zero ABA respectively].


A
B
8
6
4
2
0
0
10-7
10-6
10-5
10-4
(±)-ABA
(+)-ABA
(–)-ABA
PBI-63
PBI-51
Stomatal aperture width (um)
Concentration (M)


Stomatal aperture width (µm)
8
6
4
2
0
C
I
0
10-6
10-5
10-4
Concentration (M)
(±)-cis, trans-ABA
(±)-trans, trans-ABA

4.4 Discussion

ABA analogues (see Figure 1.4) were used as tools to investigate ABA-induced changes in guard cell *CDeT6-19* gene promoter driven GUS activity and guard cell turgor in abaxial epidermis from leaves of *Arabidopsis thaliana*. The guard cells in the abaxial epidermis of leaves of transgenic *A. thaliana* provided a suitable system in which to study the effect of ABA analogues on *CDeT6-19* driven GUS activity because: (a) the constitutive level of GUS activity in the guard cells was low; and (b) treatment with 0.1% (v/v) ethanol did not significantly affect this level whereas treatment with 10^{-5} M (±)-ABA significantly increased the level of GUS activity (Figures 4.7 and 4.11and Tables 4.3 and 4.5). This contrasts with the data described in Chapter 3 concerning the guard cells in the abaxial epidermis of leaves of *Nicotiana tabacum*.

Initially, the effects of the ABA analogues on guard cell *CDeT6-19* gene promoter activity were studied in detached epidermis from treated plants of *A. thaliana* (see Figures 4.7 and 4.8, and Tables 4.3 and 4.4). However, during the course of these investigations it became apparent that the effects of the ABA analogues on guard cell *CDeT6-19* driven GUS activity and guard cell turgor could be studied more directly in treated, detached epidermis (see Figures 4.11 and 4.12, and Tables 4.5 and 4.6). The study of two ABA responses in treated, detached epidermis was preferable to the whole plant treatment method because by treating the epidermis in isolation other plant tissues were unable to influence the effects of the ABA analogues on the guard cells (for example, metabolism, compartmentation, see Walton, 1983). Similar studies with ABA analogues have been carried out in other isolated cell types or tissues, for example barley aleurone protoplasts (Hill *et al.*, 1995). Such studies have revealed

differences in the stereospecificity of different ABA-induced responses (Hill *et al.*, 1995). In the present study, the data from treating the whole plant will be considered first, followed by those from treated, detached epidermis.

The ability of (–)-ABA to induce ABA regulated gene expression in the guard cell system correlates with its activity in other systems (Hill *et al.*, 1995; Robertson *et al.*, 1994), including its effect on the induction of transcripts hybridizing with pcC6-19 cDNA [which is encoded by the gene *CDeT6-19* (Piatkowski *et al.*, 1990; see Section 3.1.3)] in callus tissue of *Craterostigma plantagineum* (Chandler *et al.*, 1997). However, the responsiveness of the *CDeT6-19* gene promoter to (–)-ABA in guard cells is in contrast to its relative inactivity in several other systems, including the expression of storage protein genes in *Brassica napus* microspore derived embryos (Wilen *et al.*, 1993) and the *Em* gene in wheat embryos (Walker-Simmons *et al.*, 1992).

To the best of my knowledge, the effect of (±)-*trans, trans*-ABA on gene expression has not been previously investigated. In investigations of other plant responses, such as growth or transpiration rate, as long as the experiments were carried out in the dark, (±)-*trans, trans*-ABA was inactive (see Section 1.7.4(b)]. In the present study, in which experiments were carried out in the dark, (±)-*trans, trans*-ABA had no effect on *CDeT6-19* driven GUS activity in guard cells in the abaxial epidermis of leaves of *A. thaliana*. This suggests that the *trans* orientation at C-2 (see Figure 1.4) is essential for ABA-induced *CDeT6-19* driven GUS activity in guard cells.

The results from the present study concerning the biological activity of PBI-63 (in detached epidermis from a 2-day *in planta* treatment) correlate with those in other systems which show that the lack of the C-2', C-3' ring double bond and the presence of an acetylenic group in the side chain of (+)-ABA generally had little effect on ABA-

induced gene expression (Walker-Simmons *et al.*, 1992; Perras *et al.*, 1994; Dong *et al.*, 1994). In addition, the induction of transcripts hybridizing with pcC6-19 cDNA in callus tissue of *C. plantagineum* was not affected by these two changes to the structure of the ABA molecule (Chandler *et al.*, 1997). The biological activity of PBI-63 relative to (±)-ABA in the guard cell system is slightly higher than that reported in the other two systems in which this ABA analogue has been investigated [Wilen *et al.*, 1993, 1996; also see Section 1.4 7(c)]. The fact that this ABA analogue is active suggests that the C-2', C-3' ring double bond, the *trans* double bond at C-4 of the side chain and the C-1 carboxylic group are not important for ABA-induced *CDeT6-19* driven GUS activity in guard cells of *A. thaliana*. Alternatively, it is possible that the relatively high level of biological activity attributed to PBI-63 may be due to the fact that PBI-63 was metabolized to a more biologically active compound by the plant. However, it has been shown that (±)-dihydroacetylenic ABA was stable in a protoplast incubation medium for 4 days (Dong *et al.*, 1994). In addition, the effect of PBI-63 on guard cell *CDeT6-19* driven GUS activity was later studied in detached epidermis over a relatively short time period (2 h) and so it was not considered a priority to investigate the stability of PBI-63 *in planta* in the present study.

In stark contrast to the relatively high biological activity of PBI-63 was the inability of PBI-51 to induce *CDeT6-19* driven GUS activity in guard cells (Figure 4.7.E and Table 4.3). The inactivity of PBI-51 in the present study correlates with that seen for other genes in other systems (Wilen *et al.*, 1993, 1996). In addition (–)-dihydroacetylenic ABA did not induce transcripts which hybridize with pcC6-19 cDNA in *C. plantagineum* callus tissue (Chandler & Robertson, 1997). However, it has been shown that PBI-51 antagonized ABA-induced gene expression in two other systems (Wilen *et al.*, 1993, 1996; see Section 1.4). It would be particularly interesting to

determine whether PBI-51 antagonizes ABA-induced *CDeT6-19* driven GUS activity in guard cells of *A. thaliana*. If PBI-51 specifically antagonized ABA-induced changes in guard cell gene expression then it would be a useful tool for studying signalling pathways in guard cells. In this respect, the role of ABA in stress-induced gene expression in guard cells could be assessed. In addition, blocking the signalling pathways associated with ABA-induced changes in gene expression may reveal their role in the signalling pathways associated with other ABA-induced changes in guard cells such as a those terminating in a change in guard cell turgor.

It is highly advantageous to study two responses to ABA in one cell type in the same way, and even more advantageous to study the two responses in a cell type which is isolated and therefore uninfluenced by other cell types (see Walton, 1983). As described earlier, during the course of the present study it emerged that it was possible to study ABA-induced changes in guard cell *CDeT6-19* driven GUS activity and guard cell turgor in treated detached epidermis from leaves of *A. thaliana*. This was made possible by the discovery that guard cells are viable in epidermis detached from leaves of *A. thaliana* (Figure 4.10.A; Roelfsema & Prins, 1996). Both responses to ABA were studied in the guard cells of detached abaxial epidermis after incubation on solutions containing the ABA analogues. This allowed the relative stereospecificity of the two ABA response modes to be compared within a single cell type (see Figures 4.11, 4.12 and 4.14, and Tables 4.5 and 4.6). (±)- and (+)-ABA had the greatest effect on guard cell turgor and *CDeT6-19* driven GUS activity out of all the molecules investigated. They both affected the two responses to a similar degree except that 10^{-4} M (+)-ABA induced a significantly higher level of guard cell *CDeT6-19* driven GUS activity than 10^{-4} M (±)-ABA. 10^{-5} M (–)-ABA had no effect on guard cell turgor (Figure 4.14) but had a significant effect on guard cell *CDeT6-19* driven GUS activity (Figure 4.11). The

differential effectiveness of (–)-ABA at inducing these two responses suggests that there is a difference in the stereospecificity of the perception mechanism/receptor for ABA-induced changes in guard cell gene promoter activity and turgor in *A. thaliana.* However, 10^{-4} M (–)-ABA induced both responses which suggests that the stereochemical difference is not extreme but could be due to slightly different ABA receptor characteristics. (–)-ABA has also revealed differences in the perception mechanisms/receptors for different ABA signalling pathways in barley aleurone protoplasts (Hill *et al.*, 1995). In this respect, (–)-ABA was less effective at inducing *Em* gene expression than at inhibiting GA_3-induced α-amylase synthesis.

In contrast, the effectiveness of PBI-63 and PBI-51 at inducing changes in guard cell turgor was similar to that for the induction of guard cell *CDeT6-19* driven GUS activity. This suggests that the two ABA-response modes have similarities in the stereochemical requirements of their respective perception mechanisms. Both ABA-induced guard cell responses were significantly affected only by 10^{-4} M PBI-63 (Figures 4.12 and 4.15). However 10^{-4} M PBI-63 was significantly less effective than 10^{-4} M (±)-, (+)- or (–)-ABA thus suggesting that the changes to the shape of the ABA molecule apparent in PBI-63 are important for both responses in the guard cell system. PBI-51, the (–)-enantiomer of PBI-63, had no effect on either guard cell turgor or *CDeT6-19* driven GUS activity. This highlights the fact that many of the (–)-enantiomers of ABA analogues are inactive (see Section 4.1.2). There are just too many changes to the structural feature of naturally-occurring (+)-ABA for the ABA analogue PBI-51 to initiate ABA-regulated responses. It would be interesting to investigate whether PBI-51 can antagonize both ABA-induced responses in guard cells in detached epidermis from leaves of *A. thaliana* (as discussed earlier when describing the results of foliar applications of PBI-51). In Chapter 2 it was shown that PBI-51 did not antagonize (±)-

ABA-induced changes in guard cell turgor in detached epidermis from *C. Communis* or *N. tabacum*; whether this would be the case for guard cells of *A. thaliana* needs to established.

The fact that (±)-*trans, trans*-ABA did not affect either guard cell turgor or *CDeT6-19* driven GUS activity illustrates that it is not always ABA analogues with multiple structural differences to the (+)-ABA molecule that are inactive. The C-2 *cis* orientation is essential to the biological activity of (+)-ABA as shown in the present study and reported in all previous research [see Section 1.7.4(b)].

In some cases the effect of the molecules described in Figure 1.4 on stomatal aperture width in detached epidermis from leaves of *A. thaliana* differed from those reported for detached epidermis from the species investigated in Chapter 2 (Figure 4.14 and Figures 2.5, 2.6 and 2.8). (–)-ABA was more active in epidermis detached from leaves of *V. faba*, *N. tabacum* and *C. communis* than from leaves of *A. thaliana* Whether this reflects plant species differences or is due to differences in the characteristics of epidermis detached from leaves of *A. thaliana* compared with the leaves of the other species (Figure 4.10) remains unclear. However, as discussed in Section 2.4, putative plant species differences are emerging in the mechanisms by which ABA controls guard cell turgor (Armstrong *et al.*, 1995; Schmidt *et al.*, 1995; Esser *et al.*, 1997; Grabov *et al.*, 1997; Pei *et al.*, 1997). For example the *abi-1* mutant gene inhibited ABA-induced activation of slow anion channels in guard cells of *A. thaliana* (Pei *et al.*, 1997) but had no effect on these guard cell channels in transgenic tobacco carrying the mutant transgene *abi-1* (Armstrong *et al.*, 1995; Grabov *et al.*, 1997).

The effect of PBI-63 on stomatal aperture width in detached epidermis of *A. thaliana* was similar to that reported for *V. faba* and *C. communis* in that this molecule

was inactive until applied at a concentration of 10^{-4} M. The total lack of activity of 10^{-4} M PBI-51 in detached epidermis of *A. thaliana* contrasted with the low level of activity reported in detached epidermis from *V. faba* and *C. communis*. (±)-*trans, trans*-ABA was inactive in epidermis from leaves of both *A. thaliana* and *C. communis* as long as light-induced isomerization of the molecule was minimized (Figures 2.5 and 4.14).

The method of treatment application {foliar application *in planta* versus treatment of detached epidermis [see Section 4.2.3(a) and 4.2.5(b)]} had an effect on the level of *CDeT6-19* gene promoter driven GUS activity in guard cells (see Tables 4.3 and 4.5). The mean guard cell GASP for all treatments were lower in treated, detached epidermis than in epidermis from treated plants which is not surprising considering the considerably shorter exposure time to ABA and the ABA analogues in the former system. The most noticeable specific treatment difference was the decreased activity of PBI-63 in the detached epidermal system. The higher level of activity of PBI-63 when applied to the whole plant over a two day period may reflect the fact that the molecule was metabolized over this time period (as discussed earlier). To investigate this further metabolism studies could be carried out using tritiated PBI-63 (see Balsevich *et al.*, 1994). To a lesser extent (–)-ABA also had a lower level of activity in the detached epidermal system which may also reflect metabolism differences and could be investigated in the same way as described for PBI-63. Alternatively, the differences in the biological activity of PBI-63 and (–)-ABA described above could be due to a temporal effect unrelated to metabolism. Although these considerations are important it was not deemed essential that they be investigated in the present study because the major goal was to compare the biological activity of ABA analogues on two different ABA responses under the same experimental conditions.

The present study has shown that the biological activity of (–)-ABA differs between two ABA-induced responses in *A. thaliana* guard cells in detached abaxial epidermis: 10^{-5} M (–)-ABA had no effect on guard cell turgor (as measured by changes in stomatal aperture width), whereas, 10^{-5} M (–)-ABA induced *CDeT6-19* driven GUS activity in the guard cell (as determined by a quantitative GUS activity scoring method). In the future, an attractive addition to measuring stomatal aperture width would be to measure the activity of ABA-regulated ion channels in guard cells in response to ABA analogues, including (–)-ABA. This would provide more precise information on the stereospecificity of ABA-induced changes in guard cell turgor. Recently, it has been shown that ABA strongly activates slow anion channels in guard cells of *A. thaliana* (Pei *et al.*, 1997), thus making this suggestion feasible. Additionally, in the future it would be profitable to measure *CDeT6-19* gene promoter activity *in vivo*. This could be achieved by using the *in vivo* GUS assay substrate ImaGene Green (Herd *et al.*, 1997) or by using transgenic plants containing the *CDeT6-19* promoter fused to the fluorescent green protein (FGP) gene (Pang *et al.*, 1996). It would also be advantageous to quantify the level of *CDeT6-19* gene promoter activity in guard cells in detached epidermis by another method in addition to the scoring method described in Section 4.2.3(e). This could be done by carrying out a GUS assay using the fluorometric GUS assay substrate, 4-methylumbelliferryl-β-glucuronide (4-MUG) (see Naleway, 1992) possibly on treated guard cell protoplasts of *A. thaliana* to avoid any contamination from other epidermal cells.

In conclusion, the data suggest that (–)-ABA is more effective at inducing changes in ABA-responsive gene promoter activity in guard cells than at inducing changes in guard cell turgor in detached epidermis from leaves of *A. thaliana*. It was advantageous that the two responses were measured in a single cell type under the

same experimental conditions so that plant species and tissue differences, and contamination from other cell types could be avoided (see Walton, 1983; Hill *et al.*, 1995). It is now important to substantiate this finding by making more specific and quantitative measurements of these two ABA responses in guard cells of *A. thaliana* (see above). It is also of interest to investigate the effects of a wider range of ABA analogues on these two ABA-induced processes in guard cells of *A. thaliana* in order to further probe their respective perception mechanisms.

Chapter 5

The Effect of ABA Analogues on Guard Cell Cytosolic Free Calcium

5.1 Introduction

5.1.1 The role of cytosolic free calcium in ABA-induced changes in guard cell turgor

The calcium ion (Ca^{2+}) is an important component of the signalling pathway by which many stimuli, including ABA (see Section 1.5.2), induce changes in guard cell turgor and hence stomatal aperture (see reviews by Ward *et al.*, 1995; Webb *et al.*, 1996b; MacRobbie *et al.*, 1997; McAinsh *et al.*, 1997). As stated in Section 1.5.2, ABA has been shown to stimulate increases in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in guard cells (McAinsh *et al.*, 1990, 1992; Schroeder & Hagiwara, 1990; Gilroy *et al.*, 1991; Irving *et al.*, 1992; Allan *et al.*, 1994; Staxén *et al.*, 1996, 1997) which precede stomatal closure by 5-10 min (McAinsh *et al.*, 1990). However, it is important to note that the percentage of guard cells which have been reported to exhibit an increase in $[Ca^{2+}]_i$ in response to ABA varies between studies (see Section 1.5.2 for details and possible explanations for this variation). The increases in guard cell $[Ca^{2+}]_i$ that have been reported in response to ABA include those which are transient or sustained and range from 100 to 1000 nM above the resting level of $[Ca^{2+}]_i$ (McAinsh *et al.*, 1990, 1992; Schroeder & Hagiwara, 1990; Gilroy *et al.*, 1991; Irving *et al.*, 1992; Allan *et*

al., 1994; Staxén *et al.*, 1996, 1997). A similar level of variation has been reported for increases in guard cell $[Ca^{2+}]_i$ in response to other stimuli, such as oxidative stress or CO_2 (McAinsh *et al.*, 1996b; Webb *et al.*, 1996a). More recently, Staxén *et al.* (1996) have reported that ABA stimulates clearly defined oscillations in $[Ca^{2+}]_i$ in guard cells of *C. communis*. These oscillations were induced by ABA in guard cells maintained in a low (5 mM) external potassium concentration ($[K^+]_e$) (I. Staxén and M. R. McAinsh, personal communication) and will be discussed in more detail in Section 5.1.2.

Stimuli that induce opposite effects on guard cell turgor have been shown to cause an increase in guard cell $[Ca^{2+}]_i$ (see Table 5.1). For example, the plant hormones ABA and auxin both induce an increase in guard cell $[Ca^{2+}]_i$, but ABA stimulates stomatal closure (and inhibition of stomatal opening) and auxin stimulates stomatal opening (see Table 5.1). Therefore, an intriguing question has arisen as to how response specificity is encoded in stimulus-response coupling pathways in plants which make use of Ca^{2+} as a second messenger (see McAinsh *et al.*, 1997; McAinsh & Hetherington, 1997). Clearly, a mechanism (or mechanisms) must exist in the guard cell to ensure the correct coupling of different Ca^{2+}-mobilizing stimuli to their physiological responses.

5.1.2 Encoding specificity in stimulus-induced changes in guard cell $[Ca^{2+}]_i$

Several mechanisms for encoding specificity in stimulus-induced changes in $[Ca^{2+}]_i$ in guard cells have been proposed (see Webb *et al.*, 1996b; McAinsh *et al.*, 1997; McAinsh & Hetherington, 1997). These include: (i) the spatial and temporal characteristics of the increase in $[Ca^{2+}]_i$ (the "calcium signature") (McAinsh *et al.*, 1995; for reviews see Webb *et al.*, 1996b; McAinsh *et al.*, 1997); (ii) the "physiological

Stimuli	Calcium Response	Stomatal Response	Reference
ABA/ caged ABA	increase/ oscillations	closure	McAinsh *et al.*, (1990, 1992) Schroeder & Hagiwara (1990) Gilroy *et al.* (1991) Irving *et al.* (1992) Allan *et al.* (1994) Staxén *et al.* (1996, 1997)
auxin	increase	opening	Irving *et al.* (1992)
CO_2	increase	closure	Webb *et al.* (1996b)
oxidative stress	increase	closure	McAinsh *et al.* (1996b)
caged IP_3	increase	closure	Gilroy *et al.* (1990)
cADPR	increase	closure	McAinsh *et al.* (1996a) Leckie *et al.* (1997)
external calcium/ caged calcium	increase/ oscillations	closure	Gilroy *et al.* (1991) McAinsh *et al.* (1995)
mechanical stimulation	increase	---	Haley *et al.* (1995)
L (+) adenosine	increase	opening	Curvetto & Darjania (1995)
decrease in external K^+	increase	closure	Gilroy *et al.* (1991)
Triacontanol	decrease	opening	Curvetto & Darjania (1995)
EGTA	decrease	---	Gilroy *et al.* (1991) McAinsh *et al.* (1996b) Webb *et al.* (1996a)

[cADPR, cyclic-ADP-ribose; EGTA, ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid; IP_3, inositol (1,4,5) trisphosphate]

Table 5.1 Measurements of stimulus-induced increases in guard cell $[Ca^{2+}]_i$. Reproduced from McAinsh *et al.* (1997).

address" of the guard cell (McAinsh *et al.*, 1997); and (iii) the involvement of other second messengers (for reviews see Webb *et al.*, 1996b; McAinsh *et al.*, 1997). Several of these mechanisms have been suggested to be involved in encoding specificity in the Ca^{2+} signal in animal cells (for example see Berridge & Galione, 1988; Dolmetsch *et al.*, 1997; Ito *et al.*, 1997; Swanson *et al.*, 1997; for reviews see Berridge *et al.*, 1988; Fewtrell, 1993; Clapham, 1995).

Much of the evidence for the mechanisms proposed for encoding specificity in stimulus-induced changes in guard cell $[Ca^{2+}]_i$ has come from studies of ABA-induced increases in guard cell $[Ca^{2+}]_i$. Both temporal and spatial heterogeneities have been reported in the ABA-induced increase in guard cell $[Ca^{2+}]_i$ (McAinsh *et al.*, 1990, 1992; Schroeder & Hagiwara, 1990; Gilroy *et al.*, 1991; Irving *et al.*, 1992; Allan *et al.*, 1994; Staxén *et al.*, 1996, 1997). Spatial heterogeneities have been visualized using digital ratio-imaging (Irving *et al.*, 1992; McAinsh *et al.*, 1992) and appear as "hot spots" and "Ca^{2+} quiescent" regions within the guard cell following application of ABA (Irving *et al.*, 1992; McAinsh *et al.*, 1992). Other stimuli which induce stomatal closure, such as an elevation in the external Ca^{2+} concentration ($[Ca^{2+}]_e$) and $[K^+]_e$, have also been reported to induce spatial heterogeneities in guard cell $[Ca^{2+}]_i$ (Gilroy *et al.*, 1991; McAinsh *et al.*, 1995). Such heterogeneities have also been reported in animal cells (Tsien & Tsien, 1990). Localized increases in $[Ca^{2+}]_i$ which are stimulus specific may allow the activation of specific effectors and so ensure appropriate stimulus-response coupling. It has been reported that there is an asymmetric distribution of Ca^{2+} during tip growth of both *Fucus* rhizoids (Brownlee & Wood, 1986) and pollen tubes (Miller *et al.*, 1992; Malhó *et al.*, 1994, 1995; Pierson *et al.*, 1994; Franklin-Tong *et al.*, 1996). The $[Ca^{2+}]_i$ gradient in such systems was localized towards the tip. Consequently, it has been suggested that the regions with an elevated

level of $[Ca^{2+}]_i$ are associated with the control of tip growth (Battey & Blackbourn, 1993; Jackson & Heath, 1993).

Temporal heterogeneities have also been reported in the ABA-induced increase in guard cell $[Ca^{2+}]_i$, including oscillations in $[Ca^{2+}]_i$. Oscillations in $[Ca^{2+}]_i$ may provide an additional method of encoding information in the Ca^{2+} signature in both animal and plant cells (for reviews see Berridge *et al.*, 1988; Fewtrell, 1993; Webb *et al.*, 1996b; McAinsh *et al.*, 1997). Information may be encoded in both the amplitude and frequency of the oscillations in $[Ca^{2+}]_i$ (Berridge *et al.*, 1988; Fewtrell, 1993). Oscillations in guard cell $[Ca^{2+}]_i$ have been reported in response to elevated $[Ca^{2+}]_e$ (a stomatal-closing stimulus) (McAinsh *et al.*, 1995). The pattern of these oscillations was dependent on the strength of the stimulus, i.e. the concentration of $[Ca^{2+}]_e$, and correlated with the magnitude of the physiological response (McAinsh *et al.*, 1995). Thus, these oscillations in guard cell $[Ca^{2+}]_i$ may encode signalling information as has been suggested for stimulus-induced, concentration-dependent oscillations in animal cells (Berridge, 1993, 1997; Fewtrell, 1993; Clapham, 1995). In addition, McAinsh *et al.* (1995) investigated the possible sources of Ca^{2+} for the generation of oscillations in guard cell $[Ca^{2+}]_i$ induced in response to $[Ca^{2+}]_e$. It was suggested that different sources of Ca^{2+} (external and internal) may play a role in the generation of different oscillatory patterns in guard cell $[Ca^{2+}]_i$ (McAinsh *et al.*, 1995). More recently and as described briefly in Section 5.1.1, ABA has been reported to induce characteristic, regular oscillations in guard cell $[Ca^{2+}]_i$ under conditions of low $[K^+]_e$ (Staxén *et al.*, 1996; I. Staxén and M. R McAinsh, personal communication). The pattern of the (±)-ABA-induced $[Ca^{2+}]_i$ oscillations reported by Staxén *et al.* (1996) is dependent upon the concentration of (±)-ABA and can be correlated with the degree of the (±)-ABA-induced reduction in guard cell turgor (i.e. the physiological response). Therefore, this

suggests that these oscillations in guard cell $[Ca^{2+}]_i$ may encode signalling information (Staxén *et al.*, 1996) as was suggested for the oscillations in guard cell $[Ca^{2+}]_i$ reported in response to elevated $[Ca^{2+}]_e$ (under conditions of 50 mM KCl) (McAinsh *et al.*, 1995).

Previously, in a minority of guard cells (maintained in 50 mM $[K^+]_e$) ABA has been reported to induce changes in $[Ca^{2+}]_i$ akin to oscillations (McAinsh *et al.*, 1990; Gilroy *et al.*, 1991). However the characteristics of these oscillations were not at all similar to those reported by Staxén *et al.* (1996) and it has been suggested that many of these earlier reports be discounted on the basis of artifacts in signal detection (see reviews by Read *et al.*, 1992, 1993). The reason for the induction of regular oscillations in $[Ca^{2+}]_i$ in guard cells of *C. communis* by ABA under conditions of lower (5 mM) (Staxén *et al.*, 1996) as opposed to higher (50 mM) $[K^+]_e$ (see McAnish *et al.*, 1990, 1992; Gilroy *et al.*, 1991; Allan *et al.*, 1994), is as yet unclear (I. Staxén, and M. R. McAinsh, personal communication); although, it may be related to the plasma membrane potential (and hence activation of plasma membrane ion channels - see Section 1.5.2) under these two different conditions (I. Staxén and M. R. McAinsh, personal communication).

There have also been reports of stimulus-induced oscillations, spikes and "waves" of $[Ca^{2+}]_i$ in other types of plant cell. These have been reported in root hairs in response to nodulation factors (Ehrhardt *et al.*, 1996), in unicellular green algae in response to caffeine, Sr^{2+} and a "light off" stimulus (Bauer *et al.*, 1997) and in both staminal hairs (Tucker & Boss, 1996) and pollen tubes (Franklin-Tong *et al.*, 1996) in response to IP_3 and mastoparan.

The term "physiological address" was coined by McAinsh *et al.* (1997) and refers to the spectrum of signalling components which are present in a cell at a given

time. The presence of certain signalling components may depend upon the cell's local environment and also the cell's (and the plant's) history of exposure to different environmental stimuli. Amongst the evidence to support this concept in guard cells is the report by Allan *et al.* (1994) that the ABA-induced increase in guard cell $[Ca^{2+}]_i$ is dependent upon the growth temperature of the plant. The concept of physiological address may help explain some of the variation in the reports of ABA-induced increases in guard cell $[Ca^{2+}]_i$ discussed in Section 5.1.1.

Another way in which specificity may be encoded in stimulus-induced increases in guard cell $[Ca^{2+}]_i$ is through the involvement of other second messengers. Other second messengers involved in the ABA signalling pathway(s) in guard cells which terminate in a reduction in guard cell turgor have already been discussed in Section 1.6.3. Thus, Ca^{2+} mobilizing second messengers such as IP_3 (Blatt *et al.*, 1990; Gilroy *et al.*, 1990; Lee *et al.*, 1996) and cADPR (McAinsh *et al.*, 1996a), and also other second messengers which are non-Ca^{2+} mobilizing such as pH (Irving *et al.*, 1992; Blatt & Thiel, 1993; Blatt & Grabov, 1997; Grabov & Blatt, 1997) may encode an additional level of specificity in the increase in guard cell $[Ca^{2+}]_i$ induced by ABA.

5.1.3 *A. thaliana* as a model system for studying the role of $[Ca^{2+}]_i$ in ABA-induced changes in guard cell turgor

Recently, guard cells of *A. thaliana* have emerged as an attractive model system in which to investigate ABA signalling pathways in plants. This is due to the convergence of several different factors, including: (i) the discovery that the effect of ABA (and other stimuli, including ABA analogues) on guard cell turgor and gene promoter activity can be studied in isolated epidermis from *A. thaliana* (see Chapter 4; Taylor *et al.*, 1995; Roelfsema & Prins, 1995; Webb & Hetherington, 1997); (ii) the

recent report that shows that guard cells of *A. thaliana* are amenable to patch clamp techniques (Pei *et al.*, 1997) thus allowing investigations of ABA-regulated ion channel activity in the guard cells of this species; and (iii) the wealth of genetically manipulated *A. thaliana* plants including putative ABA signalling mutants such as the *abi* mutants (see Section 4.1.1; for reviews see Giraudat *et al.*, 1994; Giraudat, 1995; Merlot & Giraudat, 1997) [see Section 4.1.1(b) and (c)].

Direct investigations of the role of $[Ca^{2+}]_i$ in ABA-induced changes in the turgor of guard cells of *A. thaliana* have not yet been published. However, it has been established that increasing $[Ca^{2+}]_e$ reduced the stomatal aperture width in detached epidermis of wild type *A. thaliana* (Webb & Hetherington, 1997). As discussed earlier (see Section 5.1.2), it has been reported that an increase in $[Ca^{2+}]_e$ results in an increase in $[Ca^{2+}]_i$ in guard cells of *C. communis* (McAinsh *et al.*, 1995). Therefore, the possibility arises from the data obtained by Webb and Hetherington (1997) that $[Ca^{2+}]_i$ might also be involved in the signalling pathway by which guard cells of *A. thaliana* reduce their turgor. However, it is vital to determine directly that elevated $[Ca^{2+}]_e$ increases $[Ca^{2+}]_i$ in guard cells of *A. thaliana* as it does in *C. communis* in order to verify this suggestion. In addition, Webb and Hetherington (1997) reported that the *abi1* and *abi2* mutants [see Section 4.1.1(c)] failed to close their stomata in response to $[Ca^{2+}]_e$. This correlates with observation by Roelfsema and Prins (1995) that guard cells of the *abi1* and *abi2* mutants of *A. thaliana* fail to close their stomata in response to ABA. From these data, Webb and Hetherington (1997) have suggested that the ABA and $[Ca^{2+}]_e$ signalling pathways in guard cells of A. *thaliana* which terminate in a reduction of guard cell turgor converge on or close to the *ABI1* and *ABI2* gene products. Further suggestive evidence that $[Ca^{2+}]_i$ may be involved in ABA-induced changes in the turgor of guard cells of *A. thaliana* will be summarized. As described

earlier, it has been reported that ABA regulates the S-type anion channels of guard cells of *A. thaliana* (Pei *et al.*, 1997). It is known that these channels are activated by an increase in $[Ca^{2+}]_i$ in guard cells of *V. faba* [Schroeder & Hagiwara, 1989; Schroeder & Keller, 1992; see Section 1.5.2(a)]. In addition, the expression of the *Arabidopsis abi1* gene in *N. benthamiana* abolished ABA-induced activation of the inward and outward K^+ channels in guard cells. As described in Section 1.5.2, evidence has accumulated for the regulation of these K^+ channels in guard cells of *C. communis* and *V. faba* either directly (inward K^+) or indirectly (outward K^+) by $[Ca^{2+}]_i$ [see reviews by Ward *et al.*, 1995; McAinsh *et al.*, 1997; MacRobbie, 1997; see Section 1.5.2(a)]. Taken together, these data suggest that it is possible that $[Ca^{2+}]_i$ plays a role in the regulation of turgor by ABA in guard cells of *A. thaliana*. However, the precise role and position of $[Ca^{2+}]_i$ in the putative guard cell ABA signalling models put forward by both Pei *et al.* (1997) and Webb and Hetherington (1997) [based on work using the *abi* mutants of *A. thaliana*- see Section 4.1.1(c)] await determination. In the future it will be of interest to determine whether Ca^{2+}-responsive guard cell ion channels which have been reported to be involved in ABA-induced changes in the turgor of guard cells of *C. communis* and *V. faba* (see Section 1.5.2) other than the S-type anion channels (identified in guard cells of *A. thaliana* by Pei *et al.*, 1997) operate in guard cells of *A. thaliana*. It will then be of interest to study the regulation of such channels by ABA, $[Ca^{2+}]_i$ (and other modulators, for example pH) in guard cells of WT and *abi* mutants of *A thaliana* so that working models of ABA signalling in guard cells can be established.

5.1.4 Aims

The main aim of the present study was to investigate the effect of ABA analogues on guard cell $[Ca^{2+}]_i$. This was to determine whether $[Ca^{2+}]_i$ plays a similar role in ABA analogue-induced changes in guard cell turgor (see Chapters 2 and 4) as it does in ABA-induced changes (see Sections 5.1.1 and 1.5.2). For example, it is possible that an ABA analogue could bring about changes in guard cell turgor without affecting $[Ca^{2+}]_i$ [see Section 1.5.5(b)]. However, in the light of the evidence presented in Sections 5.1.1 and 1.5.2 this would appear rather unlikely. Alternatively, an ABA analogue that has no effect on guard cell turgor, i.e. the final stage in the signalling cascade, may induce an increase in guard cell $[Ca^{2+}]_i$. If this were the case, then it would suggest that an ABA-analogue-induced increase in guard cell $[Ca^{2+}]_i$ alone is not sufficient to stimulate a reduction in guard cell turgor; stimuli such as the plant hormone auxin have been shown to induce an increase in $[Ca^{2+}]_i$ but do not bring about a reduction in guard cell turgor (Irving *et al.*, 1992). A third alternative is that an ABA analogue may induce an increase in guard cell $[Ca^{2+}]_i$ which differs spatially and/or temporally (i.e. has a different Ca^{2+} signature, see Section 1.5.2) from that induced by ABA. As discussed previously (see Section 5.1.2), oscillations in $[Ca^{2+}]_i$ have been reported in response to ABA (Staxén *et al.*, 1996) under conditions of low $[K^+]_e$ (I. Staxén, and M. R. McAinsh, personal communication). The pattern of these oscillations is dependent upon the concentration of (±)-ABA and can be correlated with the degree of the (±)-ABA-induced reduction in guard cell turgor (i.e. the physiological response) thus suggesting that these oscillations in guard cell $[Ca^{2+}]_i$ may encode signalling information. It is possible that an ABA analogue, under conditions of low $[K^+]_e$ may induce stimulus-specific oscillations in guard cell $[Ca^{2+}]_i$. By studying these oscillations and comparing them with the extent of the physiological response

induced by the ABA analogue it may be possible to shed light on the components of the Ca^{2+} signature which are important for evoking an ABA-induced reduction in guard cell turgor.

The effect of ABA analogues on guard cell $[Ca^{2+}]_i$ was investigated initially in guard cells of *C. communis*. The techniques required for the measurement of guard cell $[Ca^{2+}]_i$ in this species are firmly established and increases in guard cell $[Ca^{2+}]_i$ in response to (±)-ABA are well documented (Gilroy *et al.*, 1990; McAinsh *et al.*, 1990, 1992; Allan *et al.*, 1994; Staxén *et al.*, 1996). In addition, it is in the guard cells of *C. communis* that clearly defined ABA-induced oscillations in guard cell $[Ca^{2+}]_i$ under low external $[K^+]_e$, have been reported (Staxén *et al.*, 1996) (I. Staxén, and M. R. McAinsh, personal communication). The pattern of these oscillations was concentration-dependent and the data suggest that such oscillations may encode signalling information (see Section 5.1.2, and above in the present Section). Therefore, it was envisaged that investigations in the present study would include an examination of the effects of ABA analogues on guard cell $[Ca^{2+}]_i$ under the same conditions (i.e. 5 mM $[K^+]_e$) used by Staxén *et al.* (1996).

A more long-term aim of the present study was to investigate the role of $[Ca^{2+}]_i$ in ABA- and ABA-analogue-induced changes in turgor and gene promoter activity in guard cells of A. *thaliana* (see Chapter 4). To date, there have been few direct studies of the involvement of Ca^{2+} in signalling pathways in guard cells of *A. thaliana.* Consequently, before attempting to make direct measurements of stimulus-induced changes in guard cell $[Ca^{2+}]_i$ in this species it is important to establish that Ca^{2+} plays a role in ABA signalling in guard cells in detached epidermis of *A. thaliana*. One way in which this can be determined is through an examination of the effect of the calcium chelator 1, 2-bis(o-aminophenoxy)ethane N', N', N', N'-tetraacetic acid (BAPTA) on

ABA-induced changes in turgor and gene promoter activity in guard cells of isolated epidermis of *A. thaliana*. BAPTA-type buffers have already been used to demonstrate the importance of Ca^{2+} in the ABA-induced reduction in guard cell turgor of *C. communis* (A. A. R. Webb, personal communication). In addition, they have provided evidence for the role of $[Ca^{2+}]_i$ gradients and $[Ca^{2+}]_e$ fluxes in the growth of pollen tubes (Pierson *et al.*, 1994). If it can be established that Ca^{2+} plays a role in ABA signalling in guard cells of *A. thaliana*, the effect of ABA on $[Ca^{2+}]_i$ might be measured directly using the techniques developed for investigating the effect of ABA on $[Ca^{2+}]_i$ in guard cells of *C. communis* (McAinsh *et al.*, 1990, 1992; Gilroy *et al.*, 1991; Allan *et al.*, 1994; Staxén *et al.*, 1996, 1997).

5.2 Materials and Methods

5.2.1 Chemicals

All chemicals were from Sigma Chemical Co. (UK) unless otherwise stated. (+)-ABA, (–)-ABA were kindly supplied by Dr. S. Abrams (Plant Biotechnology Institute, Saskatoon, Canada).

5.2.2 Growth conditions and preparation of epidermis

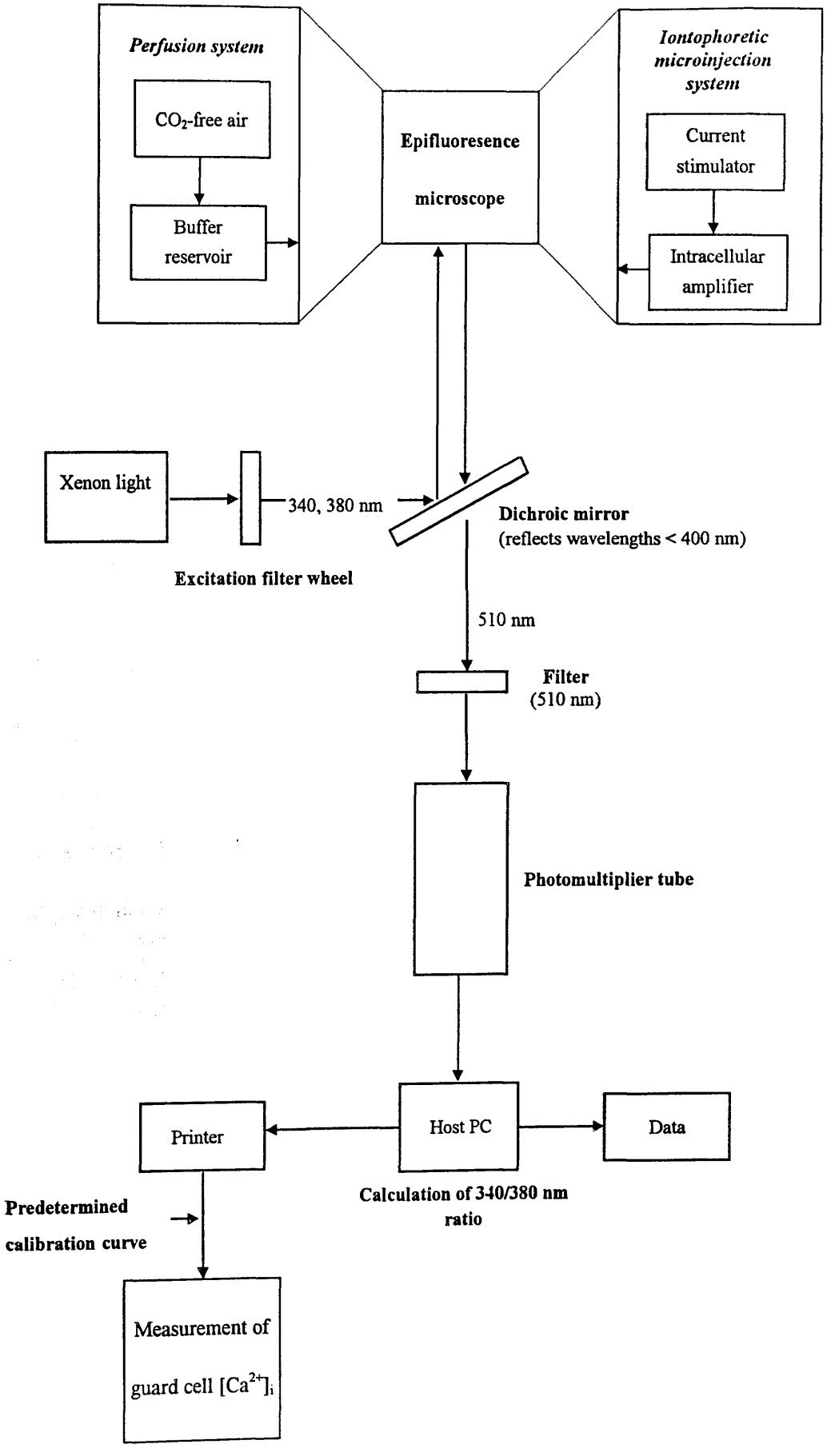
C. communis and *A. thaliana* were grown and epidermis removed from the leaves of these plants as described in Sections 2.2.2 and 4.2.2, respectively.

5.2.3 Measurement of $[Ca^{2+}]_i$ in guard cells using fura-2

A simple schematic diagram of the system used to make Ca^{2+}-dependent fluorescent measurements from fura-2-loaded guard cells is shown in Figure 5.1. The components of the system shown in Figure 5.1 are described in detail in Sections 5.2.3(a), (b), (c) and (d).

Fura-2 is a widely used fluorescent dual-excitation ratiometric Ca^{2+}-indicator dye (see review by Webb *et al.*, 1999a). Upon binding free Ca^{2+} fura-2 shifts its excitation maximum (510 nm emission) such that the fluorescence at shorter wavelengths, typically 340 nm, increases with increasing free Ca^{2+} whereas the fluorescence at longer wavelengths, typically 380 nm, decreases with increasing free Ca^{2+} (Figure 5.2A). The ratio of the fluorescence intensities at these two wavelengths (340/380 nm) is proportional to the concentration of free Ca^{2+} but is independent of the concentration of fura-2 (see review by Webb *et al.*, 1996b). This means that artifacts which may be interpreted as changes in the concentration of free Ca^{2+}, such as those caused by

Figure 5.1 A simple schematic diagram of the system used to make Ca^{2+}-dependent fluorescent measurements from fura-2-loaded guard cells. An epidermal strip was mounted on the stage of an inverted epifluorescence microscope and perfused with a MES buffer containing KCl [see section 5.2.3(a) and (b)]. Buffers were supplied under gravity and were removed by a vacuum pump (see McAinsh *et al.*, 1990, 1992, 1995, 1996; Webb *et al.*, 1996b). Guard cells in the epidermal strip were microinjected with fura-2 using iontophoresis for 1 min using 200 ms current pulses of 1.0 nA, 2 Hz. The fura-2 loaded in the cytosol of an individual guard cell was excited with light from a xenon light source. The dye was excited with 340 and 380 nm light using a filter wheel, which was operated at 64 revolutions/sec, and specific filters [see section 5.2.3(c)]. Wavelengths of excitation light below 400 nm were reflected onto the sample by use of a DM400 dichroic mirror (Nikon). The fluorescence emitted (epifluorescence) from fura-2 loaded in the guard cell cytosol passed through the dichroic mirror and fluorescence with a wavelength of 510 nm (selected using an interference filter with a 40-nm band width) was detected by a photomultiplier tube. A host computer converted the analogue data to a digitised form. The absolute fluorescence from excitation at different wavelengths, and also the calculated 340/380 nm ratio, were displayed. 340/380 nm ratios were converted into measurements of guard cell $[Ca^{2+}]_i$ (average for the whole cell) using a predetermined calibration curve (see Figure 5.3). (Redrawn from Willmer & Fricker, 1996).


Perfusion system
CO_2-free air
Buffer reservoir
Epifluoresence microscope
Iontophoretic microinjection system
Current stimulator
Intracellular amplifier
Xenon light
340, 380 nm
Excitation filter wheel
Dichroic mirror
(reflects wavelengths < 400 nm)
510 nm
Filter
(510 nm)
Photomultiplier tube
Printer
Host PC
Data
Predetermined calibration curve
Calculation of 340/380 nm ratio
Measurement of guard cell $[Ca^{2+}]_i$

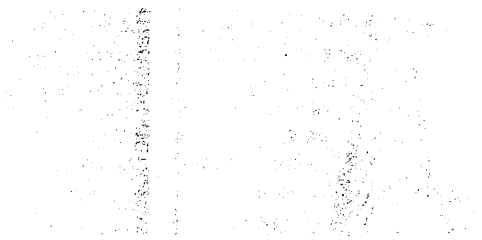
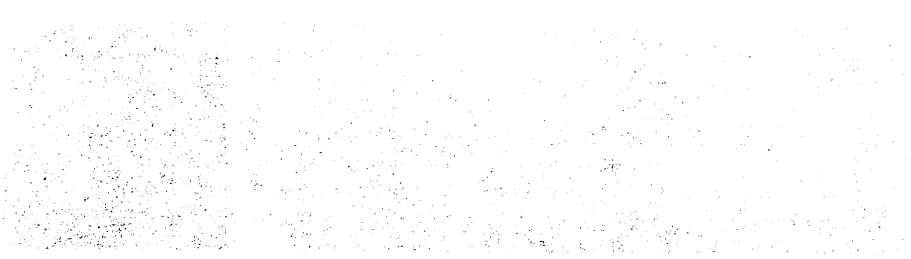
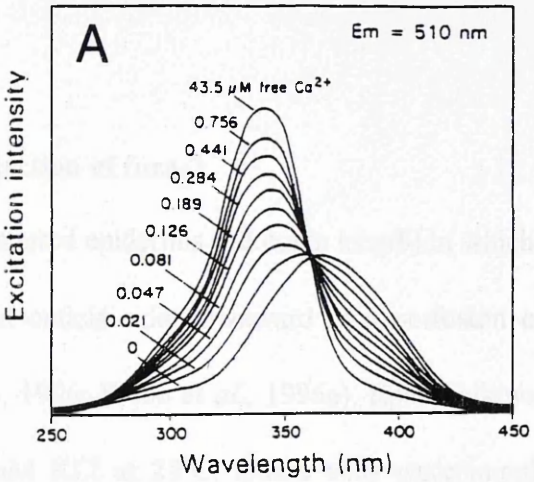


Figure 5.2 Emission spectra for the Ca^{2+}-indicator dye fura-2 (reproduced from Webb *et al.*, 1997) (**A**). The fluorescence (**B**) and bright-field (**C**) images of guard cells of *Commelina communis* loaded with the Ca^{2+}-indicator dye, fura-2, in the cytosol of a healthy guard cell (stomatal complex on the right-hand side) and the vacuole of a collapsed guard cell (stomatal complex on the left-hand side). (M.R. McAinsh & C. Brownlee, unpublished data.)




A
Em = 510 nm
43.5 μM free Ca2+
0.756
0.441
0.284
0.189
0.126
0.081
0.047
0.021
0
Excitation intensity
250
300
350
400
450
Wavelength (nm)


B


C

photobleaching, leakage of the dye out of the cell or changes in cell shape are eliminated.

5.2.3(a) Microinjection of fura-2

Freshly prepared epidermis (>2 cm in length) in which stomata were open to <1 μm were mounted cuticle side downward in a perfusion chamber (McAinsh *et al.*, 1990, 1992, 1995, 1996; Webb *et al.*, 1996a). Epidermis was perfused with CO_2-free MES/KOH, 10 mM KCl at 25°C. Guard cells were impaled with filamented glass microelectrodes (<0.25 μm tip diameter) containing 10 mM fura-2 pentasodium salt (Calbiochem-Novabiochem, Nottingham, UK) in their tips.

Fura-2 was microinjected into the guard cell cytosol iontophoretically (McAinsh *et al.*, 1990, 1992, 1995, 1996; and see review by Webb *et al.*, 1996b). The distribution of fura-2 loaded in guard cells of *C. communis* is shown in Figure 5.2B. Fura-2-loaded cells were maintained under conditions promoting stomatal opening for 45 min (McAinsh *et al.*, 1992). Fluorescence measurements were only made on stomata that opened to the same aperture as those on the rest of the epidermal strip (3 to 4 μm) and in which both the injected and non-injected cells of a single stoma exhibited the same increase in turgor. Epidermal strips in which fura-2-loaded guard cells met all the criteria for estimating viability (Gilroy *et al.*, 1991; McAinsh *et al.*, 1992) were perfused subsequently with MES/KOH, 50 mM KCl at 25°C in the presence or absence of either 10^{-4} M $CaCl_2$, 10^{-5} M (–)-*cis, trans*-ABA or 10^{-5} M (+)-*cis, trans*-ABA, and the fluorescence was monitored. In the experiments described in Section 5.3.2 the epidermal strips were perfused with MES/Tris, 15 mM KCl, 25°C to induce stomatal opening following microinjection of fura-2 into the guard cell cytosol. This epidermis was then perfused with MES/Tris, 5 mM KCl at 25°C and perfused

subsequently with either MES/Tris, 5 mM KCl, 25°C containing 10^{-6} (±)-ABA or MES/Tris, 15 mM KCl, 25°C.

5.2.3(b) Fluorescence Microscopy

The perfusion chamber was mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope (Nikon, Kingston-Upon-Thames, UK) (McAinsh *et al.*, 1992). Excitation light was provided by a 100 W Nikon xenon light source. Excitation wavelengths were specified using 20-nm bandwidth interference filters [340 and 380 nm, 400-nm dichroic mirror (Cairn Research Ltd., Faversham, UK)]. These were selected using a spinning filter changer in combination with three metal gauze neutral density filters (reducing the excitation intensity to <3%) and transmitted to the microscope via a liquid light guide (Cairn Research Ltd.). The field of excitation was limited to the area of a single stoma using the excitation diaphragm of the microscope. A Nikon CF Fluor DL 40x oil immersion lens (1.30 numerical aperture) and nonfluorescent immersion oil (Fluka, Dorset, UK) were used for all measurements. Fluorescent light emitted from the fura-2-loaded guard cells was passed to the side camera port of the microscope and focused with a 1x television relay lens (Nikon). A Nikon PFX shutter assembly and rectangular emission aperture were used to limit the area of the specimen studied to one stoma. Fluorescence emissions [510 nm, 40-nm bandwidth interference filter (Cairn research Ltd.)] were quantified using the appropriate filter and detector combination.

5.2.3(c) Photometry

Fluorescence measurements were made using a Cairn spectrophotometer system (Cairn Research Ltd.). The autofluorescence from each guard cell was

determined at each excitation wavelength prior to microinjection (McAinsh *et al.*, 1990, 1992, 1995, 1996; Webb *et al.*, 1996a). Autofluorescence subtraction was calculated on-line. The spinning filter changer was run at 64 revolutions per second in spin mode, allowing fluorescence measurements to be obtained at a rate of 64 readings per second. Values were calculated as the mean of 64 individual readings to increase the signal-to-noise ratio to give a data point every second. The ratio (340/380 nm) was calculated each second on-line (McAinsh *et al.*, 1995, 1996; Webb *et al.*, 1996a). Ratios were converted into measurements of whole-cell cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) using a predetermined calibration curve [see Section 5.2.3(d)].

5.2.3(d) Calibration

Similar results have been obtained for both *in vitro* and *in vivo* calibration of the Ca^{2+}-dependent ratio in guard cells (Gilroy *et al.*, 1991; McAinsh *et al.*, 1992). Consequently, an *in vitro* calibration was used (see Section 5.3.1). This was done by measuring the fluorescence reported from fura-2 in the presence of known concentrations of free Ca^{2+}. A range of precise concentrations of free Ca^{2+} were examined using calcium calibration buffers (10^{-8} M, 10^{-7} M, 10^{-6} M) (World Precision Instruments, Hastings, UK). A 10 µl drop of each calcium calibration buffer containing 5×10^{-7} M fura-2 was placed on a coverslip and the fluorescence measured in a manner similar to that described in Section 5.2.3(c). Calibration of the 340/380 nm Ca^{2+}-dependent fura-2 fluorescence was routinely carried out every 2 weeks and whenever a new xenon light source was installed.

5.2.4 *A. thaliana* epidermal strip bioassay

The role of Ca^{2+} in ABA-induced inhibition of stomatal opening in detached epidermis from *A. thaliana* leaves was assessed using the calcium chelator 2-bis(o-aminophenoxy)ethane N', N', N', N'-tetraacetic acid (BAPTA) (Calbiochem-Novabiochem, UK). Detached, abaxial epidermis from leaves of transgenic *A. thaliana* was incubated on MES/KOH, 50 mM KCl at 22°C containing (±)-ABA, as described in Section 4.2.5, in the presence and absence of 2 mM BAPTA. At the end of the incubation period stomatal aperture widths in the detached epidermis were determined as described in Section 4.2.5(d).

5.2.5 Statistics

Measurements of stomatal aperture widths were analyzed statistically according to the methods described in Section 2.5.2.

5.3 Results

5.3.1 Measurement of guard cell $[Ca^{2+}]_i$ using the Ca^{2+}-indicator fura-2

A typical *in vitro* calibration of the Ca^{2+}-dependent fluorescence of the Ca^{2+}-indicator fura-2 is shown in Figure 5.3. The absolute fluorescence (510 nm emission) at the two excitation wavelengths (340 nm, blue traces; 380 nm, red traces) and the Ca^{2+}-dependent ratio (340/380 nm, black traces) are given. The Ca^{2+}-dependent ratio was determined for three concentrations of free Ca^{2+} (10, 100 and 1000 nM). These reflect both the dynamic range of the dye (see Figure 5.2A) and the levels of $[Ca^{2+}]_i$ reported in guard cells (for reviews see Webb *et al.*, 1996b; McAinsh *et al.*, 1997). From Figure 5.3 it can be seen that as the concentration of free Ca^{2+} increases (from 10 nM to 1000 nM) so does the 340 nm (excitation wavelength) fluorescence. This is accompanied by a decrease in the 380 nm (excitation wavelength) fluorescence. These observations are consistent with the fura-2 excitation spectra shown in Figure 5.2. The changes in the absolute fluorescence (510 nm emission) are logarithmic. Consequently, the Ca^{2+}-dependent ratio also increases logarithmically with the concentration of free Ca^{2+}, from approximately 0.9 at 10 nm to approximately 1.4 at 100 nm to approximately 7.3 at 1000 nM free Ca^{2+}.

The measurement of guard cell $[Ca^{2+}]_i$ employs difficult methodologies (see Section 5.2.3 and Figure 5.1). Therefore, initial investigations were concerned with ensuring that guard cell $[Ca^{2+}]_i$ was being measured correctly using the system described in Figure 5.1. The viability of fura-2-loaded guard cells and the ability of fura-2 to report real changes in guard cell $[Ca^{2+}]_i$ were examined. Guard cell viability was assessed using the criteria described by Gilroy *et al.* (1991) and McAinsh *et al.* (1992). In addition, the capacity of guard cells to generate oscillations in $[Ca^{2+}]_i$ under

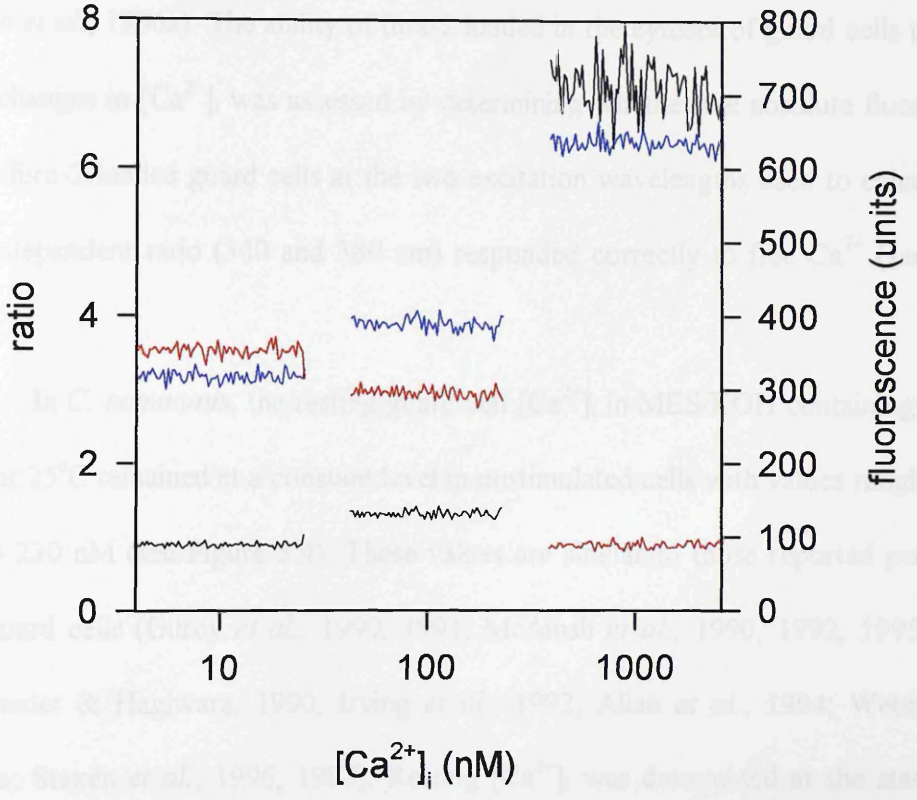
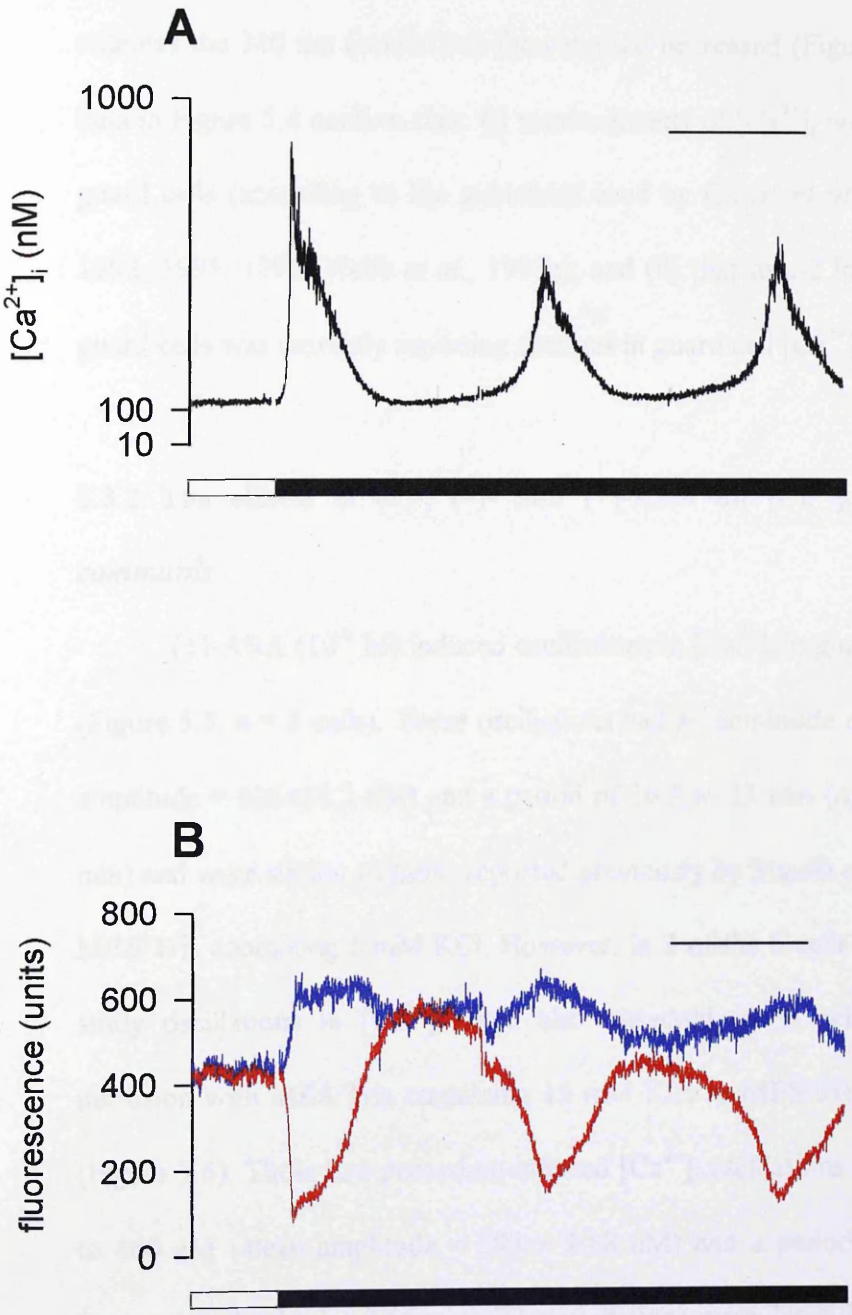


Figure 5.3 Calibration of the Ca^{2+}-indicator dye, fura-2. The absolute fluorescence (510 nm emission) (right-hand y axis) reported from fura-2 at excitation wavelengths of 340 nm (blue) and 380 nm (red) and the 340/380 nm ratio (left-hand y axis) (black) of these two wavelengths at three concentrations of free Ca^{2+} (10, 100 and 1000 nM).

conditions of elevated $[Ca^{2+}]_e$ (McAinsh *et al.*, 1995) was used as an indicator that guard cells were undamaged. This indicator has been used to determine the viability of fura-2-loaded guard cells in several previous studies (McAinsh *et al.*, 1995, 1996; Webb *et al.*, 1996a). The ability of fura-2 loaded in the cytosol of guard cells to report real changes in $[Ca^{2+}]_i$ was assessed by determining whether the absolute fluorescence from fura-2-loaded guard cells at the two excitation wavelengths used to calculate the Ca^{2+}-dependent ratio (340 and 380 nm) responded correctly to free Ca^{2+} (see Figure 5.3).

In *C. communis*, the resting guard cell $[Ca^{2+}]_i$ in MES/KOH containing 50 mM KCl at 25°C remained at a constant level in unstimulated cells with values ranging from 90 to 230 nM (see Figure 5.4). These values are similar to those reported previously for guard cells (Gilroy *et al.*, 1990, 1991; McAinsh *et al.*, 1990, 1992, 1995, 1996; Schroeder & Hagiwara, 1990; Irving *et al.*, 1992; Allan *et al.*, 1994; Webb *et al.*, 1996a; Staxén *et al.*, 1996, 1997). Resting $[Ca^{2+}]_i$ was determined at the start of all subsequent experiments and always fell within the range described above (total n = 10 cells). It was found that 10^{-4} M $[Ca^{2+}]_e$ induced oscillations in $[Ca^{2+}]_i$ in guard cells of *C. communis* (Figure 5.4, n = 3 cells). These $[Ca^{2+}]_e$-induced oscillations were similar to those reported previously by McAinsh *et al.* (1995) and had an amplitude of 300 to 870 nM (mean amplitude = 474 ± 70.6 nM) and a period of 18.3 min to 20 min (mean period of 19.2 ± 0.9 min). The amplitude of the first oscillation in guard cell $[Ca^{2+}]_i$ after the application of 10^{-4} M $[Ca^{2-}]_e$ was often larger than all the subsequent $[Ca^{2+}]_i$ oscillations (see Figure 5.4). This observation has also been reported by McAinsh *et al.* (1995). The absolute fluorescence at the excitation wavelengths, 340 nm (blue traces) and 380 nm (red traces) used to calculate the Ca^{2+}-dependent ratio shown in Figure 5.4.A are given in Figure 5.4B. The changes in guard cell $[Ca^{2+}]_i$ correspond to

Figure 5.4 Oscillations in $[Ca^{2+}]_i$ in guard cells of *Commelina communis* induced by 10^{-4} M $[Ca^{2+}]_e$. Guard cells of closed stomata were microinjected with fura-2 into the cytosol, and the stomata opened to 3-4 μm. Resting $[Ca^{2+}]_i$ was determined in MES/KOH, 50 mM KCl, 25°C (open box). Guard cells were subsequently perfused with MES/KOH, 50 mM KCl, 25°C containing 10^{-4} M $CaCl_2$ (solid box). The 340/380 nm Ca^{2+}-dependent fura-2 ratio (**A**) and the fluorescence reported from fura-2 at the two excitation wavelengths 340 nm (blue) and 380 nm (red) (**B**) were monitored (n = 3 cells). Bar = 10 min.


A
1000
100
10
$[Ca^{2+}]_i$ (nM)
B
800
600
400
200
0
fluorescence (units)

the changes in absolute fluorescence seen at the excitation wavelengths 340 and 380 nm (Figure 5.4B) in accordance with the fura-2 excitation spectra given in Figure 5.2. Thus, as guard cell $[Ca^{2+}]_i$ increased so did the 340 nm (excitation) fluorescence, whereas the 380 nm (excitation) fluorescence decreased (Figure 5.4B). Therefore, the data in Figure 5.4 confirm that: (i) measurements of $[Ca^{2+}]_i$ were being made on viable guard cells (according to the guidelines used by Gilroy *et al.*, 1991; McAinsh *et al.*, 1992, 1995, 1996; Webb *et al.*, 1996a); and (ii) that fura-2 loaded into the cytosol of guard cells was correctly reporting changes in guard cell $[Ca^{2+}]_i$.

5.3.2 The effects of (±)-, (–)- and (+)-ABA on $[Ca^{2+}]_i$ in guard cells of *C. communis*

(±)-ABA (10^{-6} M) induced oscillations in $[Ca^{2+}]_i$ in guard cells of *C. communis* (Figure 5.5, n = 3 cells). These oscillations had an amplitude of 550 to 700 nM (mean amplitude = 628±18.2 nM) and a period of 16.5 to 23 min (mean period = 18.8 ± 0.5 min) and were similar to those reported previously by Staxén *et al.* (1996) measured in MES/Tris containing 5 mM KCl. However, in 2 of the 5 cells examined in the present study oscillations in $[Ca^{2+}]_i$ were also observed when cells were switched from perfusion with MES/Tris containing 15 mM KCl to MES/Tris containing 5 mM KCl (Figure 5.6). These low potassium-induced $[Ca^{2+}]_i$ oscillations had an amplitude of 310 to 460 nM (mean amplitude = 398 ± 20.8 nM) and a period of 16.25 to 21.25 min (mean period = 18.3 ± 0.66 min). The oscillations were dependent on the presence of 5 mM KCl and were abolished when the cells were returned to MES/Tris containing 15 mM KCl (Figure 5.6). Low potassium-induced oscillations in guard cell $[Ca^{2+}]_i$ have also been observed by I. Staxén and M. R. McAinsh (personal communication).

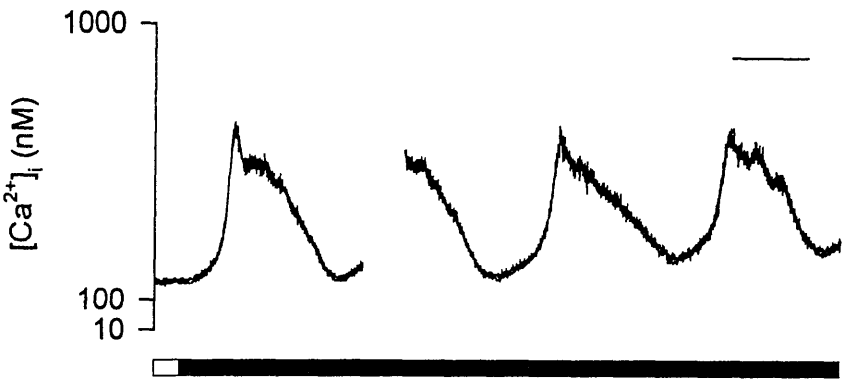


Figure 5.5 The effect of 10^{-6} M (±)-ABA on $[Ca^{2+}]_i$ in guard cells of *Commelina communis* maintained in MES/Tris containing 5 mM KCl, 25°C. Guard cells of closed stomata were microinjected with fura-2 into the cytosol, and the stomata opened to 3-4 μm. Resting $[Ca^{2+}]_i$ was determined in MES/Tris, 5 mM KCl, 25°C (open box). Guard cells were subsequently perfused with MES/Tris, 5 mM KCl, 25°C containing 10^{-6} M (±)-ABA (solid box) (n = 3 cells). Bar = 10 min.

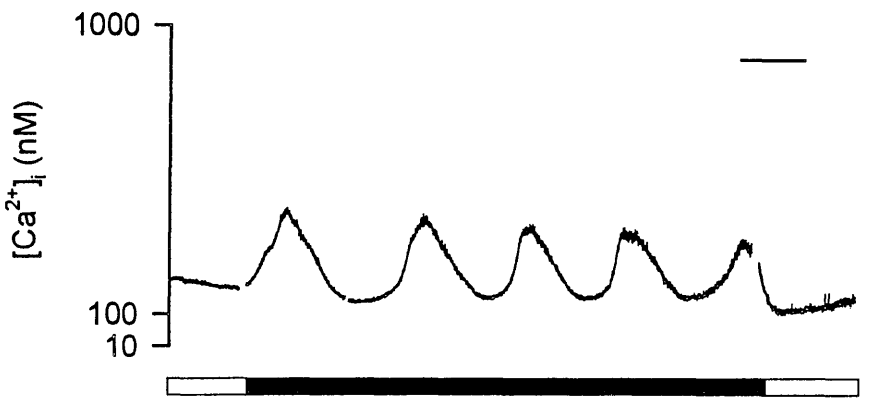


Figure 5.6 The effect of reducing the external potassium concentration from 15 mM to 5 mM on $[Ca^{2+}]_i$ in guard cells of *Commelina communis*. Guard cells of closed stomata were microinjected with fura-2 into the cytosol, and the stomata opened to 3-4 μm. Resting $[Ca^{2+}]_i$ was determined in MES/Tris, 15 mM KCl, 25°C (open box). Guard cells were subsequently perfused with MES/Tris, 5 mM KCl, 25°C (solid box) and then returned to perfusion with MES/Tris, 15 mM KCl, 25°C (open box) (n = 2 cells). Bar = 10 min.

Low potassium buffers have been used previously to study ABA-induced oscillations in guard cell $[Ca^{2+}]_i$ (Staxén *et al.*, 1996). However, in the light of the low potassium (i.e. "background")-induced oscillations observed in this study (Figure 5.6) it was decided to conduct further studies into the effects of (±)-, (–)- and (+)-ABA on $[Ca^{2+}]_i$ in guard cells of *C. communis* using epidermis perfused with MES/KOH containing 50 mM KCl (McAinsh *et al.*, 1990, 1992, 1995, 1996; Allan *et al.*, 1994; Webb *et al.*, 1996a), as no "background" oscillations have been reported in this system.

In two guard cells it was shown that (–)-ABA (10^{-5} M) induced oscillations in $[Ca^{2+}]_i$ (Figure 5.7, n = 2 cells). These oscillations had an amplitude of 365 to 440 nM (mean amplitude = 396 ± 8 nM) and a period of 13.3 to 16.7 min (mean period = 15 ± 0.75 min). (–)-ABA (10^{-5} M) also led to a reduction in the turgor of fura-2 loaded guard cells and in epidermal bioassays (see Section 2.3.1). Subsequent perfusion of the same guard cell with 10^{-5} M (+)-ABA induced an oscillation which had an amplitude of 615 or 650 nM (mean amplitude = 632 ± 26.9 nM) and a period of approximately 13.3 or 15.5 min (mean period = 14.4 ± 1.1 min). It was only possible to measure one oscillation in response to 10^{-5} M (+)-ABA in both of the cells examined due to a reduction in the amount of fura-2 in the guard cell cytosol and the associated decrease in signal-to-noise ratio towards the end of the $[Ca^{2+}]_i$ trace (Figure 5.7).

5.3.3 The role of Ca^{2+} in guard cells of *A. thaliana*

The Ca^{2+}-chelator BAPTA abolished completely (±)-ABA-induced inhibition of stomatal opening in detached epidermis from *A. thaliana* (Figure 5.8). This effect was apparent at all concentrations of (±)-ABA (10^{-7} to 10^{-4} M) investigated. These data

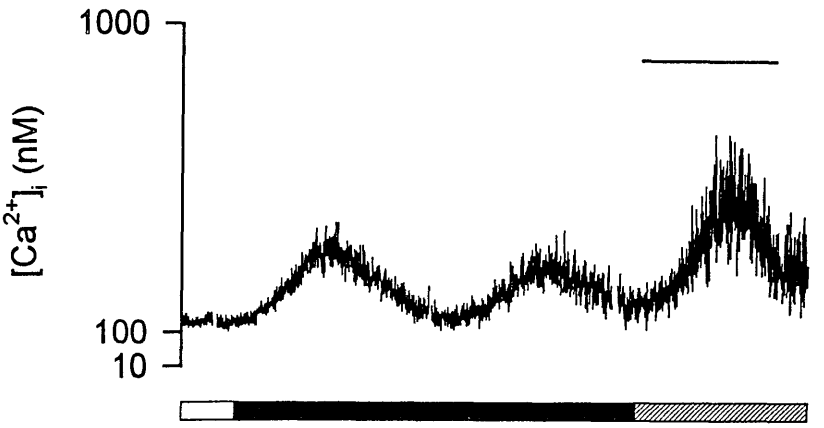


Figure 5.7 The effect of 10^{-5} M (–)-ABA and (+)-ABA on $[Ca^{2+}]_i$ in guard cells of *Commelina communis* maintained in MES/KOH containing 50 mM KCl at 25°C. Guard cells of closed stomata were microinjected with fura-2 into the cytosol, and the stomata opened to 3-4 μm. Resting $[Ca^{2+}]_i$ was determined in MES/KOH, 50 mM KCl, 25°C (open box). Guard cells were subsequently perfused MES/KOH, 50 mM KCl, 25°C containing 10^{-5} M (–)-ABA (solid box). This was followed by perfusion with MES/KOH, 50 mM KCl, 25°C containing 10^{-5} M (+)-ABA (hatched box) (n = 2 cells). Bar = 10 min.

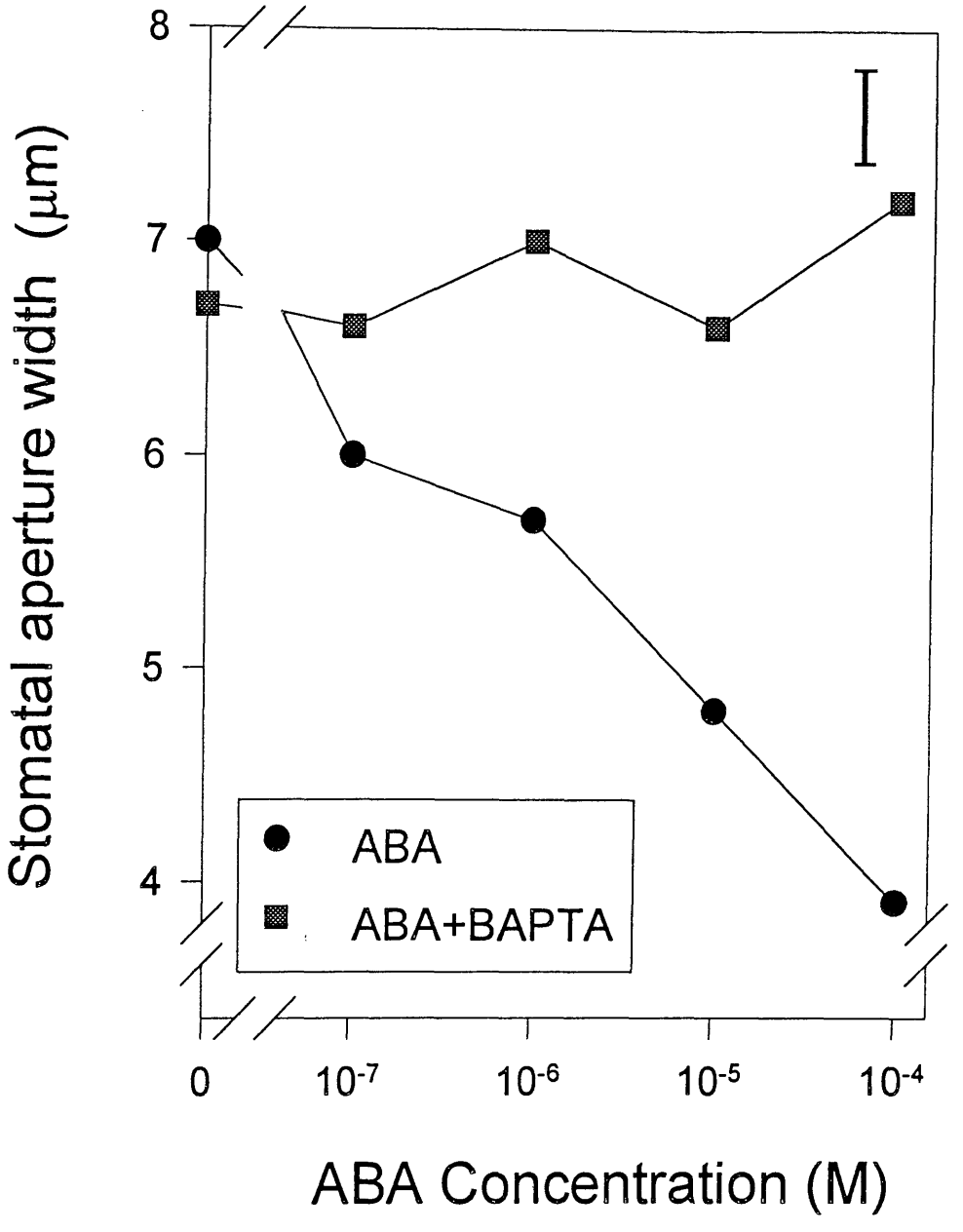


Figure 5.8 The effect of 2 mM BAPTA on (±)-ABA-induced inhibition of stomatal opening in detached, abaxial epidermis from leaves of *Arabidopsis thaliana* (n = 120). The vertical bar represents the LSD.

suggest a role for Ca^{2+} in the inhibition of stomatal opening of *A. thaliana* observed in response to ABA.

5.4 Discussion

ABA analogues can be used to determine the structural features of the ABA molecule which are important for initiating ABA signalling pathways in guard cells. In Chapters 2 and 4 the effects of ABA analogues on guard cell turgor and gene promoter activity were examined. However, it is also of interest to determine how these ABA analogues affect the earlier events in ABA signalling cascades which lead to these two end responses. These events include an increase in guard cell $[Ca^{2+}]_i$ which is believed to act in the capacity of a second messenger (for reviews see Webb *et al.*, 1996a; McAinsh *et al.*, 1997).

Investigations of (±)-ABA-induced changes in $[Ca^{2+}]_i$ have played an important role in the dissection of the ABA signal transduction pathway in guard cells which terminates in a change in guard cell turgor (see Sections 5.1.1 and 1.5.2). The present study describes preliminary data concerned with examining the effect of (±)-,(+)-, and (–)-ABA on $[Ca^{2+}]_i$ in guard cells of *C. communis*. Guard cells of *C. communis* have been extensively used in investigations of ABA-induced changes in $[Ca^{2+}]_i$ (McAinsh *et al.*, 1990, 1992; Gilroy *et al.*, 1991; Allan *et al.*, 1994; Staxén *et al.*, 1996, 1997). Therefore, it was envisaged that initial investigations of the effect of ABA analogues on guard cell $[Ca^{2+}]_i$ would be carried out using guard cells of this species. The effect of (±)-ABA on $[Ca^{2+}]_i$ in guard cells of *C. Communis* was investigated to act as a positive control because all previous studies in *C. communis* have examined the effect of this form of ABA on guard cell $[Ca^{2+}]_i$ (McAinsh *et al.*, 1990, 1992; Gilroy *et al.*, 1991; Allan *et al.*, 1994; Staxén *et al.*, 1996, 1997). The ABA analogue, (–)-ABA, (see Figure 1.4 and Section 1.7) had a clear effect on the turgor of guard cells of *C. communis*, inhibiting stomatal opening and promoting stomatal closure (see Chapter

2). (–)-ABA was therefore a good initial candidate for studying ABA analogue-induced changes in guard cell $[Ca^{2+}]_i$ (also see Section 1.8). The effect of (+)-ABA on $[Ca^{2+}]_i$ in guard cells of *C. Communis* was investigated because this is the naturally-occurring form of ABA [see Section 1.7.4(a)] and acts as a positive control to the effect of (–)-ABA in the present study and to the effect of other ABA analogues investigated in the future.

The techniques available to study guard cell $[Ca^{2+}]_i$ (Webb *et al.* 1996a) are well established but nevertheless are not simple or easy. It is important that the correct methodology is employed so that real changes in $[Ca^{2+}]_i$ in fully viable guard cells are reported (see Section 5.3.1). In the present study, the viability of fura-2-loaded guard cells was assessed using the criteria for viability established by Gilroy *et al.* (1991) and McAinsh *et al.* (1992) together with the ability of undamaged guard cells to generate oscillations in $[Ca^{2+}]_i$ under conditions of elevated $[Ca^{2+}]_e$ (McAinsh *et al.*, 1995). Oscillations in $[Ca^{2+}]_i$ in response to elevated $[Ca^{2+}]_e$ have also been used as an indicator of the viability of guard cells of *C. communis* in several other studies (McAinsh *et al.*, 1995, 1996; Webb *et al.*, 1996a). As described in Section 5.2.3, fura-2 is ratiometric indicator dye. The use of a ratiometric Ca^{2+}-indicator reduces artifacts due to variations in the fluorescence signal emitted from this indicator which might otherwise be misinterpreted as changes in $[Ca^{2+}]_i$. However, as an extra precaution to check that the fura-2 loaded into the cytosol of guard cells was reporting $[Ca^{2+}]_i$ correctly, the changes in absolute fluorescence at the two excitation wavelengths (340 and 380 nm) used to calculate the Ca^{2+}-dependent ratio were monitored during measurement of guard cell $[Ca^{2+}]_i$. As a result, it was established that changes in guard cell $[Ca^{2+}]_i$ corresponded to the changes in the 340 and 380 nm absolute fluorescence from the fura-2 loaded guard cells (Figure 5.4) in accordance with the reported fura-2

excitation spectra shown in Figure 5.2. Once it had been established that guard cell $[Ca^{2+}]_i$ was being measured accurately, including the replication of previous reports of 10^{-4} M $[Ca^{2+}]_e$-induced oscillations in guard cell $[Ca^{2+}]_i$ (McAinsh *et al.*, 1995) which acted as an indicator of viability and a positive control in the present study (see Figure 5.4A and B), it was deemed possible to investigate the effect of the ABA analogue, (–)-ABA, on guard cell $[Ca^{2+}]_i$.

Recently, it has been reported that (±)-ABA induces clearly defined oscillations in $[Ca^{2+}]_i$ in guard cells maintained under low (5 mM) $[K^+]_e$ (Staxén *et al.*, 1996; I. Staxén and M. R. McAinsh, personal communication). Changes in $[Ca^{2+}]_i$ akin to oscillations in guard cells maintained in 50 mM $[K^+]_e$ have previously been reported in response to (±)-ABA (McAinsh *et al.*, 1990; Schroeder & Hagiwara, 1990; Gilroy *et al.*, 1991). However, the pattern of these differs from the clearly defined oscillations reported by Staxén *et al.* (1996) and it is possible that many of these earlier reports can be discounted on the basis of artifacts in signal detection (see review by Read *et al.*, 1993). It has been suggested that the pattern of stimulus-induced oscillations in $[Ca^{2+}]_i$ observed in both animal and plant cells may encode signalling information (see Section 5.1.2; for reviews see Berridge *et al.*, 1988; Berridge, 1993; Fewtrell 1993; Clapham, 1995; Webb *et al.*, 1996b; McAinsh *et al.*, 1997). The pattern of the (±)-ABA-induced oscillations in $[Ca^{2+}]_i$ reported by Staxén *et al.* (1996) is dependent upon the concentration of (±)-ABA and can be correlated with the degree of the (±)-ABA-induced reduction in guard cell turgor (i.e. the physiological response). This correlation suggests that the oscillations in guard cell $[Ca^{2+}]_i$ induced in response to (±)-ABA under low $[K^+]_e$, in common with those induced by elevated $[Ca^{2+}]_e$ (McAinsh *et al.*, 1995; see Section 5.1.2) may encode information which leads to the appropriate physiological response. Therefore, it was decided to investigate the effect of (–)-ABA

on guard cell $[Ca^{2+}]_i$ under the same experimental protocol used by Staxén *et al.* (1996) in order to determine whether (−)-ABA, a less potent stomatal-closing (inhibition-of-stomatal-opening) stimulus than (±)- or (+)-ABA, induces characteristic oscillations in guard cell $[Ca^{2+}]_i$. These (−)-ABA-induced oscillations in guard cell $[Ca^{2+}]_i$ may shed light on the signalling information contained in the (±)-ABA-induced oscillations reported by Staxén *et al.*, (1996).

It is important to note that the results from the present study on the effects of (±), (+)-, and (−)-ABA on $[Ca^{2+}]_i$ in guard cells of *C. communis* are of a preliminary nature. Therefore, the data in the present study will not be subject to over-interpretation and until further confirmatory experiments have been conducted shall be treated with caution.

10^{-6} M (±)-ABA induced oscillations in $[Ca^{2+}]_i$ in guard cells maintained in low external $[K^+]_e$ similar to those reported by Staxén *et al.* (1996) (Figure 5.5, n = 3). The mechanism(s) for the generation of these ABA-induced oscillations are as yet unclear, although they may be related to the guard cell plasma membrane potential prevalent under low $[K^+]_e$ (see Section 5.1.2). Figure 5.6 shows that the low $[K^+]_e$ (5 mM) used in these experiments also stimulated oscillations in guard cell $[Ca^{2+}]_i$ in the absence of (±)-ABA. These were observed in 2 of the 5 cells investigated (see Figure 5.6). Such oscillations have also been seen by I. Staxén and M. R. McAinsh (personal communication) in 30% of the cells that they examined, although the precise reason(s) for such a phenomenon is unclear (I. Staxén and M. R. McAinsh, personal communication). It is possible that reducing $[K^+]_e$ may affect the plasma membrane potential such that plasma membrane Ca^{2+} channels are activated (I. Staxén and M. R. McAinsh, personal communication). Indeed, it has been shown previously that lowering the external potassium concentration (from 50 to 25 mM) results in an

increase in $[Ca^{2+}]_i$ in the guard cells of *C. communis*, although oscillations in $[Ca^{2+}]_i$ were not observed (Gilroy *et al.*, 1991). Thus, the results from the present study and those of Gilroy *et al.* (1991) suggest that lowering the external potassium concentration has the potential to affect $[Ca^{2+}]_i$, although the precise mechanism by which this occurs remains to be investigated. A decrease in external potassium results in a reduction of the turgor of fura-2-loaded guard cells (I. Staxén and M. R. McAinsh, personal communication) and guard cells in epidermal bioassays (for example see Travis & Mansfield, 1979). Therefore, a decrease in external potassium can be considered a closing stimulus for stomata in detached epidermis. Consequently, it is not so surprising that a decrease in external potassium, along with other stomatal-closing stimuli (see Table 5.1), results in an increase in guard cell $[Ca^{2+}]_i$.

(–)-ABA (10^{-5} M) induced oscillations in $[Ca^{2+}]_i$ in two guard cells in detached epidermis perfused with MES/KOH at 25°C containing 50 mM KCl (Figure 5.7). (–)-ABA also led to a reduction in the guard cell turgor (see Section 5.3.2). An attempt was made to determine the effect of (+)-ABA on these (–)-ABA induced oscillations, but no conclusions can be drawn because it was only possible to measure one oscillation in response to 10^{-5} M (+)-ABA in both of the cells examined (see Section 5.3.2). Distinct oscillations have not been observed previously in response to (±)-ABA in 50 mM KCl (McAinsh *et al.*, 1990; Schroeder & Hagiwara, 1990; Gilroy *et al.*, 1991), although changes in $[Ca^{2+}]_i$ akin to oscillations were reported in guard cells in these studies. However, the (–)-ABA-induced oscillations in $[Ca^{2+}]_i$ were more closely related to those observed by Staxén *et al.* (1996) in response to (±)-ABA under low $[K^+]_e$ than the changes in $[Ca^{2+}]_i$ akin to oscillations reported by McAinsh *et al.* (1990), Schroeder and Hagiwara (1990) and Gilroy *et al.* (1991). Clearly, further work is required to determine the characteristics of these (–)-ABA induced oscillations. It is

also vital to determine the effect of (±)- and (+)-ABA on $[Ca^{2+}]_i$ in guard cells of *C. communis* so as to have positive controls with which to compare the effect of (−)-ABA and other ABA analogues. In the future, it will be of interest to examine the spatial characteristics of ABA and ABA analogue-induced changes in guard cell $[Ca^{2+}]_i$ using digital ratio-imaging (Irving *et al.*, 1992; McAinsh *et al.*, 1992) in order to shed light on the signalling information contained in the (±)-ABA-induced spatial heterogeneities in guard cell $[Ca^{2+}]_i$ reported by Irving *et al.* (1992) and McAinsh *et al.* (1992).

The guard cells of *C. communis* have been extensively used in the dissection of the ABA signalling pathway which leads to a reduction in guard cell turgor. This is especially true for the examination of the role of $[Ca^{2+}]_i$ in guard cell signal transduction (McAinsh *et al.*, 1990, 1992; Gilroy *et al.*, 1991; Allan *et al.*, 1994; Staxén *et al.*, 1996, 1997). However, recent advances mean that it is now possible to use *A. thaliana* as a new model system in which to investigate ABA signalling pathways in guard cells. As well as being able to study changes in turgor (see Chapter 4; Roelfsema & Prins, 1995; Webb & Hetherington, 1997), ion channel activity (Pei *et al.*, 1997) and gene promoter activity (see Chapter 4; Taylor *et al.*, 1995) in guard cells of *A thaliana*, the availability of mutants [*abi* mutants see reviews by Giraudat *et al.*, 1994; Giraudat, 1995; Merlot & Giraudat, 1997; see Section 4.1.1(c)] of putative components of ABA signalling pathways makes them a particularly attractive model system for teasing out the intricacies of guard cell ABA signalling pathways. Investigations of ABA signalling pathways in guard cells of genetically manipulated *A. thaliana* are in their infancy [see Section 4.1.1(c)]. In addition, the precise role of $[Ca^{2+}]_i$ in these pathways remains to be established. In the present study, preliminary experiments were conducted to assess the role of $[Ca^{2+}]_i$ in ABA signalling pathways in guard cells of *A. thaliana*. These data are an essential prerequisite to studies of the

effects of genetically manipulating putative components of the ABA signalling cascade on ABA-induced changes in guard cell $[Ca^{2+}]_i$. In addition, they provide the basis for a double barrelled approach in the future using ABA analogues as tools to probe the ABA perception mechanism and involvement of $[Ca^{2+}]_i$ in guard cells of genetically manipulated *A. thaliana*.

Prior to direct investigations of ABA- and ABA analogue-induced changes in guard cell $[Ca^{2+}]_i$ in this species, it was deemed important to establish firmly that Ca^{2+} is involved in ABA-induced changes in guard cells in detached epidermis from *A. thaliana*. This question was examined using the Ca^{2+}-chelator BAPTA. It was observed that BAPTA abolished completely (±)-ABA-induced inhibition of stomatal opening (Figure 5.8). Webb and Hetherington (1997) have reported $[Ca^{2+}]_e$-induced stomatal closure in detached epidermis of *A. thaliana*. These data, in conjunction with those from the present study suggest that $[Ca^{2+}]_i$ plays a role in the ABA signalling pathway terminating in a change in the turgor of guard cells of *A. thaliana*. Recently, it has also been reported that $[Ca^{2+}]_i$ plays an important role in ABA regulated gene promoter activity in guard cells of *A. thaliana* (M. Larman and A.M. Hetherington, personal communication). The enhancement of GUS activity driven by the *CDeT6-19* gene promoter in response to ABA in these cells (see Chapter 4) was significantly inhibited by BAPTA (M. Larman and A. M. Hetherington, personal communication). Taken together, these observations suggest that $[Ca^{2+}]_i$ is a common component of the ABA signalling pathways in guard cells which terminate in changes in turgor and gene promoter activity. However, to date there have been no direct measurements of stimulus-induced changes in $[Ca^{2+}]_i$ in guard cells of *A. thaliana*. The present study shows that it is possible to microinject fura-2 into the cytosol of guard cells of *A. thaliana* by iontophoresis and measure resting $[Ca^{2+}]_i$ (Figure E.1). In the future, it is

envisaged that it will be possible to measure changes in $[Ca^{2+}]_i$ in guard cells of *A. thaliana* in response to ABA and ABA analogues using Ca^{2+}-indicator dyes such as fura-2. As described in Appendix E, fura-2 was loaded into the cytosol of a guard cell of *A. thaliana* but subsequently moved into the vacuole. This problem may be overcome by the microinjection of fura-2 or another Ca^{2+}-indicator dye conjugated to a dextran into the cytosol using pressure. Such compounds are unable to move out of the cell compartment into which they have been microinjected (Haugland, 1996) thus preventing dye loss from the guard cell cytosol. The use of dextran-linked-Ca^{2+}-indicator dyes has been successfully employed in other plant cell types (Taylor *et al.*, 1996). Recently, guard cells of *C. communis* have been successfully microinjected with Ca^{2+}-indicator dyes using pressure (Leckie *et al.*, 1997). This suggests that such an approach could also be applied to guard cells of *A. thaliana*.

If it is possible to successfully measure $[Ca^{2+}]_i$ in guard cells of *A. thaliana* (see Appendix E.1) in the future, then the importance of ABA-induced changes in $[Ca^{2+}]_i$ in two types of ABA response in guard cells may be examined (i.e. changes in turgor and gene promoter activity). ABA analogues could be used in such investigations. If ABA analogues induce different Ca^{2+} signatures, differing in temporal (i.e. pattern of oscillations in $[Ca^{2+}]_i$) and/or spatial aspects [determined using Ca^{2+} imaging techniques, see McAinsh *et al.* (1992); Irving *et al.*, (1992)]), then these Ca^{2+} signatures could be correlated to the effect of the ABA analogues on an ABA-induced response in the guard cell. Several scenarios can be envisaged by which ABA analogues would provide information on the role of changes in $[Ca^{2+}]_i$ in ABA-induced changes in guard cell turgor and gene expression (see below).

An ABA analogue may induce characteristic oscillations in $[Ca^{2+}]_i$ but have no effect on guard cell turgor or gene expression, thus implying that the oscillations in

$[Ca^{2+}]_i$ alone are not sufficient to induce the physiological response or indeed may not at all be causally related to it. Another ABA analogue may induce an ABA response in the guard cell but have no effect on guard cell $[Ca^{2+}]_i$. This would suggest that it is evoking the response by an ABA perception mechanism which doesn't stimulate Ca^{2+} mobilization. This may mean that ABA can initiate effective signalling pathways in guard cells which are Ca^{2+} independent (see Allan *et al.*, 1995). Alternatively, an ABA analogue may induce both oscillations in $[Ca^{2+}]_i$ and the final response. This suggests that the oscillations in $[Ca^{2+}]_i$ are important for the final response and the ABA analogue is interacting with a perception mechanism which leads to the mobilization of Ca^{2+}.

The requirement of ABA-induced oscillations in $[Ca^{2+}]_i$ for the induction of the physiological response could be further assessed by examining the effect of blocking the oscillations in $[Ca^{2+}]_i$ induced by an ABA analogue (using a Ca^{2+} chelator or Ca^{2+} channel blockers) on the final response. In addition, ABA analogues which have differential effects on the two ABA-induced responses in guard cells and induce stimulus-specific Ca^{2+} signatures could be used to investigate the characteristics of the different ABA-induced Ca^{2+} signatures which are involved in changes in guard cell turgor and changes in guard cell gene expression. In this respect, if one ABA analogue induced gene expression in the guard cell but had no effect on cell turgor [for example 10^{-5} M (–)-ABA in the present study], and another ABA analogue induce a change in guard cell turgor but had no effect on gene expression, then the Ca^{2+} signatures induced by these two analogues could be compared and their importance for the induction of the response investigated by blocking a change in $[Ca^{2+}]_i$ (in the manner described above).

In addition, and as discussed earlier, extending investigations of the involvement of $[Ca^{2+}]_i$ in ABA signalling cascades in guard cells to include those of *A. thaliana* will help to uncover further the intricacies of ABA signalling in this cell type due to the availability of mutants of ABA signalling. Guard cells of the *abi* mutants of *A. thaliana* have already been used to investigate the role of the some of the *ABI* gene products in the regulation of guard cell turgor by ABA [Roelfsema & Prins, 1995; Pei *et al.*, 1997; see Section 4.1.1(c)]. It is of extreme interest to investigate the role of $[Ca^{2+}]_i$ in ABA signalling pathways in the guard cells of these mutants in order to determine the position of the *ABI1* and *ABI2* gene products in the signalling pathway relative to changes in $[Ca^{2+}]_i$.

In summary, although the data in this chapter are of a preliminary nature they do allow some conclusions to be drawn. Firstly, they suggest that caution is needed when examining ABA-induced oscillations in guard cell $[Ca^{2+}]_i$ in low $[K^+]_e$ due to the fact that low $[K^+]_e$ can also induce such oscillations in the absence of ABA. Secondly, the measurement of the effects of (–)-ABA on guard cell $[Ca^{2+}]_i$, although limited in number, suggest that it may be possible in the future to use ABA analogues to investigate the role of $[Ca^{2+}]_i$ in ABA signalling pathways in guard cells. Finally, a preliminary measurement of resting $[Ca^{2+}]_i$ in a guard cell of *A thaliana* points to the possibility of carrying out exciting research in the future using the ABA signalling mutants of *A. thaliana* to further define the role of $[Ca^{2+}]_i$ in ABA signalling pathways in guard cells.

Chapter 6

Conclusions

The main conclusions from this thesis are:

(1) (–)-ABA, hitherto believed to have little or no effect on stomatal aperture (see Milborrow, 1980; Walton, 1983; Hornberg & Weiler, 1984) significantly inhibited stomatal opening in *C. communis*, *V. faba*, *N. tabacum* and *A. thaliana* (see Chapters 2 and 4).

(2) PBI-51 [a competitive inhibitor of ABA-induced gene expression (Wilen *et al.*, 1993, 1996)] had no effect on ABA-induced inhibition of stomatal opening (determined in *C. communis* and *N. tabacum*) or ABA-induced promotion of stomatal closure in (determined in *C. communis* only) (see Chapter 2).

(3) It is vital to carry out investigations in a single plant species when comparisons are to be made between the effect of ABA analogues on ABA-induced changes in stomatal aperture and gene expression in guard cells. This is due to differences in the effectiveness of PBI-63, PBI-51 and (–)-ABA at inhibiting stomatal opening being discovered between plant species (see Chapter 2).

(4) (±)-ABA consistently enhanced *CDeT6-19*/GUS activity in guard cells of *A. thaliana* but not *N. tabacum* (see Chapters 3 and 4).

(5) The ABA analogues that inhibited stomatal opening also enhanced *CDeT6-19*/GUS activity in guard cells of *A. thaliana* (see Chapter 4). The relative biological activity of the ABA analogues in detached epidermis of *A. thaliana* was (+)-ABA > (±)-ABA > (–)-ABA > PBI-63. PBI-51 and (±)-*trans, trans*-ABA had no effect on stomatal opening or *CDeT6-19*/GUS activity in guard cells. A differential effect of

10^{-5} M (–)-ABA on the two ABA-induced responses was reported; 10^{-5} M (–)-ABA enhanced *CDeT6-19*/GUS activity in guard cells but had no effect on stomatal in detached epidermis of *A. thaliana* (see Chapter 4).

(6) Preliminary data showed that (–)-ABA induced oscillations in $[Ca^{2+}]_i$ in guard cells of *C. communis*. They also demonstrate the possibility of microinjecting guard cells of *A. thaliana* with fura-2 and provide a measurement of resting $[Ca^{2+}]_i$ in this species.

The overriding aim of this thesis was to use ABA analogues to determine whether a common ABA "receptor" is utilized in the signalling pathway by which ABA evokes changes in stomatal aperture and that by which it induces changes in gene expression. The data concerning 10^{-5} M (–)-ABA described in point (4) (see above) suggests that the signalling pathways by which ABA inhibits stomatal opening and enhances gene promoter activity utilize an ABA receptor with different stereochemical requirements. However, additional studies are required to confirm this result. These could include determining the effect of ABA analogues on ion channel activity in guard cells of *A. thaliana* (see Chapter 4). Such an approach would provide a more sensitive assay than stomatal aperture measurements and would complement and confirm the stomatal aperture data presented in this thesis. In addition, the use of a more quantitative and also *in vivo* measurement of *CDeT6-19* gene promoter activity would provide a more sensitive assay of the effects of ABA analogues on gene expression in guard cells (see Chapter 4).

A guard cell specific promoter would provide a more direct system in which to measure ABA and ABA analogue-induced changes in guard cell gene expression. Under these conditions, any changes in gene expression measured in epidermal tissue

or in the whole plant would be due exclusively to changes in gene expression in guard cells. Using this system, changes in gene expression in guard cells could be measured without the need to isolate or visualize the guard cells. In addition, the use of a gene that is more tightly regulated by ABA than the *CDeT6-19* gene would reduce variation between individual guard cells in the magnitude of ABA-induced increases in gene expression and in the level of endogenous level of gene expression (see Chapter 4). This approach would be important for single cell studies such as those that examine the involvement of $[Ca^{2+}]_i$ in ABA-induced changes in gene expression in guard cells (see Chapter 5).

A guard cell specific gene that is tightly controlled by ABA has not yet been isolated. In the future, the expression of such a gene could be examined by several methods, including an *in vivo* analysis using plants transformed with the gene promoter linked to GFP (see Chapter 4) and also the determination of transcript and protein levels. Until such a gene is isolated, it would be of interest to determine the effect of ABA analogues on the accumulation of (±)-ABA-responsive dehydrin-like mRNA in isolated guard cells of *V. faba* and *Pisum sativum L. Argenteum* (Shen *et al.* 1995; Hays *et al.* 1997; see Table 1.1 and Section 1.7.4). These data could be compared with the effect of ABA analogues on stomatal opening in detached epidermal strips in these two species. The effect of several ABA analogues on stomatal opening in *V. faba* has already been investigated in the present study (see Chapter 2). In addition, it would be interesting to determine the effect of ABA and ABA analogues on the genes reported recently to be drought-responsive in guard cells of potato (Kopka *et al.*, 1997). This system could be used to assess whether there are different modes of ABA-regulated gene expression in guard cells, comparable to those revealed using ABA analogues in

other cell types and tissues, for example wheat embryos (Walker-Simmons *et al.*, 1992).

In general, the ABA analogues that inhibited stomatal opening also enhanced *CDeT6-19*/GUS activity in guard cells of *A. thaliana*. However, as described above, 10^{-5} M (–)-ABA had a differential effect on these two ABA-induced responses. These data suggest that the ABA "receptors" utilized in these two ABA signalling pathways in guard cells have similar but not identical stereochemical requirements. To test the similarity (difference) of these stereochemical requirements further, the effect of ABA analogues with different permutations to the structure of naturally occurring (+)-ABA could be tested on ion channel activity (and stomatal apertures) and gene expression in guard cells.

This study has shown that ABA analogues have the potential to be used as tools which can be used to help establish a clearer picture of the "receptor(s)" utlized in ABA signalling pathways in guard cells. An understanding of the initiation of these pathways offers opportunities for the manipulation of ABA/stress-induced responses of plants. Such manipulations may be vital for the survival of an ever-increasing human population on an earth whose plant resources are being continually stretched to the limit.

Appendices

Appendix A

The Effect of Ethanol on Stomatal Aperture Width

Ethanol was the solvent in which (±)-, (+)-, (−)-ABA, (±)-*trans, trans*-ABA, PBI-63 and PBI-51 were dissolved. Therefore, it was essential to determine that the ethanol concentration present in the incubation medium during stomatal bioassays had no effect on stomatal aperture width in the abaxial epidermis of the three species investigated in Chapter 2.

C. communis, *V. faba* and *N. tabacum* was grown and epidermis removed from the abaxial surface of their leaves as described in Sections 2.2.2 and 2.2.3. Epidermal strip bioassays were carried out as described in Section 2.2.4, except that, when the ABA molecules or ABA analogues would have been present in the incubation medium, in the present study only the ethanol concentration which would have accompanied these molecules was added. The highest ethanol (v/v) concentrations were chosen for each experimental protocol, namely, 1%, 0.15% and 0.105% (v/v).

In summary:

(i) For *C. communis*, the effect of 1% (v/v) on stomatal opening and 0.105% (v/v) on open stomata was investigated. And also the effect of a 3 h incubation, under opening conditions in 0.1% (v/v) ethanol, followed by a 1 h incubation in 0.105% (v/v) ethanol [see Section 2.2.4(iv)].

(ii) For *V. faba*, the effect of 1% (v/v) ethanol on stomatal opening was investigated.

(iii) For *N. tabacum*, the effect of 1% (v/v) ethanol on stomatal opening; and the effect of a 1 h pre-incubation in 0.1% (v/v) ethanol followed by a 3 h incubation, under opening conditions in 0.15% (v/v) ethanol [see Section 2.2.4(iv)] was investigated.

Stomatal aperture widths were determined (see Section 2.2.5) and AOV and LSD tests (using the whole data sets) carried out as described in Section 2.2.6.

The data (see Tables A.1, A.2, A.3) show that ethanol [1%, 0.15% and 0.105% (v/v) ethanol] had no effect on stomatal aperture in epidermis from all species and in all experimental protocols (see Section 2.2.4). This means that the effects of the ABA molecules and ABA analogues reported in Chapter 2 were intrinsic to these molecules and were not due to the ethanol in the incubation medium.

	Mean stomatal aperture width (μm)		
Effect of ethanol [% (v/v)] on:	Control	Ethanol	LSD
Stomatal opening [1%(v/v)]	17.4	17.0	0.71
Open stomata [0.105%(v/v)]	18.4	19.1	0.81
Stomatal opening [0.1% (v/v)] followed by open stomata [0.105% (v/v)]	17.6	18.2	0.7

Table A.1 The effect of ethanol on stomatal aperture width in detached abaxial epidermis of *C communis*. The LSD is shown for each experimental protocol. The control incubation medium for the epidermal bioassay in all cases was MES/KOH, 50 mM KCl at 25°C. There were no significant differences between the control and the ethanol treatments in all cases, as determined by AOV and LSD tests (see Section 2.2.6).

	Mean stomatal aperture width (μm)		
Effect of 1% (v/v) ethanol on:	Control	Ethanol	LSD
Stomatal opening	14.0	13.7	0.5

Table A.2 The effect of 1% (v/v) ethanol on stomatal opening in detached abaxial epidermis of *V. faba*. The LSD is shown. The control incubation medium for the epidermal bioassay was MES/Tris, 10 mM KCl at 25°C. There was not a significant difference between the control and 1% (v/v) ethanol treatment, as determined by AOV and LSD tests (see Section 2.2.6)

	Mean stomatal aperture width (μm)		
Effect of ethanol [% (v/v)] on:	Control	Ethanol	LSD
Stomatal opening (1%)	8.5	8.9	0.5
1 h Pre-incubation in the dark [0.1% (v/v)] followed by stomatal opening [0.15% (v/v)]	7.7	7.3	0.5

Table A.3 The effect of ethanol on stomatal aperture width in detached abaxial epidermis of *N. tabacum*. The LSD is shown for each experimental protocol. The control incubation medium for the stomatal opening bioassay was MES/KOH, 100 mM KCl at 25°C; and for the 1 h pre-incubation in the dark was MES/KOH, at 25°C. There were no significant differences between the control and the ethanol treatments in all cases, as determined by AOV and LSD tests (see Section 2.2.6).

Appendix B

The Effect of an Incubation in 10^{-5} M PBI-51 in the Dark on Stomatal Opening in Abaxial Epidermis of *N. tabacum*

Experiments were designed to test the antagonist properties of the ABA analogue, PBI-51 on (±)-ABA-induced inhibition of stomatal opening in abaxial epidermis of *N. tabacum* [see Section 2.2.4(iv)]. One such protocol involved a 1 h pre-incubation in 10^{-5} M PBI-51 in the dark prior to a concurrent application of 10^{-5} M PBI-51 and (±)-ABA in the light for 3 h [see Section 2.2.4(iv)]. Therefore, it was important to establish that a 1 h pre-incubation in 10^{-5} PBI-51 in the dark had no effect on stomatal opening under the control opening conditions (see below).

N. tabacum was grown and epidermis removed from the abaxial surface of leaves as described in Sections 2.2.2 and 2.2.3, respectively. An epidermal strip bioassay which involved a pre-incubation in 10^{-5} M PBI-51 for 1 h in the dark followed by a 3 h incubation period in the light in the control treatment (i.e. MES/KOH, 100 mM KCl, 25°C [see Section 2.2.4(iv)]. Stomatal aperture width measurements were made at the end of the 3 h incubation period in the light (see Section 2.2.5) and AOV and LSD tests carried out (see Section 2.2.6).

The mean stomatal aperture width for the control treatment (i.e. 1 h in the dark and 3 h in the light in the control treatment solution) was 7.7 μm ± SE 0.2 μm; and for the pre-incubation in 10^{-5} M PBI-51 in the dark followed by a 3 h incubation in the light in the control treatment (in the absence of PBI-51) was 7.8 μm ± SE 0.2 μm. AOV and LSD tests (see Section 2.2.6) (on the whole data set, see Figure 2.10)

showed that these two means were not statistically different from one another ($p = 0.05$).

In conclusion, a pre-incubation in 10^{-5} M PBI-51 in the dark had no effect on stomatal aperture after 3 hours under opening conditions. This means that the effect of 10^{-5} M PBI-51 on (±)-ABA-induced inhibition of stomatal opening in abaxial epidermis of *N. tabacum* could be assessed (see Figure 2.10 and Section 2.3.4).

Appendix C

The Effect of 10^{-9} M PBI-51 on ABA-Induced Inhibition of Stomatal Opening in Detached Abaxial Epidermis of *C. communis*

The effect of 10^{-9} M PBI-51, a potential ABA antagonist (see Section 1.8), was investigated on ABA-induced inhibition of stomatal opening in detached epidermis of *C. communis*. A high concentration of PBI-51 (10^{-5} M) was shown previously to have no effect on ABA-induced changes in guard cell turgor (see Section 2.3.4). However, it was considered necessary to investigate the possible antagonistic properties of lower concentrations of PBI-51 because concentration-dependent antagonism by certain agents has been seen in biological systems (e.g. calcium channel blocker activity in guard cells - McAinsh *et al.*, 1991a).

C. communis was grown and epidermis removed from the abaxial surface of leaves as described in Sections 2.2.2 and 2.2.3, respectively. An epidermal strip bioassay was carried out as described in Section 2.2.4(iv); a concurrent application of 10^{-5} M PBI-51 and (±)-ABA in a 3 h stomatal opening protocol [see Section 2.2.4(i)] was used. Stomatal aperture widths (n = 40) were determined (see Section 2.2.5) and AOV and LSD tests were carried out as described in Section 2.2.6. The results are from one experiment.

The data (see Figure C.1) show that 10^{-9} M PBI-51 affected the (±)-ABA dose-response curve for inhibition of stomatal opening. 10^{-9} M PBI-51 completely abolished the effect of 10^{-8} M (±)-ABA on stomatal opening. However, 10^{-9} M PBI-51 had no effect on the inhibition of stomatal opening induced by concentrations of (±)-ABA higher than 10^{-8} M. Interestingly, a solution containing both 10^{-9} M ABA and 10^{-9} M

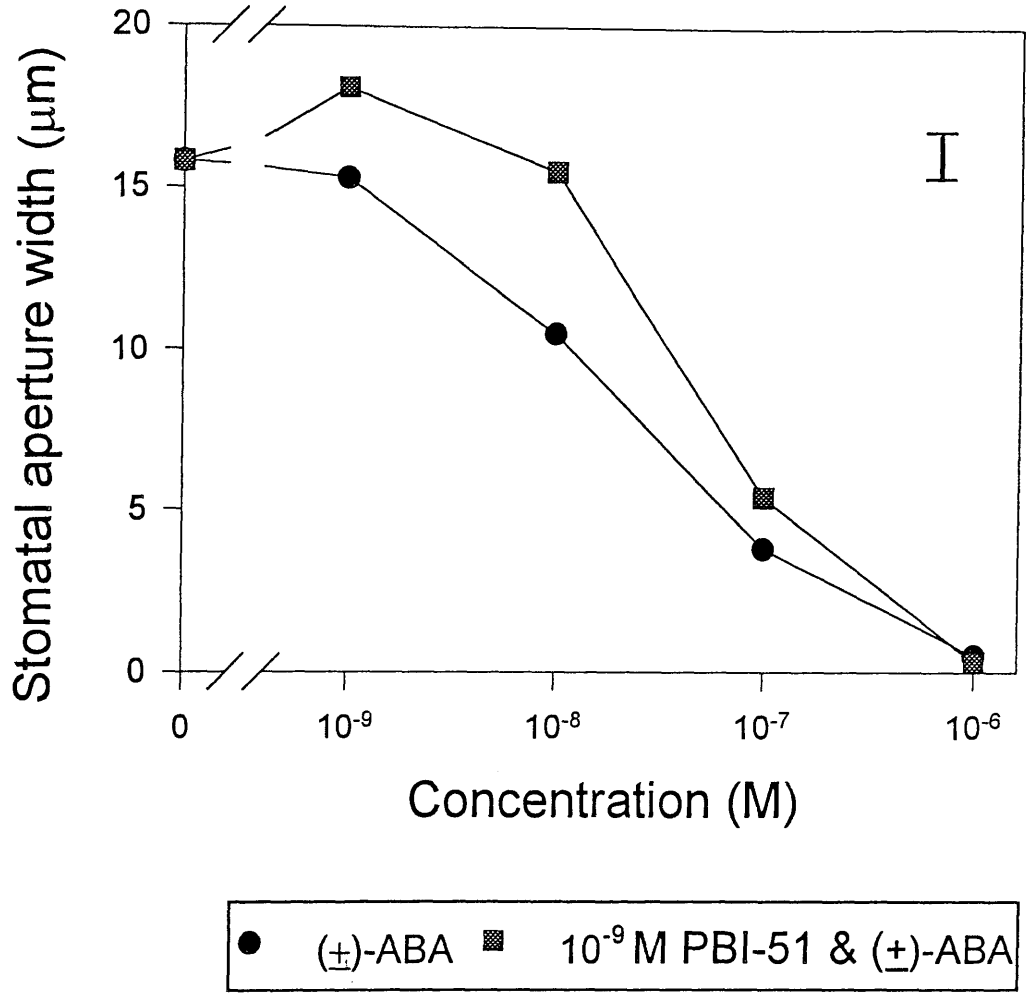


Figure C.1 The effect of 10^{-9} M PBI-51 on (±)-ABA-induced inhibition of stomatal opening in detached abaxial epidermis of *Commelina communis*. Each point represents an average of 40 measurements; the bar represents the LSD. [Positive and negative controls are (±)-ABA and zero ABA respectively].

PBI-51 promoted stomatal opening, whereas 10^{-9} M (±)-ABA had no effect on stomatal aperture.

Although preliminary, these results suggest that a low concentration of the ABA analogue PBI-51 antagonizes the effect of low concentrations (10^{-8} M) of (±)-ABA on stomatal opening. The fact that this antagonism is only seen at low and not high concentrations of PBI-51 suggests that PBI-51 displays concentration-dependent antagonism (see McAinsh *et al.*, 1991a). In the future, it would be advisable to increase the number of stomatal aperture measurements for the present data set to 120 to further investigate and validate the effects shown in Figure C.1. In addition, the effect of 10^{-9} M PBI-51 on ABA-induced promotion of stomatal closure could be investigated. It would also be of interest to determine whether 10^{-9} M PBI-51 antagonizes ABA-induced changes in stomatal aperture in abaxial epidermis of *N. tabacum*. However, the present data do not suggest that 10^{-9} M PBI-51 is a powerful ABA antagonist because it had no effect on the inhibition of stomatal opening by concentrations of 10^{-7} M (±)-ABA and above. It may be fruitful, in the future, to investigate the effect of higher concentrations of PBI-51 (from 10^{-8} to 10^{-6} M) on ABA-induced changes in stomatal aperture in these species [although it must be borne in mind that 10^{-5} M PBI-51 did not act as an antagonist of (±)-ABA]. If PBI-51 can totally inhibit the action of ABA, it could be used as a powerful tool in investigations of the role of ABA in guard cell stimulus-response coupling, for example in the CO_2 signalling pathway (see Webb *et al.*, 1996a; Webb & Hetherington, 1997) (see Section 1.8).

Appendix D

The Effect of Ethanol on Stomatal Aperture Width in *A. thaliana*

Ethanol was the solvent in which (±)-, (+)-, (−)-ABA, (±)-*trans, trans*-ABA, PBI-63 and PBI-51 were dissolved. Therefore, it was essential to determine that the ethanol concentration present in the incubation medium during stomatal bioassays had effect on stomatal aperture width in the abaxial epidermis of *A. thaliana*

A. thaliana. was grown and epidermis removed from the abaxial surface of the leaves as described in Sections 4.2.2 and 4.2.5(a) respectively. The effect of 0%, 0.01%, 0.1% or 1% (v/v) ethanol on stomatal opening was investigated by carrying out epidermal strip bioassays as described in Section 4.2.5(b). Stomatal aperture widths were determined (see Section 2.2.5) and AOV and LSD tests carried out as described in Section 4.2.6.

The data (see Table D.1) show that both 0.01% and 0.1% (v/v) ethanol had no effect on stomatal aperture in epidermis from *A. thaliana*. 1% (v/v) ethanol resulted in a reduction in stomatal aperture width (Table D.1). These data show that final concentrations of ethanol of 0.1% (v/v) and below in the incubation medium in stomatal bioassays have no adverse effects on stomatal aperture in detached epidermis of *A thaliana*. However, concentrations of ethanol as high as 1% (v/v) should not be included in the incubation medium in such assays because 1% (v/v) ethanol induced a reduction in stomatal aperture width.

Ethanol concentration in the incubation medium (%)	Mean stomatal aperture width (μm)	SE	Significantly different from 0% ethanol [LSD = 0.48 μm (p = 0.05)]
0	7.6	0.12	-
0.01	7.3	0.14	No
0.1	7.5	0.11	No
1.0	6.1	0.12	Yes

Table D.1 The effect of 0.01%, 0.1% and 1% (v/v) ethanol on stomatal aperture width in detached abaxial epidermis of *A. thaliana*. The control incubation medium (0% (v/v) ethanol) for the epidermal bioassay was MES/KOH, 50 mM KCl at 22±1°C.

Appendix E

Measurement of $[Ca^{2+}]_i$ in Guard Cells of *A. thaliana* Using Fura-2

A. thaliana was grown and epidermis removed from the leaves as described in Section 4.2.2. Measurements of $[Ca^{2+}]_i$ in guard cells of *A. thaliana* was performed in the same manner as that described for guard cells of *C. Communis* (see Section 5.2.3). However, the perfusion chamber was slightly smaller (10 mm x 7 mm) than that used for *C. communis* epidermis (McAinsh *et al.*, 1990, 1992) and the epidermis was perfused with MES/KOH at 22°C.

Preliminary studies showed that it was possible to apply the same techniques used to measure $[Ca^{2+}]_i$ in the guard cells of *C. communis* to the considerably smaller and less accessible guard cells of *A thaliana*. The $[Ca^{2+}]_i$ in a guard cell of *A. thaliana* microinjected iontophoretically with the Ca^{2+}-indicator fura-2 is shown in Figure E.1. Resting $[Ca^{2+}]_i$ was approximately 210 nm. However, it was not possible to measure the effects of ABA on $[Ca^{2+}]_i$ in this cell due to movement of fura-2 from the cytosol into the vacuole (Figure 5.2B shows fura-2 loaded into the vacuole of a guard cell of *C. communis*). This was accompanied by the collapse of the cell approximately 7 min after the start of the $[Ca^{2+}]_i$ trace shown in Figure E.1.

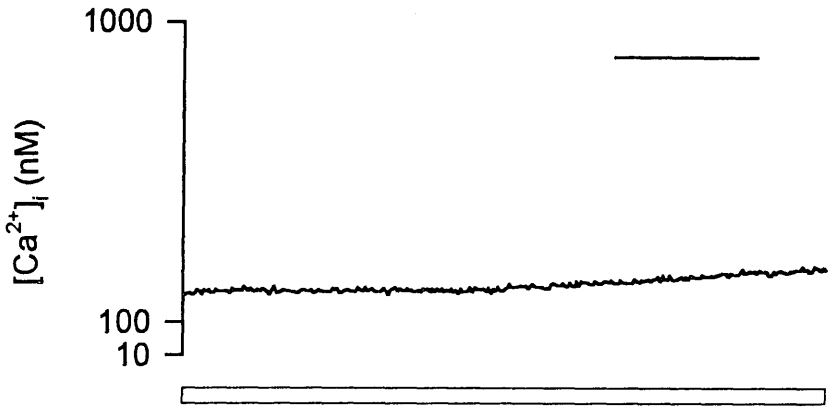


Figure E.1 Resting $[Ca^{2+}]_i$ in a guard cell of *A. thaliana.* Guard cells of closed stomata were microinjected with fura-2 into the cytosol. Resting $[Ca^{2+}]_i$ was determined in MES/KOH at 22°C (open box) (n = 1 cell). Bar = 1 min.

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