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The activity of 1,3-β-D-glucan synthase in aluminum-sensitive and aluminum-resistant cultivars of *Triticum aestivum* L.

by

Paulus Bhuja

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

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Sikumana- Kupang, NTT,
Indonesia 85225

Date: December 6, 1999.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The activity of 1,3-β-D-glucan synthase II in aluminum-sensitive and aluminum-resistant cultivars of *Triticum aestivum* L." submitted by Paulus Bhuja in partial fulfillment of the requirements for the degree of *Doctor of Philosophy in Physiology and Cell Biology.*

Gregory J. Taylor (supervisor)

David J. Gifford (Member)

Arnost Horak (Member)

John Hoddinott (Internal Examiner)

David D Cass (Internal Examiner)

Susanne Widell (External Examiner)

Date: December 6, 1999

Abstract

Differences in the behavior of 1,3-β-D-glucan synthase in isolated membranes and intact plant systems have not been resolved. Aluminum has been observed to stimulate callose accumulation in intact roots, but inhibit the activity of 1,3-β-D-glucan synthase (the enzyme mediating callose synthesis) in isolated plasma membrane (PM) vesicles. I postulated that these differences reflect access of Al and Ca to the cytosolic face of the enzyme. Experiments using Al-activated affinity columns showed that Al binds to 1,3-β-D-glucan synthase. Ninety six % of recovered 1,3-β-D-glucan synthase activity was eluted from Al-activated affinity columns by EDTA, while, 99.7% was detected in chelex-H_2O and K-phthalate washes from uncharged columns. A strong reduction in total 1,3-β-D-glucan synthase activity from Al-charged columns (81.7%) raised the possibility that binding of Al to 1,3-β-D-glucan synthase inhibits activity. This was confirmed in experiments with right-side-out (RSO) and inside-out (ISO) PM vesicles, where the presence of Al strongly inhibited the activity of 1,3-β-D-glucan synthase. The degree of inhibition was greater in ISO vesicles, perhaps reflecting better access of Al to the active site of this enzyme.

Efforts were made to demonstrate the inhibitory effect of Al on 1,3-β-D-glucan synthase in *in vivo* systems by adding detergents and the ionophore A23187, each of which increases membrane permeability to Ca, an activator of 1,3-β-D-glucan synthase. Under these conditions, the inhibitory effects of Al might then be observed. Digitonin, CHAPS, and A23187 increased callose accumulation in root tips of Al-sensitive and Al-

resistant cultivars, but not to the same extent as Al. The failure of these agents to fully engage 1,3-β-D-glucan synthase in the absence of Al could not be explained by a failure to trigger an increase in $[Ca^{2+}]_{cyt}$. Experiments using confocal laser scanning microscopy demonstrated that A23187 increased $[Ca^{2+}]_{cyt}$ to a greater extent than Al, while Al induced a 3-fold higher callose accumulation than A23187. In all *in vivo* experiments, the stimulatory effects of Al overshadowed the inhibitory effect. However, these experiments demonstrate that other factors must also be involved in modulating an increase in callose production *in vivo*, or the differences in behavior of *in vitro* and *in vivo* systems.

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List of abbreviations

Al: Aluminum
AM: Acetoxymethyl
ATPase: Adenosine triphosphatase
ATP: Adenosine triphosphate
Ca: Calcium
$[Ca^{2+}]_{cyt}$: cytosolic free calcium concentrations
CCD: Counter current distribution
CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CLSM: Confocal laser scanning microscopy
DMSO: Dimethyl sulfoxide
DTT: Dithiothreitol
EDTA: Ethylenediamine tetra acetic acid
ER: Endoplasmic reticulum
FW: fresh weight (mass)
GTP: Guanosine triphosphate
HEPES: 4-(-2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICM: Intracellular membranes
ISO: Inside-out
Kb: Kilobase
Km: Michaelis-Menten's constant
MES: 2-[N-Morpholino]ethanesulfonic acid
MF: Microsomal fraction
MOPS: 3-(N-morpholino)propanesulfonic acid
mV: milivolt
NADH: Nicotinamide adenine dinucleotide (reduced)
NAD(P)H: Nicotinamine adenine dinucleotide phosphate (reduced)
PEG: Polyethylene glycol
PM: Plasma membrane
PMSF: Phenylmethylsulfonyl fluoride
PPF: Photosynthetic photon flux
PVP: Poly(vinylpyrrolidone)
RSO: Right-side-out
UDP-glucose: Uridine diphosphate-glucose

1. Introduction

1.1 General

Aluminum (Al) is the most abundant metal in the earth's crust, comprising approximately 7.5% by weight (Haug, 1984). In soils with a pH of ≥ 5, Al is predominantly bound as insoluble oxides and complex aluminosilicates. Aluminum is not strongly toxic at this range of pH, but can have some negative effects, including changes in membrane electrical potential, the activities of several enzymes in the plasma membrane (PM) (Lindberg *et al.*, 1991; Lindberg and Griffiths, 1993; Widell *et al.*, 1994), and the function of GTP-proteins that have been shown to stimulate calcium (Ca) fluxes across the PM (Elliot and Yao, 1989). However, as soil pH drops, there is a release of ionic Al^{3+} and a reduction in the availability of exchangeable cations such as Ca^{2+}, Mg^{2+}, and K^{+} (Kinraide and Parker, 1989). Under these conditions, Al can be strongly phytotoxic. For example, Al has been shown to rapidly inhibit both primary root and root hair growth, resulting in poor nutrient acquisition and shoot nutrient deficiencies (Taylor, 1991; Kochian, 1995). Consequently, excess available Al may determine the distribution of plant species and ecotypes, and limit growth and yield of crops (Kinzel, 1983; Taylor and Foy, 1984).

Externally applied Al rapidly binds to sites within the apoplast (Zhang and Taylor, 1989; Blamey *et al.*, 1990). The extent to which Al is bound depends on the cation exchange capacity of plant roots resulting from negative charges carried on pectins and proteins in the cell wall. In addition, interactions of Al with other cell-wall constituents such as enzymes, extensins, and xyloglucans likely alter not only the physical, but also

the functional integrity of the cell wall. For example, a modification of the charge distribution in the cell wall affects uptake of cations (Rengel, 1990; Godbold *et al.*, 1988) and anions (Nichol *et al.*, 1993).

Aluminum not only rapidly affects the cell wall (Le Van *et al.*, 1994), but also the PM. This may include modification of membrane structure (Zhang *et al.*, 1997), membrane fluidity (Vierstra and Haug, 1978; Zel *et al.*, 1993a,b), H^+-ATPase (Widell *et al.*, 1994), membrane potential (Gassmann and Schroeder, 1994; Lindberg et al., 1991), induction of callose synthesis (Wissemeier *et al.*, 1987; Schreiner *et al.*, 1994; Zhang *et al.*, 1994); reduction of K^+ efflux (Horst *et al.*, 1992; Cakmak and Horst, 1991), and inhibition of Ca^{2+} influx (Lindberg, 1990; Huang *et al.*, 1992a,b; Rengel, 1992 a,b,c; Rengel and Elliot, 1992). Although the actively dividing and expanding cells of the root apex have been identified as the principal site of toxicity (Ryan *et al.*, 1993), the causes of Al toxicity have remained evasive.

Any investigation of the interaction of Al^{3+} with biological membranes is tangled by the difficulties in distinguishing between ion binding to lipids and proteins. Kinraide and Parker (1987) suggested that Al^{n+} may either displace Ca^{2+} from PM binding sites or bind to some other site from which it can be displaced by Ca^{2+} and other cations. It is possible that Al-sensitive genotypes have a greater number of Al-binding sites at the cell surface, or that the binding sites have a higher binding affinity. Caldwell (1989) demonstrated that the PM proteins of an Al-sensitive wheat genotype bound Al^{3+} more tightly than those of an Al-resistant genotype. This greater binding capacity may or may

not be associated with a greater negative cell-surface charge density. Yermiyahu *et al.* (1997) suggested that cell-surface charge is not the only factor to differ the sensitivity or resistance of wheat to Al^{3+}. In addition to cell-surface charge, Al may bind to uncharged (zwitterionic) ligands (Akeson *et al.*, 1989), and other ligands of dissimilar charge which may have different binding strength for Al^{3+}.

1.2 Effect of aluminum on 1,3-β-D-glucan synthase and callose production

Several authors have speculated that an apoplastic lesion might be capable of mediating the toxic effects of Al (Taylor, 1995; Kochian, 1995). While debate on this issue is likely to continue, it is clear that biological processes within the apoplast are rapidly affected. One of the most well documented changes is an increase in synthesis of 1,3-β-D-glucans or callose (Kauss, 1996; Zhang *et al.*, 1994). Synthesis of callose is normally favored by high concentrations of UDP-glucose (Bulone *et al.*, 1990; Girard *et al.*, 1991, 1992) and is rapidly triggered under stressful conditions involving perturbation of the PM (Fincher and Stone, 1981). Callose forms a more irregular, space-filling helix than cellulose. It is also more vulnerable to chemical and enzymatic degradation than cellulose. Thus, it is unsuitable as a building material for highly organized structures such as the cell wall. However, it seems that callose is well suited as a temporary wall material in situations where a high degree of organization and stability are not the primary importance. In these circumstances, callose can play a role as a support on which

cellulose microfibrils and other cell wall polymers can associate to form a more complicated network (Fredrikson, 1993).

Callose is synthesized by a few cell types at specific stages of wall development (such as that formed in growing pollen tubes and in phragmoplasts of dividing cells) and by most cells in response to wounding (Delmer, 1991). Several authors have named developmentally-regulated 1,3-β-D-glucan synthase as glucan synthase I (Fink *et al.*, 1978; Van Der Woude *et al.*, 1974), while wound-activated 1,3-β-D-glucan synthase is called glucan synthase II (EC 2.4.1.34; Van Der Woude, 1974).

Wound-activated 1,3-β-D-glucan synthase is a Ca^{2+}-dependent enzyme with a low Km for UDP-glucose (0.45 mM), and is generally unaffected or only partially inhibited by trypsin (Fink *et al.*, 1987; Hayashi *et al.*, 1987; Sloan and Wasserman, 1989; Read and Delmer, 1990; Wu and Wasserman, 1993; Kauss, 1996). The activity of wound-regulated 1,3-β-D-glucan synthase is regulated *in vitro* by Ca^{2+}, polyamines, lipids and proteases and uses UDP-glucose as a substrate (Aist, 1983; Kauss *et al.*, 1983; Kauss and Jeblick, 1985; Fredriksson and Larsson, 1989; Lin and Lee, 1991). Calcium activates wound-regulated 1,3-β-D-glucan synthase at μM concentrations in a rapid and reversible manner, both by lowering the Km and by increasing the V_{max} (Fredrikson, 1993). Thus, Ca seems to play a role as an allosteric effector of the enzyme (Kauss, 1987). These properties distinguish the wound-regulated 1,3-β-D-glucan synthase from the developmentally-regulated 1,3-β-D-glucan synthase, which has a higher Km for UDP-glucose (2.5 mM), is activated by trypsin, and lacks a requirement for Ca^{2+} (Schlupmann *et al.*, 1994). Both

enzymes, however, can be activated by β-glucosides (Schlupmann *et al.*, 1994; Hayashi *et al.*, 1987; Callaghan *et al.*, 1988; Kauss and Jeblick, 1991; Ohana *et al.*, 1992), which may play a role as a primer for callose synthesis (Fredrikson, 1993). This suggests that they share common regulatory features.

Cellulose synthase (UDP-glucose: 1,4-β-D-glucan glucosyl transferase; EC 2.4.1.12) and wound-regulated 1,3-β-D-glucan synthase are each integral components of the PM and, with the exception of possible activity in the Golgi (Gibeaut and Carpita, 1994a), are not located in intracellular membranes (Fredrikson, 1993). Polysaccharide synthesis mediated by these enzymes may involve several independent steps such as chain initiation, chain extension (polymerization), and chain termination (Iiyama *et al.*, 1993; Fincher and Stone, 1982). Any factor that affects one of these steps can potentially increase or decrease the amount of reaction product. Because of the C-3 hydroxyl position of glucose, (1,3)-linked chains can be formed without substantial reorientation of the polymer chain or the synthase during polymerization. Thus, it has been postulated that a disorganized PM cellulose synthase could be capable of catalyzing synthesis of wound-activated callose (normally associated with 1,3-β-D-glucan synthase; Gibeaut and Carpita, 1994b; Delmer, 1999). Polymerization of glucans requires coordinated transport of substrates across the PM and may rely on a delicate membrane orientation of the synthase complex. This may account for difficulties encountered in conserving natural glucosyl transferase activities (cellulose synthase activity) *in vitro* (Gibeaut and Carpita, 1994b).

Isolated plant PM contains an active 1,3-β-D-glucan synthase, which has been used as a marker for the PM (Van Der Woude *et al.*, 1974; Ray, 1977; Widell *et al.*, 1994). The active site of the enzyme is located on the cytoplasmic surface of the PM (Larsson *et al.*, 1984), while the product (cell wall callose) is deposited on the apoplastic side of the membrane (Fredrikson and Larsson, 1992). Work with 1,3-β-D-glucan synthase *in vitro* has shown that UDP-glucose is available to the enzyme in right-side-out (RSO) vesicles in the presence of detergents such as digitonin, which permeabilize the membranes (Eiberger and Wasserman, 1987; Hayashi *et al.*, 1987; Read and Delmer, 1987; Dhugga and Ray, 1994; Widell *et al.*, 1994; Fredriksson, 1993). However, digitonin and some other amphipathic substances such as CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) may also interact directly with 1,3-β-D-glucan synthase to activate the enzyme (Kauss and Jeblick, 1986; Fredrikson and Larsson, 1989).

Recent studies indicate that the activities of cellulose synthase (Huck, 1972) and 1,3-β-D-glucan synthase (Widell *et al*, 1994) are affected by aluminum. If these two activities do indeed reflect that activity of a single enzyme, such a result would not be surprising. As of yet, an effect of Al on developmentally-regulated 1,3-β-D-glucan synthase has not been reported.

1.3 Effect of aluminum on cytosolic calcium homeostasis

Aluminum-stressed plants typically contain low concentrations of Ca and symptoms of Al toxicity frequently resemble those of Ca deficiency (Foy, 1988). Low concentrations of Ca in Al-stressed plants may be due to direct interference of Al with membrane-transport mechanisms (Keltjens, 1988; Rengel and Robinson, 1989). Furthermore, under acidic conditions, polyvalent cationic species of Al tend to bind very tightly to negative charges in the apoplastic space, thus greatly reducing the amount of Ca^{2+} in the Donnan free space (Rengel and Robinson, 1989). These physicochemical reactions may partly explain the deleterious effect of Al on Ca^{2+} accumulation in cells.

Research has also demonstrated that Al toxicity is linked to changes in cellular Ca homeostasis and the blockage of Ca^{2+}-permeable channels in the PM. Working with BY-2 tobacco cell cultures, Jones *et al.* (1998) demonstrated that Al exposure resulted in a prolonged reduction in cytosolic free calcium concentrations ($[Ca^{2+}]_{cyt}$), suggesting that Al may act to block Ca^{2+} channels in the PM. While Al has been shown to block Ca^{2+} channels in the root-cell PM (Kochian, 1995; Rengel and Elliot, 1992) and reduce calcium influx (Ryan and Kochian, 1993), increase in cytosolic Ca^{2+} have nonetheless been observed after short term exposure of *Triticum aestivum* (Lindberg and Strid, 1997) and *Hordeum vulgare* (Nichol and Oliviera, 1995) to Al. Changes in $[Ca^{2+}]_{cyt}$ may play a crucial role in the phytotoxic activity of Al. It has been postulated by numerous authors that Al may interfere with cellular Ca^{2+} homeostasis, leading to a breakdown of the Ca^{2+}-dependent signal transduction cascades that may be necessary for both cell division and elongation (Haug, 1984; Taylor, 1990; Rengel, 1992a; Delhaize and Ryan, 1995;

Kochian, 1995). Irrespective of the proposed role of Ca^{2+} in regulating cell expansion and division, homeostatic control of $[Ca^{2+}]_{cyt}$ is known to be essential for continued cell viability (Bush, 1995).

1.4 Contradictory evidence

Callose formation is driven by the activity of the Ca-dependent, 1,3-β-D-glucan synthase (EC 2.4.1.34). Quantitative studies conducted by Zhang *et al.* (1994) showed that synthesis of callose in roots (*in vivo*) of Al-sensitive cultivars of *Triticum aestivum* increased 38-fold after 48 hours of exposure to 75 μM Al. While, this result is consistent with a that of Nichol and Oliveira (1995), it is somewhat surprising since Al also interferes with the absorption of Ca^{2+} by roots (Taylor, 1988). Using calcium-sensitive microelectrodes, Ryan and Kochian (1993) documented a 40% reduction of calcium influx in root tips of Al-sensitive cultivars of *Triticum aestivum* after exposure to 50 μM Al. Additional concern also arises from studies with purified PM (*in vitro*), which have shown that Al is a powerful inhibitor of 1,3-β-D-glucan synthase activity (Widell *et al.*, 1994).

Differences in the behavior of 1,3-β-D-glucan synthase in isolated membranes and intact systems have not been resolved. The stimulatory effect of Al *in vivo* could be facilitated by an Al-induced increase in $[Ca^{2+}]_{cyt}$ (Lindberg and Strid, 1997), which is known to stimulate 1,3-β-D-glucan synthase activity. Similar increases in Ca would not be observed in isolated membrane vesicles. Another important difference between *in vivo*

and *in vitro* systems may be the access of Al to the cytoplasmic face of 1,3-β-D-glucan synthase. In *in vitro* systems, access of Al to the cytoplasmic face of 1,3-β-D-glucan synthase is frequently facilitated by the presence of detergents and ionophores. The situation may be different *in vivo*. Several studies have suggested that Al can cross the PM into the cytoplasm (Zhang *et al.*, 1994; Archambault *et al.*, 1996; Lazof *et al.*, 1997; Taylor *et al.*, 1999), however, the activity of free Al inside the cytoplasm is likely limited to 10^{-10} M by formation of insoluble hydroxy-Al-phosphate compounds (Taylor, 1995). Thus, Al may have limited access to the cytoplasmic face of 1,3-β-D-glucan synthase. Under these conditions, the effect of Al on cytosolic Ca^{2+} may dominate the *in vivo* response of 1,3-β-D-glucan synthase.

1.5 Objective

The primary objective of my research is to test potential mechanisms that regulate the synthesis of 1,3-β-D-glucan under conditions of Al stress. I have hypothesized that Al inhibits the activity of 1,3-β-D-glucan synthase, but this effect may not be detected *in vivo*. Aluminum may be able to cross the PM to gain access to the cytosolic face of 1,3-β-D-glucan synthase, but its activity may be too low to have a powerful inhibitory effect. Consequently the stimulatory effect of Al resulting from an Al-induced increase in cytosolic Ca^{2+} is greater than the direct inhibitory effect on 1,3-β-D-glucan synthase activity. In contrast, inhibition of 1,3-β-D-glucan synthase activity *in vitro* may reflect better access of this metal to the cytosolic side of PM and the lack of internal sources of

calcium such as the endoplasmic reticulum, mitochondria and vacuoles. If my hypothesis is correct, it should be possible to verify the following predictions:

a. The 1,3-β-D-glucan synthase enzyme binds to Al *in vitro*.

b. The activity of 1,3-β-D-glucan synthase in right-side-out (RSO) and inside-out (ISO) PM vesicles (*in vitro*) isolated from Al-sensitive and Al-resistant cultivars of *Triticum aestivum* is inhibited by Al, regardless the presence of detergents and ionophores.

c. *In vivo*, 1,3-β-D-glucan synthase activity is inhibited by Al when the enzyme is initially stimulated by high cytosolic Ca^{2+} levels (induced by calcium ionophores such as A23187). The extent of inhibition may increase under conditions promoting free access of Al to the cytoplasm (induced by detergents).

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2. Plasma membrane isolation and markers: technical approaches

2.1 Introduction

Plant plasma membranes (PM) not only form a living barrier between cells and their surroundings, but are also their "eyes and ears" (Fosket, 1994) as membrane-bound receptors monitor signals coming from the external environment. Preparation of plant PM is essential to elucidating the role of membrane-bound enzymes such as 1,3-β-D-glucan synthase in response to environmental stresses.

Numerous cytochemical and enzymological techniques have been developed to isolate and identify PM vesicles. Polysaccharides present on the external face of the PM can be stained using periodic acid oxidation and reaction of the aldehyde groups with phosphotungstic and chromic acids (Roland *et al.*, 1972; Hodges *et al.*, 1972; Leonard and Van Der Woude, 1976; Van Der Woude, 1974; Nagahasi *et al.*, 1978) or with silicotungstic acid (Widell *et al.*, 1982). Alternatively, cytochemical localization of PM-specific enzymes such as 1,3-β-D-glucan synthase, an enzyme which catalyses incorporation of glucose from UDP-glucose into 1,3-β-D-polyglucans (Widell and Larsson, 1990; Larsson *et al.*, 1994), and vanadate-sensitive ATPase (Widell and Larsson, 1990; Serrano, 1990) have been widely used as the markers for the PM. Intracellular enzymes, such as the nitrate-sensitive ATPase and antimycin A-sensitive NAD(P)H-cytochrome c reductase (localized at the tonoplast and endoplasmic reticulum, respectively) can be assayed to detect contamination of PM preparations with endomembranes.

In addition to its use as a marker of the PM, the role of 1,3-β-D-glucan synthase in response to biological, physical and chemical stresses (Schreiner, 1992; Kauss, 1990; Stone, 1984) has been intensively investigated. The activity of 1,3-β-D-glucan synthase has been correlated with aluminum (Al) application in *Glycine max* (Wissemeier *et al.*, 1993, Wissemeier and Horst, 1995), *Picea abies* (Jorns *et al.*, 1991), *Triticum aestivum* (Schreiner *et al.*, 1994; Zhang *et al.*, 1994), and *Zea mays* (Llugany *et al.*, 1994). Interestingly, this membrane-bound enzyme has its active site facing the cytosolic side of the PM (Fredrikson, 1995). Depending on the orientation of the vesicle, the active site may either be exposed to the bulk solution or hidden inside the vesicle. In an inside-out (ISO) orientation, the active site of the enzyme is in direct contact with substrates, cofactors, and inhibitors in the bulk solution. Conversely, in a right-side-out (RSO) orientation, access to the active site may be limited. When working with RSO vesicles, detergents and ionophores (such as digitonin and A23187 respectively) can be used to facilitate access of substrates, cofactors, and inhibitors to the active site on the cytosolic face of the enzyme. Given the importance of vesicle sidedness in relation to enzyme activity, preparation of PM vesicles of known sidedness is necessary for my experiments.

The purpose of these experiments was to develop reliable techniques for isolation and purification of RSO and ISO PM vesicles that can be subsequently used to characterize the effect of Al on the activity of 1,3-β-D-glucan synthase. Using an aqueous two-phase system (Larsson *et al.*, 1994), I have isolated highly-purified, RSO, PM vesicles from roots of *Triticum aetivum* cv. Alikat and Katepwa. I then used multiple freeze thaw treatments to invert vesicle sidedness (from RSO to ISO) and Counter-

Current Distribution (CCD; Larsson *et al.*, 1994) to isolate fractions enriched in ISO (67%) and RSO (93.9%) PM vesicles.

2.2 Materials and methods

2.2.1 Plant material

Seeds of Al-tolerant (Alikat) and Al-sensitive (Katepwa) cultivars of *Triticum aestivum* were surface sterilized in 1.2% sodium hypochlorite for 20 min and germinated overnight in solution of 0.005 g L^{-1} Vitavax to limit fungal growth. Seedlings were then grown for 5 days on nylon mesh suspended over 15 L of full nutrient solution containing (mM): 3.30 NO_3^--N, 0.30 NH_4^+-N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S; and (μM): 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo (pH 4.4) in a growth chamber with 16 h of light (20 °C, 68% relative humidity) and 8 h of darkness (16 °C, 85% relative humidity). The photosynthetic photon flux (PPF) was 335 ± 12 μmol m^{-2} s^{-1} at plant base level. After 5 days of growth, whole roots were harvested for PM isolation.

2.2.2 Isolation of plasma membrane vesicles

Roots (100 g fresh weight) were homogenized in 220 ml (1:2.2 w/v ratio) of a homogenization buffer consisting of 0.25 M sucrose, 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-KOH (pH 7.5), 5 mM EDTA, 5 mM dithiothreitol (DTT), 5 mM ascorbic acid, 0.2% (w/v) bovine serum albumin, 0.2% casein (enzymatic hydrolyzate boiled for 8 min), and 0.6% insoluble poly(vinylpyrrolidone) (PVP) (Larsson *et al.*, 1994), using a Proctor Silex instablend. The

homogenate was filtered through 240-µm nylon mesh, and plastids, mitochondria, and cell debris were pelleted at 10,000 g (Beckman JA-17 rotor) for 10 min. Microsomal membranes were pelleted from the supernatant by centrifugation at 80,000 g (Beckman SW28) for 1h. Pellets were then resuspended in 10 ml in of a resuspension buffer containing 0.25 M sucrose, 5 mM potassium phosphate buffer (pH 7.8), 5 mM KCl, 0.1 mM EDTA, and 2 mM DTT. Nine ml of resuspended microsomal membranes were added to 27 ml of a two-phase mixture (Larsson *et al.*, 1994) with a final composition of 6.2% (w/w) each of dextran T500 and poly(ethylene glycol) (PEG) 3350 in 0.25 M sucrose, 5 mM potassium phosphate buffer (pH 7.8), 5 mM potassium chloride (KCl), 1 mM DTT, and 0.1 mM EDTA. Separation of the phases was achieved by low speed centrifugation (1,500 g) for 3 min. Once a clear interface had formed, approximately 90% of the upper phase (U1) was carefully removed to a second tube without disturbing the interface. The upper phase (U1) and the remaining lower phase (L1) were diluted with fresh lower and upper phase (respectively). These fresh phases were prepared by partitioning a bulk phase mixture containing 97.5 g of 20% Dextran T500, 48.75 g of 40% PEG 3350, 25.68 g sucrose, 7.5 ml of 5 mM potassium phosphate buffer (pH 7.8), 750 µl of 5 mM KCl, 750 µl of 0.1 mM EDTA, and 0.005g DTT adjusted to 300 ml with ddH_2O. After the two tubes were inverted twenty four times, they were centrifuged at the same speed and time as before. Approximately 90% of the upper phase of U1 tube was removed (U2) to a third tube containing fresh lower phase, and the upper phase of L1 tube (U1') was added to U1 tube's remaining lower phase (L1'). The remaining lower phase of L1 tube (L2) was collected as the intracellular membrane (ICM) source. After centrifugation, the upper phase of the third tube was collected as U3, and the upper phase

of L1' tube (U2') was added to the third tube's remaining lower phase and collected as U3' after centrifugation.

The rest (approximately 1 ml) of resuspended microsomal fraction (MF) and the combined upper phases (U3 + U3') containing PM vesicles were diluted five times with a dilution buffer containing 0.25 M sucrose, 5 mM 2-[N-Morpholino] ethanesulfonic acid (MES)-Tris, (pH 7.0), 2 mM DTT, 1 mM Phenylmethyanesulfonyl fluoride (PMSF), and 5 mM EDTA. The L2 fraction was diluted eight to ten times in dilution buffer. After centrifuging at 100,000 g (Beckman SW 40) for 1h, pellets from these three preparations were diluted in an appropriate volume of dilution buffer and collected as microsomal membranes (MF), PM, and intracellular membranes (ICM) fractions. Membrane protein from each fraction was assayed according to Bradford (1976), using bovine serum albumen as a standard.

2.2.3 Enzyme markers

In order to positively identify the plasma membrane and assess the extent of contamination by other membrane components, specific markers are required. In this experiment, 1,3-β-D-glucan synthase (EC 2.4.1.34) and vanadate-sensitive ATPase (EC 3.1.6.35) were used as positive markers (localized at PM), whereas nitrate-sensitive ATPase (EC 3.6.1.3) (mainly localized at tonoplast), azide-sensitive ATPase (EC 3.6.1.34) and cytochrome c-oxidase (EC 1.9.3.1) (mainly localized at mitochondria), and cytochrome c-reductase (EC 1.6.99.3) (mainly localized at endoplasmic reticulum) were used as negative markers.

2.2.3.1 1,3-β-D-glucan synthase

This assay was carried out according to Larsson *et al.* (1994) in a 100 µL total volume containing 5-10 µg protein from fresh isolated membrane vesicles, 0.33 M sucrose, 50 mM 4-(-2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (pH 7.25), 2 mM spermine, 20 mM cellobiose, 0.1 mM $CaCl_2$, and 2 mM DTT, with or without 0.01% (w/v) digitonin to determine enzyme latency. Latency was measured as the increase in 1,3-β-D-glucan synthase activity observed in the presence of 0.010 % digitonin. The reaction was started by addition of UDP[^{14}C]glucose (20 Gbq/mol) to a final concentration of 2 mM. The reaction was run for 45 min at room temperature and was terminated by immersing in boiling water for 5 min. The samples were then transferred to paper filters (25-mm circles, Whatman, 3 MM), dried, and washed 2 times for 1 hr each in 0.35 M ammonium acetate (pH 3.6), and 30% (v/v) ethanol (60 filters per 0.5 L). Filters were dried and the radioactivity measured by liquid scintillation counting (Beckman LS6000TA). Background was determined by running blanks without protein.

2.2.3.2 Total ATPase

Total ATPase activity in the PM vesicles was measured according to Larsson *et al.* (1994) with several modification. The assay was run in a total volume of 1 ml containing 50-100 µg membrane protein, 0.33 M sucrose, 50 mM MES-KOH (pH 6.5), 4 mM $MgSO_4$, 0.1 mM sodium molybdate, 0.1 mM EDTA, 3 mM ATP, and 1 mM DTT. The assay was run at 37 °C for 30 min in the absence of orthovanadate, with and without 0.01% digitonin. The reaction was started by adding ATP and blanks lacking $MgSO_4$

were run in parallel. The enzyme reaction was stopped by adding 500 μL of Ames (1966) reagent, which contains six parts of 0.42% (w/v) ammonium molybdate in 1 N H_2SO_4 mixed with one part of 10 % (w/v) ascorbic acid (Briskin *et al.*, 1987). After incubation at room temperature for 20 min, the optical density was determined at 700 nm.

2.2.3.3 Vanadate-sensitive ATPase

The assay for vanadate-sensitive ATPase was the same as the assay for total ATPase (2.2.3.2 above), except that the assay was run in a total volume of 1 ml containing 50-100 μg membrane protein, 0.33 M sucrose, 50 mM MES-KOH (pH 6.5), 4 mM $MgSO_4$, 50 mM KNO_3, 1 mM sodium azide, 0.1 mM sodium molybdate, 0.1 mM EDTA, 3 mM ATP, and 1 mM DTT. The assay was run at 37 °C for 30 min in the presence and absence of 0.1 mM orthovanadate, with and without 0.01% digitonin.

2.2.3.4 Nitrate-sensitive and azide-sensitive ATPases

These reactions were performed as for the vanadate-sensitive ATPase assay, except that they were run in 30 mM Tris-MES (pH 8), in the presence and absence of 50 mM KNO_3 for the nitrate-sensitive ATPase and 30 mM Tris-MES (pH 8.5), in the presence or absence of 1 mM sodium azide for the azide-sensitive ATPase (Briskin *et al.*, 1987; Larsson *et al.*, 1994).

2.2.3.5 Cytochrome c-reductase

NADH-cytochrome c-reductase activity was measured as $\Delta(A_{550} - A_{600})$ using 40 μM cytochrome-c as an electron acceptor instead of 0.2 mM $K_3[Fe(CN)_6]$, and with 0.4 μM antimycin A and 1 mM KCN present in the assay medium. The assay was run at 25 °C in 1 ml of 0.33 M sucrose, 25 mM HEPES-KOH (pH 7.3), 0.25 mM NADH, and 10 μg protein in the absence of digitonin. Since this enzyme is localized at the cytoplasmic face of the plasma membranes, cytochrome c-reductase can only be used as a marker for the endoplasmic reticulum in the absence of detergent (Briskin *et al.*, 1987).

2.2.3.6 NADH-cytochrome c-oxidase

Cytochrome c-oxidase activity was measured using 0.2 mM K_3 $[Fe(CN)_6]$ instead of 40 μM cytochrome-c as an electron acceptor. The assay mixture contained the same components as for cytochrome c-reductase, but without 0.4 μM antimycin and 1 mM KCN present in the medium. The activity was recorded at A_{550}, and in the presence and absence of 0.025% (w/v) Triton X-100 (Larsson *et al.*, 1994). The reaction was initiated by the addition of NADH.

2.2.4 Separation of inside-out vesicles from right-side-out vesicles using counter current distribution.

Highly purified, RSO, PM vesicles obtained using the two-phase system were frozen and thawed to produce a mixture of ISO and RSO vesicles. Aliquots of 0.8 ml (4-6

mg protein) were repeatedly (5-7 times) frozen in liquid N_2 and thawed in water at 20 °C. The freeze-thawed plasma membranes were then subfractionated using the CCD procedure (Askerlund and Albertson, 1994; Larsson *et al.*, 1994) to produce one fraction enriched in ISO vesicles, another enriched in RSO vesicles, and two intermediate fractions. Aliquots (0.8 ml) of freeze-thawed plasma membranes were added to a phase mixture (7.2 g) to give a phase system (8.0 g) with a final composition of 6.2% (w/w) Dextran T500, 6.2% (w/w) PEG 3350, 0.33 M sucrose, 5 mM potassium phosphate (pH 7.8), 5 mM KCl, 1 mM DTT, and 0.1 mM EDTA (at 4 °C). The phase system was shaken and centrifuged for approximately 3 min at 1500 g to promote phase separation. Approximately 90% of the upper phase was removed without disturbing the interface and transferred to a second tube containing fresh lower phase plus fresh upper phase corresponding to the 10% upper phase not removed from tube 1. Fresh upper phase was added to tube 1 and mixing and centrifuging were repeated. Approximately 90% of the upper phase in tube 2 was transferred to a third tube that also contained fresh lower phase plus the additional 10% of the fresh upper phase. After that, approximately 90% of the upper phase in tube 1 was transferred to tube 2 a second time, and fresh upper phase was added to tube 1 for the third time. The procedure was repeated once again; approximately 90% of the third tube's upper phase was transferred to a new tube (tube 4). At this stage, the lower phase of tube 1 was enriched in ISO vesicles, while the upper phase in tube 4 was enriched in RSO PM vesicles. Enriched fractions were diluted with 0.33 M sucrose, 10 mM MOPS-KOH (pH 7.5), 0.1 mM EDTA, and 1 mM DTT dilution buffer. The plasma membranes from these two fractions were pelleted at 100,000 g for 1 hr and the pellets were resuspended in the same medium (without DTT).

2.3 Results

After performing the two-phase partitioning procedure, the PM fraction accounted for 9.7% of total microsomal protein (16.6 mg) and the intracellular membrane (ICM) fraction accounted for 77.2% of the total microsomal protein. Thus 86.9% of total protein was recovered (Table 2.1). Different species, varieties, and organs produce more or less PM. For example, Larsson *et al.* (1994) recovered 17% of microsomal membrane protein in the PM fraction and 65% in the ICM fraction in their work with leaves of sugar beet, whereas Galtier *et al.* (1988) recovered 9.5% of microsomal membrane protein in the PM fraction from roots of corn. It is therefore not surprising that 77.2% the total protein was attributed to ICM protein, which may contain the membrane proteins from endoplasmic reticulum, mitochondria, tonoplast and Golgi apparatus.

The most reliable marker for the plant PM is 1,3-β-D-glucan synthase. Analyses of 1,3-β-D-glucan synthase activity showed that 82.0% of total activity was located in the plasma membrane fraction, while 4.5% was located in the intracellular membranes. In addition, the specific activity of 1,3-β-D-glucan synthase in the PM was approximately 144-fold (360 ± 16 nmol mg protein^{-1} min^{-1}) higher than that in the ICM (2.5 ± 0.1 nmol mg protein^{-1} min^{-1}; Table 2.1).

Vanadate-sensitive ATPase, which is insensitive to azide, molybdate and NO_3^- (Widell and Larsson, 1990), is also a classical marker for the plant plasma membrane (Hodges *et al.*, 1972). The vanadate-sensitive ATPase forms a phosphorylated

Table 2.1. Enzyme marker assays to test the purity of membrane fractions from roots of *Triticum aestivum* cv. Katepwa. 1,3-β-D-glucan synthase provides a marker for the plasma membrane[1], cytochrome c-oxidase provides a marker for the mitochondria, and cytochrome c-reductase provides a marker for the endoplasmic reticulum. Values are means ± SE of three replicates.

	Total Protein		1,3-β-D-glucan synthase			Cytochrome c-oxidase			Cytochrome c-reductase		
Faction	(mg)	% recovery	Total A. nmol min^{-1}	% recovery	S.A. nmol mg^{-1} min^{-1}	Total A. µmol min^{-1}	% recovery	S.A. µmol mg^{-1} min^{-1}	Total A. µmol min^{-1}	% recovery	S.A. µmol mg^{-1} min^{-1}
Microsomal membranes	16.6	100	706 ± 35	100	43 ± 5	9.30 ± 0.50	100	0.56 ± 0.03	8.50 ± 0.33	100	0.51 ± 0.02
Plasma membrane	1.6	9.7	580 ± 12	82.0 ± 0.1	360 ± 16	0.10 ± 0.01	1.1	0.06 ± 0.01	0.12 ± 0.01	1.4	0.07 ± 0.01
Intracellular membranes	12.8	77.2	32 ± 3	4.5 ± 0.1	2.5 ± 0.1	8.20 ± 0.26	88.2	0.64 ± 0.02	7.56 ± 0.26	88.9	0.59 ± 0.02
PM+ ICM	14.4	86.9	608 ± 15	86.5 ± 0.2		8.30 ± 0.27	89.3		7.68 ± 0.27	90.3	

[1] Plasma membrane fractions show 90.6 % latency (right-side-out) as determined by 1,3-β-D-glucan synthase activity in the presence and absence of 0.01% digitonin.

intermediate during ATP hydrolysis, a property not shared by ATPases found in the tonoplast, mitochondria and plastids (Macara, 1980). From the total activity of vanadate-sensitive ATPase, 84.1% was localized in the PM fraction, compared with 5.6% in the ICM fraction (Table 2.2). The specific activity of the vanadate-sensitive ATPase was 120-fold higher in the PM fraction (2.4 ± 0.20 μmol mg protein^{-1} min^{-1}) than in the ICM fraction (0.02 ± 0.1 μmol mg protein^{-1} min^{-1}), even though total ATPase activity in these two different protein fractions was approximately equal (Table 2.2).

Cytochrome c-oxidase (mainly localized in mitochondria), cytochrome c-reductase (mainly in endoplasmic reticulum), nitrate-sensitive ATPase (mainly in tonoplast) and azide-sensitive ATPase (in inner membrane mitochondria) have all been used as negative markers of the plasma membrane or as indicators of contaminated membrane preparations. My PM fraction contained less than 2.0% (Table 2.1, 2.2) of the total activities of each of these negative markers. The bulk of cytochrome c-oxidase (88.2%), cytochrome c-reductase (88.9%), nitrate-sensitive ATPase (88.6%), and azide-sensitive ATPase (90.3%) activity was located in the ICM fraction.

In order to understand the localization of 1,3-β-D-glucan synthase activity, preparation of vesicles of known sidedness is required. This requires estimation of the latency of 1,3-β-D-glucan synthase activity. It is important to recognize that the amount of protein in the 1,3-β-D-glucan synthase assay may affect not only the specific activity of 1,3-β-D-glucan synthase, but also the % latency of PM. For example, increasing the amount of protein from 8.7 to 87.3 μg sample^{-1} reduced the specific activity of 1,3-β-D-

Table 2.2. Enzyme marker assays to test the purity of membrane fractions from roots of *Triticum aestivum* cv. Katepwa. Vanadate-sensitive ATPase provides a positive marker for the plasma membrane, nitrate-sensitive ATPase provides a positive marker for the tonoplast, and azide-sensitive ATPase provides a marker for mitochondrial membranes. Values are means ± SE of three replicates.

Fraction	Total Protein		Total ATPase			Vanadate-inhibited ATPase			
	(mg)	% recovery	Total A. µmol min^{-1}	% recovery	S.A. µmol mg^{-1} min^{-1}	Total A. µmol min^{-1}	% recovery	% of total ATPase	S.A. µmol mg^{-1} min^{-1}
Microsomal membranes	16.6	100	102.6 ± 6.1	100	6.2 ± 0.4	4.7 ± 0.05	100	4.5 ± 0.05	0.3 ± 0.01
Plasma membrane	1.6	9.7	9.6 ± 0.5	9.3 ± 0.1	5.9 ± 0.3	3.9 ± 0.25	84.1 ± 5.4	41 ± 2.62	2.4 ± 0.20
Intracellular membranes	12.8	77.2	80 ± 5.0	77.9 ± 0.2	6.2 ± 0.4	0.3 ± 0.01	5.6 ± 0.3	0.3 ± 0.02	0.02 ± 0.01
PM + ICM	14.4	86.9	89.5 ± 5.5	87.2 ± 0.3		4.2 ± 0.26	89.7 ± 5.7		

Fraction	Nitrate-sensitive ATPase				Azide-sensitive ATPase			
	Total A. µmol min^{-1}	% recovery	% of total ATPase	S.A. µmol mg^{-1} min^{-1}	Total A. µmol min^{-1}	% recovery	% of total ATPase	S.A. µmol mg^{-1} min^{-1}
Microsomal membranes	44.4	100.0	25.0	1.3 ± 0.2	39.1 ± 0.2	100	22.0 ± 0.2	1.1 ± 0.01
Plasma membrane	nil	0.0	0.0	0.0	0.2 ± 0.01	0.5	2.0 ± 2.62	0.1 ± 0.01
Intracellular membranes	39.3	88.6	30.0	1.6 ± 0.3	35.3 ± 0.5	90.3	27.0 ± 0.8	1.4 ± 0.04
PM + ICM		88.6			35.5 ± 0.5	90.8		

glucan synthase and the latency in the PM vesicles by 70% (from 386 to 114 nmol mg protein^{-1} min^{-1}) and 31.9% (from 91.0 to 58.1%; Table 2.3) respectively. Thus, I decided to use 5 to 10 μg protein sample^{-1} in all future experiments.

The presence of 0.010% digitonin increased the specific activity of 1,3-β-D-glucan synthase from 29.0 ± 0.7 to 334.3 ± 15.6 nmol mg protein^{-1} min^{-1} (11.5 fold) and H^+-ATPase from 0.7 ± 0.08 to 6.9 ± 0.6 μmol mg protein^{-1} min^{-1} (9.8 fold) (Table 2.4). Digitonin provided similar latent activities of 1,3-β-D-glucan synthase (91.3 ± 2.2%) and H^+-ATPase (90.4 ± 2.0%), indicating that the purified PM fraction was approximately 90.6% RSO.

The polyoxyethylene acyl ether detergent, Brij-58, has been used as a tool to obtain nearly 100% of ISO vesicles (Johansson *et al.*, 1995). Treatment of PM vesicles isolated from the roots of *Triticum aestivum* cv. Katepwa with Brij-58 (0.01%, w/v) in the presence and absence of 0.010% digitonin indicated that this detergent strongly inhibits both the specific activity and latency of 1,3-β-D-glucan synthase, but it had relatively little effect on the activity of H^+-ATPase (Table 2.4). The specific activity of 1,3-β-D-glucan synthase was reduced from 334.3 ± 15.6 nmol mg protein^{-1} min^{-1} (in the presence of digitonin only) to 47.7 ± 2.3 nmol mg protein^{-1} min^{-1} (in the presence of Brij-58 only) or by 85.7%. Brij-58 also inhibited the latent activity of 1,3-β-D-glucan synthase by 77.1 ± 2.4% (Table 2.4). In contrast, Brij-58 had a strong positive effect on the specific activity of H^+-ATPase and little effect on its latency. In the presence of Brij-58, the specific

Table 2.3. The effect of changes in the amount of protein (μg plasma membrane protein sample^{-1}) on the activity of 1,3-β-D-glucan synthase in the presence and absence of 0.010 % of digitonin and its latency (%) in purified plasma membranes prepared from roots of *Triticum aestivum* cv. Katepwa. Values are means ± SE of three replicates.

Amount of protein (μg sample^{-1})	1,3-β-D-glucan synthase s.a. (nmol mg^{-1} min^{-1})		Latency (%)
	- Digitonin	+ Digitonin	
8.7	35.2 ± 1.9	386 ± 18	91.0 ± 0.8
17.5	38.0 ± 2.1	235 ± 15	83.9 ± 0.7
35.0	41.3 ± 2.4	195 ± 15	78.8 ± 0.6
52.4	43.8 ± 2.9	169 ± 14	74.0 ± 0.6
69.8	46.8 ± 2.9	133 ± 12	64.7 ± 0.6
87.3	47.9 ± 3.0	114 ± 12	58.1 ± 0.7

Table 2.4. The effect of Brij-58 on the activity of 1,3-β-D-glucan synthase and H^+-ATPase from plasma membrane vesicles isolated from roots of *Triticum aestivum* cv. Katepwa in the presence and absence of 0.010 % digitonin. Brij-58 inhibition of latent activity (%) was measured by subtracting activity in the presence of digitonin from the activity in the presence of both digitonin and Brij-58 and dividing by the activity in the presence of digitonin. Values are means ± SE of three replicates.

Marker enzyme	Total protein (mg)	Specific activity [a,b]				Latency (%)		Brij-58 inhibition of latent activity (%)
		- Dig., - Brij	+ Dig., - Brij	- Dig., + Brij	+ Dig., + Brij	Dig.	Brij	
1,3-β-D-glucan synthase[a]	0.82	29.0 ± 0.7	334.3 ± 15	47.7 ± 2.3	77.0 ± 5.3	91.3 ± 2.2	39.3 ± 2.0	77.1 ± 2.4
H^+-ATPase[b]	0.82	0.7 ± 0.08	6.9 ± 0.6	6.5 ± 0.5	6.1 ± 0.5	90.4 ± 2.0	89.7 ± 2.4	11.5 ± 2.1

[a] Specific activity of 1,3-β-D-glucan synthase in nmol mg protein^{-1} min^{-1}
[b] Specific activity of H^+-ATPase in μmol mg protein^{-1} min^{-1}

activity of H^+-ATPase was 6.5 ± 0.5 μmol mg protein^{-1} min^{-1} and the latent activity was 89.7 ± 2.4%, similar to the effect of digitonin (Table 2.4). Due to my interest in 1,3-β-D-glucan synthase, Brij-58 was not suitable for preparing ISO vesicles. Thus, I decided to use freeze-thaw treatments to generate ISO PM vesicles and CCD procedure (Larsson *et al.*, 1994) to separate them from RSO vesicles.

Before performing the counter-current distribution procedure to yield preparations enriched in ISO vesicles, a series of freeze-thaw cycles were conducted to find the appropriate frequency for subsequent experiments. After seven freeze-thaw cycles, non-latent activity of 1,3-β-D-glucan synthase increased from 25.0 ± 1.5 to 144.2 ± 3.8 nmol mg protein^{-1} min^{-1}, but the maximum activity declined from 423.3 ± 7.9 to 403.3 ± 4.7 nmol mg protein^{-1} min^{-1} (approximately 5%) (Table 2.5). Five freeze-thaw cycles increased non-latency to 34.1 ± 0.7%, a figure approximately similar to that produced by seven freeze-thaw cycles (35.7 ± 0.8%). In this case, maximum activity declined by only 3%. Thus, five freeze-thaw cycles were used in subsequent experiments.

Application of the CCD procedure provided a significant enrichment of ISO vesicles. Latent activity (RSO) of 1,3-β-D-glucan synthase decreased from 65.6 ± 0.8% (after five freeze-thaw cycles) to 33.0 ± 0.6% after CCD (lower fraction; Table 2.6). In other words, the enrichment of ISO PM orientation increased from 34.4 to 67% after performing CCD procedure. Interestingly, CCD could also be used to recover the enrichment of RSO vesicles. In this experiment, I successfully increased the preparation

Table 2.5. The relationship between freeze-thaw frequency and latency of 1,3-β-D-glucan synthase activity in plasma membrane fractions isolated from roots of *Triticum aestivum* cv. Katepwa. Latency was measured as the increase in 1,3-β-D-glucan synthase activity observed in the presence of 0.010 % digitonin (expressed as a % of total 1,3-β-D-glucan synthase activity). Values are means ± SE of three replicates.

Number of freeze-thaws	1,3-β-D-glucan synthase s.a. (nmol mg^{-1} min^{-1})		Latency (%)	Non-latency (%)
	- digitonin	+ digitonin		
1	25.0 ± 1.5	423.3 ± 7.9	94.1 ± 0.6	5.9 ± 0.7
2	40.3 ± 1.2	418.7 ± 2.3	90.5 ± 0.6	9.5 ± 0.6
3	69.3 ± 3.5	415.3 ± 4.2	83.3 ± 0.5	16.7± 0.6
4	137.5± 6.0	411.8 ± 4.3	66.6 ± 0.5	33.4± 0.5
5	139.6± 4.6	409.2 ± 4.1	65.9 ± 0.8	34.1± 0.7
6	143.2± 4.8	405.5 ± 5.5	64.7 ± 0.5	35.3± 0.9
7	144.2± 3.8	403.3 ± 4.7	64.3 ± 0.5	35.7± 0.8

Table 2.6. The activity of 1,3-β-D-glucan synthase in the presence and absence of 0.010% digitonin and % latency of PM vesicles isolated from roots of *Triticum aestivum* cv. Katepwa using the CCD. Values are means ± SE of three replicates.

Fraction	μg of protein	Recovery (%)	1,3-β-D-glucan synthase s.a. (nmol mg^{-1} min^{-1}) - Digitonin	+ Digitonin	Latency (%)
Free-thawed	468.6	100	122 ± 6	354 ± 6	65.6 ± 0.8
5x					
Upper	216.2	46.1	22 ± 3	353 ± 6	93.9 ± 0.6
Lower	123.5	26.4	96 ± 4	143 ± 6	33.0 ± 0.6
Inter	82	17.5	80 ± 4	207 ± 7	61.5 ± 0.8

of RSO vesicles (latent activity) from 65.6%, after five freeze-thaw cycles, to 93.9% (upper fraction), after performing CCD (Table 2.6).

2.4 Discussion

The aqueous polymer two-phase partitioning technique has become the standard for isolation of plant PM (Larsson *et al.*, 1987, 1994; Sandelius and Morre, 1990) from a variety of biological materials, including roots of *Triticum aestivum*. The isolated PM obtained from my experiments were highly enriched in 1,3-β-D-glucan synthase and vanadate-sensitive ATPase activities. Plasma membrane vesicles isolated using this technique were primarily RSO vesicles (90.6%) as determined by 1,3-β-D-glucan synthase activity in the presence and absence of 0.010% (w/v) digitonin (Table 2.1). These results were consistent with experiments conducted by Larsson *et al.* (1994). Less than 2.0% of the total activity (MF) of the negative marker enzymes, cytochrome c-oxidase, cytochrome c-reductase, nitrate-sensitive ATPase, and azide-sensitive ATPase were located in the PM, suggesting some contamination of the PM by intracellular membranes. This may also be due to the existence of enzymes such as cytochrome c-reductase, in the PM (Askerlund *et al.*, 1988).

Since the active sites of 1,3-β-D-glucan synthase and vanadate-sensitive ATPase are located on the cytosolic side of the PM, the presence of digitonin in the assay media plays an important role in facilitating the access of substrates (UDP-glucose for 1,3-β-D-glucan synthase, ATP for ATPases) and cofactors (such as calcium for 1,3-β-D-glucan

synthase) to the active sites of enzymes. The relative abundance of enzyme, digitonin (detergent) and substrate are other factors that can affect enzyme activity. This premise is supported by my experimental results in which increasing the amount of PM protein in constant levels of digitonin (0.010%, w/v) and substrate (2mM UDP-glucose) significantly reduced 1,3-β-D-glucan synthase specific activity (Table 2.3). While I did not explore the cause of this effect, it did not reflect a substrate limitation. The reaction assay contained 10 μg plasma membrane protein and the specific activity of 1,3-β-D-glucan synthase after one freeze-thaw cycle was 423.3 nmol mg protein^{-1} min^{-1} (Table 2.5). Thus, after 45 min, the total amount of UDP-glucose consumed in this reaction was 190.5 nmol, less than 0.01% of the total 2 mmol UDP-glucose in the assay buffer. All subsequent experiments were performed with a constant amount of protein in the assay.

Most plant PM vesicles formed upon aqueous two-phase partitioning are in the RSO orientation (Palmgren *et al.*, 1990; Larsson *et al*, 1994). However, most PM enzymes such as 1,3-β-D-glucan synthase, H^+-ATPase and Ca^{2+}-ATPase (Fredrikson, 1993; Johansson *et al.*, 1995) expose their active sites towards the cytoplasm. This means that these vesicles have to be inverted to ISO orientation for optimal activity. Nearly 100% of vesicles can be converted to ISO orientation by adding the detergent, Brij-58 (0.05%, w/v), into the assay medium (Johansson *et al.*, 1995). However, the presence of 0.01% Brij-58 inhibited the activity of 1,3-β-D-glucan synthase in my system (Table 2.4). Due to time limitations, the effect of only one form of Brij-58 on the activity of 1,3-β-D-glucan synthase was examined. More research on the effect of various members of Brij-

58 series, particularly from lower sizes, may be worthwhile. Changes in the sizes of Brij-58 may reveal its positive effects on 1,3-β-D-glucan synthase activity.

Since Brij-58 inhibited 1,3-β-D-glucan synthase, the freeze-thaw/CCD procedure was used as an alternative means for preparing ISO PM vesicles. Five freeze-thaw cycles were used to provide PM vesicles with a suitable amount of ISO orientation (34.1 ± 0.7%), sacrificing only a 3% (instead of 5% after seven freeze-thaw cycles) reduction in the activity of 1,3-β-D-glucan synthase (Table 2.6). The Counter-Current Distribution (CCD) procedure provided a means of isolating fraction enriched in ISO vesicles. It decreased the percentage of latent activity by approximately two-fold from 65.6% (after five freeze-thaw cycles) to 33.0% (after CCD; Table 2.6). In other words, the CCD procedure increased the proportion of ISO PM vesicles from 34% to 67%. Nonetheless, the presence of RSO vesicles (33%) in these preparations (latent activity; Table 2.6) may influence the overall calculation of 1,3-β-D-glucan synthase activity specifically related to a particular PM sidedness. The CCD procedure can provide not only a relatively high percentage of ISO vesicles enrichment; it can also be used to recover a higher proportion of RSO PM vesicles (93.9%; Table 2.6) than present in fresh PM isolates (90.6%; Table 2.1).

With these experiments, I have developed reliable techniques for the studying 1,3-β-D-glucan synthase from RSO-enriched as well as ISO-enriched PM vesicles derived from roots of wheat (*Triticum aestivum* cv. Alikat and Katepwa). The aqueous polymer two-phase partitioning technique lived up to its reputation as the standard for isolation of

plant PM. Using this technique, approximately 82 and 84% of the total positive marker enzyme activities were recovered from PM fractions, but less than 2.0% of the activities of each of the negative marker enzymes were obtained from this fraction. Because Brij-58 inhibited the activity of 1,3-β-D-glucan synthase, freeze-thaw and CCD were chosen as the means to generate the enrichment ISO vesicles from RSO vesicles. In addition, CCD can also be used to improve the enrichment of RSO vesicles (93.9%). The ability to manipulate the specific orientation of root PM vesicles (RSO and ISO), while maintaining the optimal conditions for 1,3-β-D-glucan synthase enzyme, will allow exploration of the effect of Al on the activity of this enzyme in the presence and absence of detergents, such as digitonin, and ionophores.

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3. Aluminum-chelate affinity chromatography demonstrates that 1,3-β-D-glucan synthase forms a stable complex with aluminum

3.1 Introduction

1,3-β-D-glucan synthase (EC 2.4.1.34) is widely described in plants and is responsible for synthesis of ubiquitous cell wall 1,3-β-glucans (callose) using uridine diphosphateglucose (UDP-glucose) as a substrate (Delmer 1987; Zhang *et al.*, 1994). Besides being described as a developmentally-regulated enzyme (Fincher and Stone, 1981; Stone and Clarke, 1992; Schlupmann *et al.*, 1993), it has been widely accepted as a general response to wounding, physiological stress, and infection (Fincher and Stone, 1981; Aist, 1983; Skou *et al.*, 1984). Numerous studies (Schreiner *et al.*, 1994; Zhang *et al.*, 1994; Wissemeier *et al.*, 1987) have also shown that metal stresses such as aluminum (Al) toxicity increase the activity of 1,3-β-D-glucan synthase as indicated by high accumulation of callose. However, in a study of the activities of plasma membrane-bound enzymes isolated from roots of spruce (*Picea abies*) grown in the presence of Al, Widell *et al.* (1994) demonstrated that the activity of 1,3-β-D-glucan synthase was severely inhibited by this metal *in vitro*. It has been suggested that Al in the cytosol may bind directly to 1,3-β-D-glucan synthase, thereby inhibiting its activity. Aluminum would have to cross the lipid bilayer of the plasma membrane (PM) for this effect to be observed.

Rapid accumulation of Al in the symplast of wheat root-tip cells has been detected by Tice *et al.* (1992) using sequential washing and a fluorescence (morin) staining procedure, and in the symplast of soybean (*Glycine max*) roots (Lazof *et al.*, 1994) using

secondary ion mass spectrometry (SIMS). However, the activity of free Al inside the cytoplasm is likely limited to 10^{-10} M by formation of insoluble hydroxy-Al-phosphate compounds (Taylor, 1995). These low levels could be physiologically active and interactions of Al with cell constituents may alter both their physical and functional integrity (Deeleers, 1985; Kinraide and Parker, 1987; Caldwell, 1989; Yermiyahu, 1997). For example, Al may occupy Ca^{2+} binding sites in Ca^{2+}-requiring enzymes (Haug, 1984; Taylor, 1990; Rengel, 1992; Delhaize and Ryan, 1995) such as 1,3-β-D-glucan synthase.

I have predicted that Al binds to 1,3-β-D-glucan synthase and inhibits the activity of this enzyme. To test this prediction, I have used Al-chelate affinity chromatography to determine whether or not 1,3-β-D-glucan synthase forms a stable complex with Al. This approach is based on numerous studies using metal ion affinity chromatography. The studies demonstrate that certain metal ions such as Al, Cu and Ni are able to bind to specific amino acids (e.g. histidine, and tryptophan) in peptides (Hochuli *et al*, 1987; Monjon and Solms, 1987; Yip and Hutchens, 1989; Yip *et al.*, 1989; Hutchens and Yip, 1990) and on protein surfaces (Sulkowski, 1985; Hutchens and Li, 1988; Mantovaara-Jonsson *et al.*, 1989). If 1,3-β-D-glucan synthase binds Al, this protein will be retained by columns that are charged with Al. The bulk of 1,3-β-D-glucan synthase activity will be detected only after charged columns are eluted with a strong chelator such as EDTA. In contrast, the bulk activity of 1,3-β-D-glucan synthase will be detected after uncharged-columns are eluted with H_2O. This approach is possible since solubilization of the PM fraction with digitonin or CHAPS does not inhibit the activity of this enzyme (Fredrikson, 1993; Kudlicka and Brown, 1997).

3.2 Materials and methods

3.2.1 Plant material

Seeds of an Al-sensitive (Katepwa) cultivar of *Triticum aestivum* were surface sterilized in 1.2% sodium hypochlorite for 20 min, and germinated overnight in a solution of 0.005 g L^{-1} Vitavax to limit fungal growth. Seedlings were then grown for 5 days on nylon mesh suspended over 15 L of full nutrient solution containing (mM): 3.30 NO_3^--N, 0.30 NH_4^+-N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S; and (μM): 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo (pH 4.4) in a growth chamber with 16 h of light (20 ^{0}C, 68% relative humidity) and 8 h of darkness (16 °C, 85% relative humidity). The photosynthetic photon flux (PPF) was 335 ± 12 μmol m^{-2} s^{-1} at plant base level. After 5 days of growth, whole roots were harvested for PM isolation using the techniques described in chapter 2.2.2.

3.2.2 Solubilization of plasma membrane proteins

Preliminary experiments indicated that the response of solubilized 1,3-β-D-glucan synthase protein from Al-resistant cv. Alikat to an Al-charged column was similar to that from Al-sensitive cv. Katepwa. Therefore, only the response of 1,3-β-D-glucan synthase from Al-sensitive cv. Katepwa was described in this chapter. Plasma membrane vesicles (5 to 5.5 mg protein in 4 to 5 ml aliquots) isolated from whole roots of *Triticum aestivum* cv. Katepwa were first solubilized in 2% digitonin containing 10 mM $MgCl_2$ and 100 mM Tris-HCl (pH 7.5). The suspension was incubated at room temperature (22 °C) for 30 minutes and centrifuged at 100,000 g in a SW 56 rotor for 2 hours. The supernatant was collected and the pellet was resuspended to a concentration of 2 to 3 mg protein ml^{-1} in

50 mM HEPES (pH 7.5). An equal volume of a second solubilization buffer containing 1% digitonin, 2.0 mM EDTA, 2.0 mM EGTA and 100 mM Tris-HCl (pH 7.5) was added to the resuspended protein (Lawson *et al.*, 1989). The suspension was once again centrifuged at 100,000 g for 2 hours. The supernatant was collected separately and the amount of protein from each step of solubilization was determined according to Bradford (1976). 1,3-β-D-glucan synthase activity was assayed as described in section 3.2.4 below.

3.2.3 Protein separation in an uncharged and an Al-activated affinity column

Aluminum-activated metal chelate affinity columns were prepared by packing Iminodiacetic Sepharose 6B in 20 ml columns (up to 10 cm height) and washing with 50 to 60 ml chelex-purified H_2O. An Al reference solution (5 ml, 1000 μg ml^{-1}) was loaded onto the column and incubated for one hour. The column was subsequently washed with 50 to 60 ml of chelex-purified H_2O to removed unbound Al. A total of 300 to 350 μg protein in 150 to 200 μl solution and a further 200 to 300 μl of chelex-purified H_2O were added to the column and incubated for another hour. The column was then sequentially eluted with 10 ml of chelex-purified H_2O, 10 ml of potassium phthalate buffer (pH 4.0), 10 ml of 20 mM EDTA, and 10 ml of 100 mM EDTA. All fractions were dialyzed against a 10 Kb exclusion limit membrane overnight. Protein was then concentrated to 50 μL using a Speed Vac SC 100 concentrator (Savant) and the activity of 1,3-β-D-glucan synthase from all fractions was measured to determine enrichment. The same procedure was followed to collect the protein from an uncharged column, except that there was no Al loaded onto the column.

3.2.4 1,3-β-D-glucan synthase assay

Proteins collected from various fractions (10 μg for each sample) were assayed according to Larsson *et al.* (1994) in a 100 μL total volume containing, 0.33 M sucrose, 50 mM 4-(-2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (pH 7.25), 2 mM spermine, 20 mM cellobiose, 0.1 mM $CaCl_2$, and 2 mM DTT, without 0.01% (w/v) digitonin. The reaction was started by addition of UDP[^{14}C]glucose (20 Gbq/mol) to a final concentration of 2 mM. The reaction was run for 45 min at room temperature and was terminated by immersing in boiling water for 5 min. The samples were then transferred to paper filters (25-mm circles, Whatman, 3 MM), dried, and washed 2 times for 1 hr each in 0.35 M ammonium acetate (pH 3.6), and 30% (v/v) ethanol (60 filters per 0.5 L). Filters were dried and the radioactivity was measured by liquid scintillation counting (Beckman LS6000TA). Background was determined by running blanks without protein.

3.3 Results

Before separating proteins using Al-activated chelate affinity chromatography, PM proteins from roots of the Al-sensitive cv. Katepwa were solubilized in 2% digitonin and 1% digitonin plus 2.0 mM EDTA, 2.0 mM EGTA and 100 mM Tris-HCl (pH 7.5), consecutively. Twenty six ± 2 % of total protein (5 mg crude protein) was solubilized in the first step, and 15 ± 2 % was solubilized in the second step. Most of the proteins (58 ± 4 %) were retained in the pellet (99% recovery; Table 3.1).

1,3-β-D-glucan synthase from these four fractions showed a wide range of activities. The specific activity of 1,3-β-D-glucan synthase in the pellet (315 ± 24 nmol mg protein^{-1} min^{-1}) was 90% of that from the crude fraction (350 ± 21 nmol mg protein^{-1} min^{-1}). In contrast, the specific activity of 1,3-β-D-glucan synthase solubilized in the second step was only 26.3% of that from the crude fraction and approximately 50% of the specific activity from the first solubilization step (Table 3.1). The fraction arising from the first solubilization step was chosen for subsequent experiments, because it had a higher protein recovery and a higher specific activity of 1,3-β-D-glucan synthase.

Aluminum-activated metal chelate affinity chromatography was performed using the solubilized protein and the specific activity of 1,3-β-D-glucan synthase from each affinity fraction was subsequently determined. The percentage of total protein recovered in each fraction varied from 28 ± 3 % in chelex-H_2O to 25 ± 4 % in potassium phthalate, 15 ± 2 % in 20 mM EDTA, and 31 ± 4 % in 100 mM EDTA fraction (99% recovery; Table 3.2). The highest specific activity of 1,3-β-D-glucan synthase was obtained after the column was eluted by 100 mM EDTA (90 ± 5 nmol mg protein^{-1} min^{-1}) and to a lesser extent by 20 mM EDTA (40 ± 5 nmol mg protein^{-1} min^{-1}). Even though 28 ± 3 % of the total protein eluted in the H_2O fraction, 1,3-β-D-glucan synthase was not detected in this fraction. The total activity of 1,3-β-D-glucan synthase in the K-phthalate wash (25 ± 4 % of total protein) was less than 1% (2 ± 1 nmol min^{-1}) of the total activity from the first solubilized step or only 4% from the total recovered activity (46 nmol min^{-1}). These data suggest that 1,3-β-D-glucan synthase was tightly bound to Al.

Table 3.1. 1,3-β-D-glucan synthase activity of different fractions of solubilized-plasma membrane protein from roots of *Triticum aestivum* cv. Katepwa. Values are means ± SE of three replicates.

Fractions	Total Protein (mg)	Recovery (%)	Total activity (nmol min^{-1})	Specific activity (nmol mg^{-1} min^{-1})
Crude Protein	5 ± 0.5	100	1750 ± 32	350 ± 21
Pellet	2.90 ± 0.2	58 ± 4	914 ± 27	315 ± 24
1st solubilization step[a]	1.30 ± 0.1	26 ± 2	241 ± 19	185 ± 19
2nd solubilization step[b]	0.75 ± 0.1	15 ± 2	69 ± 10	92 ± 15
Recovery of Total	4.95	99	1224	

[a] in the presence of 2% digitonin; [b] in the presence of 1% digitonin plus 2.0 mM EDTA, 2.0 mM EGTA and 100 mM Tris-HCl (pH 7.5)

Table 3.2. 1,3-β-D-glucan synthase activity of solubilized-plasma membrane protein isolated from roots of *Triticum aestivum* cv. Katepwa separated by Al-affinity chromatography. Values are means ± SE of three replicates.

Fractions	Total Protein (mg)	Recovery (%)	Total activity (nmol min^{-1})	Specific activity (nmol mg^{-1} min^{-1})
1st solubilization step	1.30 ± 0.07	100	241 ± 21	185 ± 16
H_2O	0.36 ± 0.04	28 ± 3	ND	ND
K-phthalate	0.32 ± 0.05	25 ± 4	2 ± 1	5 ± 1
20 mM EDTA	0.19 ± 0.03	15 ± 2	8 ± 2	40 ± 5
100 mM EDTA	0.40 ± 0.05	31 ± 4	36 ± 4	90 ± 5
Recovery of Total	1.27	99	46	

ND Not detectable

To confirm that these results reflected Al-binding capacity, solubilized protein was passed through metal chelate affinity columns, but this time the columns were not charged with Al. Approximately 54 ± 8 % of the total protein was recovered in the H_2O fraction, 23 ± 8 % in the K-phthalate fraction, 13 ± 5 % in 20mM EDTA fraction and 9 ± 3 % in 100 mM EDTA fraction (Table 3.3). The highest specific activity (295 ± 25 nmol mg protein^{-1} min^{-1}) was observed in the H_2O fraction (99% recovery; Table 3.3). In contrast to the high specific activity of 1,3-β-D-glucan synthase eluted from Al-activated columns by 20 mM and 100 mM EDTA, there was little (4 ± 1 nmol mg protein^{-1} min^{-1}) to no specific activity detected in the 20 mM and 100 mM EDTA fractions from columns lacking Al-activation (Table 3.3).

3.4 Discussion

A crucial problem in any attempt to purify a membrane bound enzyme is the difficulty of solubilizing the enzyme from the membrane without severe loss of activity (Fredrikson, 1993). In these experiments, PM proteins from roots of Al-sensitive Katepwa were solubilized in two steps with 2% digitonin and 1% digitonin plus 2.0 mM EDTA, 2.0 mM EGTA and 100 mM Tris-HCl (pH 7.5). The first solubilization of 1,3-β-D-glucan synthase in 2% digitonin resulted in recovery of 26% total protein. The specific activity of this fraction was 53% (185 nmol mg protein^{-1} min^{-1}) of that in the crude fraction. In contrast, the second solubilization step recovered only 15% of the total protein, while its specific activity was 26% (92 nmol mg protein^{-1} min^{-1}) of that in the crude fraction. A considerable amount of 1,3-β-D-glucan synthase activity was retained in

Table 3.3. 1,3-β-D-glucan synthase activity of solubilized-plasma membrane protein isolated from roots of *Triticum aestivum* cv. Katepwa separated by uncharged Al-affinity chromatography. Values are means ± SE of three replicates.

Fractions	Total Protein (mg)	Recovery (%)	Total activity (nmol min^{-1})	Specific activity (nmol mg^{-1} min^{-1})
1st solubilization step	1.20	100	321.70	183.80
H_2O	0.65 ± 0.1	54 ± 8	192 ± 16	295 ± 25
K-phthalate	0.28 ± 0.1	23 ± 8	15 ± 3	52 ± 10
20 mM EDTA	0.15 ± 0.06	13 ± 5	0.7 ± 0.2	4 ± 1
100 mM EDTA	0.11 ± 0.03	9 ± 3	ND	ND
Recovery of Total	1.19	99	207.7	

ND Not detectable

the insoluble fraction (pellet). The specific activity of the enzyme in the pellet was similar to that of the crude protein (untreated root PM protein). In fact, the specific activity of 1,3-β-D-glucan synthase from this fraction declined by only 10% (Table 3.1), indicating that the enzyme was resistant to solubilization. The reduction in 1,3-β-D-glucan synthase specific activity in the solubilized fractions (Table 3.1) could reflect differences in the solubility of various membrane proteins or a modification of the functional structure of this enzyme. An association of the enzyme with the lipid bilayer of the PM may be required for its stability.

I hypothesized that Al inhibits the activity of 1,3-β-D-glucan synthase by binding directly to the protein. This possibility was investigated using Al-activated metal chelate affinity column chromatography (Porath and Olin, 1983; Hutchens and Yip, 1990; Yip and Hutchens, 1992). After measuring the activity of GS II from four different eluted fractions (Table 3.2), the present experiments demonstrated that the highest specific activity of 1,3-β-D-glucan synthase was obtained from the fraction eluted by 100 mM EDTA followed by the 20 mM EDTA fraction. No 1,3-β-D-glucan synthase activity was detected in the H_2O fraction, even though it contained 28% of the total recovered protein. Similarly little specific activity (5 nmol mg protein^{-1} min^{-1}) was detected in the K-phthalate fraction, even though it contained 25% of the total recovered protein. In contrast, the bulk of activity was eluted by H_2O from uncharged columns. Thus, the assumption that Al binds to 1,3-β-D-glucan synthase in order to inhibit the activity of this enzyme was supported by the present experiments.

Results shown in Table 3.2 not only reveal that Al binds to 1,3-β-D-glucan synthase, but also that this association causes a significant reduction in enzyme activity. With the Al-activated metal chelate affinity column, the highest total and specific activity of 1,3-β-D-glucan synthase (90 nmol mg protein^{-1} min^{-1}) was detected in 100 mM EDTA eluted fraction. In the uncharged column, the highest total and specific activities of 1,3-β-D-glucan synthase (295 nmol mg protein^{-1} min^{-1}) were observed in the H_2O fraction. The strong reduction of 1,3-β-D-glucan synthase specific activity in fractions eluted from the Al-activated column could be due to an interaction between the 1,3-β-D-glucan synthase protein and free Al (Al^{3+}), EDTA, or Al-EDTA. Future studies are required to disclose these possibilities.

In conclusion, the present experiments provided a fast and a simple means of demonstrating that Al does bind to 1,3-β-D-glucan synthase, while increasing the purity of this enzyme. This technique is reproducible and could be used with any other enzyme that Al binds to. It remains to be explored whether Al binds to the active site or other parts of the enzyme.

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4. Characterizing the effects of aluminum on the activity of 1,3-β-D-glucan synthase *in vitro*

4.1 Introduction

Quantitative studies by Zhang *et al.* (1994) have shown that synthesis of 1,3-β-D-glucan (callose), which is driven by the activity of 1,3-β-D-glucan synthase (EC 2.4.1.34), increased 38-fold after 48 hours of exposure to 75 μM Al in roots of aluminum (Al)-sensitive cultivars of *Triticum aestivum*. While this result is consistent with a number of other more recent studies (Delhaize and Ryan, 1995; Horst, 1995; Papernik and Kochian, 1997; Archambault *et al.*, 1996), it is somewhat surprising. Aluminum has been found to interfere with the absorption of Ca^{2+}, which is required for callose synthesis (Taylor, 1988; Nichol *et al.*, 1993; Rengel, 1992a, 1992b). For example, Ryan and Kochian (1993) found a 40% reduction in calcium (Ca) influx into root tips of Al-sensitive cultivars of *Triticum aestivum* in the presence of Al. Aluminum may interfere directly with Ca^{2+} uptake by displacing Ca^{2+} from binding sites in the cell wall (Godbold, 1991) and/or by blocking Ca^{2+}-channels in the plasma membrane (Huang *et al.*, 1992a, 1992b, 1995; Rengel and Elliot, 1992; Rengel, 1994; Nichol and Oliveira, 1995). It may also disrupt uptake indirectly by interfering with the action of inositol-1,4,5-triphosphate or GTP-binding proteins or by disrupting cellular Ca^{2+} homeostasis (Huang *et al.*, 1995; Nichol and Oliveira, 1995).

Increased activity of 1,3-β-D-glucan synthase in the face of reduced Ca^{2+} influx across the PM seems contradictory at first sight. Recent reports, however, have demonstrated that treatment with Al increases cytosolic Ca ($[Ca^{2+}]_{cyt}$) (Lindberg and

Strid, 1997). It is possible that increasing $[Ca^{2+}]_{cyt}$ are not driven by changes in Ca^{2+} fluxes across the PM, but by changes in fluxes across internal membranes. Release of endostorage Ca^{2+} to the cytoplasm may have an effect on 1,3-β-D-glucan synthase activity, perhaps triggered through the phosphatidyl-inositol cycle or inositol 1,4,5-triphosphate. Another apparent contradiction arises from studies with purified PM, which have shown that Al is a powerful inhibitor of 1,3-β-D-glucan synthase. Widell *et al.* (1994) demonstrated that 1,3-β-D-glucan synthase activity in PM isolated from roots of *Picea abies* and *Triticum aestivum* was inhibited approximately 65% after treatment with Al.

Previous experiments (Chapter 3) demonstrated that Al binds tightly to the 1,3-β-D-glucan synthase protein and the activity of enzyme eluted from an Al-activated metal chelate affinity column was lower than that from an uncharged column. This would suggest that the direct effect of Al on 1,3-β-D-glucan synthase is inhibitory. I have hypothesized that Al inhibits the activity of 1,3-β-D-glucan synthase, but this effect may not be detected *in vivo*. Aluminum may be able to cross the PM to gain access to the cytosolic face of 1,3-β-D-glucan synthase, but its activity may be low. Consequently the stimulatory effect of Al resulting from an Al-induced increase in $[Ca^{2+}]_{cyt}$ is greater than the direct inhibitory effect on 1,3-β-D-glucan synthase activity. In contrast, inhibition of 1,3-β-D-glucan synthase activity *in vitro* may reflect better access of this metal to the cytosolic side of PM and the lack of internal sources of calcium such as from the endoplasmic reticulum, mitochondria and vacuoles.

With this hypothesis in mind, I predicted that the *in vitro* activity of 1,3-β-D-glucan synthase in RSO and ISO PM-vesicles should be inhibited by Al, regardless the presence of detergents and A23187, which can speed up the delivery of Ca to the vesicle interior. While the inhibitory effects of Al might be expected to be greater in the presence of detergents, which should speed the delivery of Al to the vesicle interior, the net effect should always be inhibitory. The following experiments were designed to test these predictions and to explore whether or not the response of 1,3-β-D-glucan synthase to Al, detergents, and A23187 varies when membrane protein is isolated from Al-resistant and Al-sensitive cultivars.

4.2 Materials and methods

4.2.1 Plant material

Seeds of Al-tolerant (Alikat) and Al-sensitive (Katepwa) cultivars of *Triticum aestivum* were surface sterilized in 1.2% sodium hypochlorite for 20 min, and germinated overnight in a solution of 0.005 g L^{-1} Vitavax to limit fungal growth. Seedlings were then grown for 5 days on nylon mesh suspended over 15 L of full nutrient solution containing (mM): 3.30 NO_3^--N, 0.30 NH_4^+-N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S; and (μM): 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo (pH 4.4) in a growth chamber with 16 h of light (20 ^{0}C, 68% relative humidity) and 8 h of darkness (16 ^{0}C, 85% relative humidity). The photosynthetic photon flux (PPF) was 335 ± 12 μmol m^{-2} s^{-1} at plant base level. After 5 days of growth, whole roots were harvested for PM isolation using the techniques described in Chapter 2.

4.2.2 Experimental design

My experimental approach made use of detergents and A23187 to vary the access of Al and/or Ca across the PM. Preliminary experiments using PM vesicles (RSO and ISO) isolated from Al-sensitive cv. Katepwa were conducted to find optimal concentrations of digitonin, A23187, and Al. Working concentrations of digitonin varied from 0 - 0.016% (w/v), A23187 varied from 0 - 250 nmol mg protein^{-1} and Al varied from 0 - 100 μM. A23187 was dissolved in DMSO and an equivalent concentration of this solvent was added to controls to avoid variation due to the presence of this solvent in the assay buffer. Using the optimal concentrations of digitonin, A23187, and a combination of digitonin and A23187, a series of experiments was conducted to find the effects of Al (0, 10, 20, 30, 40, 50, 75, and 100 μM) in the presence digitonin, A23187, or the combination of both, on the activity of GS II from RSO and ISO PM vesicles isolated from roots of Al-resistant cv. Alikat and Al-sensitive cv. Katepwa. Each treatment included three replicates and was repeated three times independently.

4.2.3 1,3-β-D-glucan synthase assay

Right-side-out (RSO) or inside-out (ISO) PM vesicles were prepared from roots of *Triticum aestivum* cv. Alikat and Katepwa using the techniques described in Chapter 2. The amount of PM protein in RSO and ISO preparations was determined according to Bradford (1976). Vesicles (5 - 10 μg protein) were pre-incubated for 10 min in the presence and absence of Al and/or A23187. The assay was carried out according to Larsson *et al.* (1994) in a 100 μL total volume containing, 0.33 M sucrose, 50 mM 4-(-2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (pH 7.25), 2 mM spermine, 20 mM cellobiose, 0.1 mM $CaCl_2$, and 2 mM DTT. The reaction was started by addition of UDP[^{14}C]glucose (20 Gbq/mol) to a final concentration of 2 mM. The reaction was run for 45 min at room temperature and terminated by immersing in boiling water for 5 min. The samples were then transferred to paper filters (25-mm circles, Whatman, 3 MM), dried, and washed 2 times for 1 hr each in 0.35 M ammonium acetate (pH 3.6), and 30% (v/v) ethanol (60 filters per 0.5 L). Filters were dried and the radioactivity was measured by liquid scintillation counting (Beckman LS6000TA). Background was determined by running blanks without protein.

4.3 Results

Before investigating the effect of Al on the activity of 1,3-β-D-glucan synthase in isolated PM vesicles, it was necessary to find the optimal concentrations of A23187 and digitonin needed to maximize activity of the enzyme. The activity of 1,3-β-D-glucan synthase in response to digitonin was dependent on the sidedness of PM vesicles. In RSO vesicles, the activity of 1,3-β-D-glucan synthase was strongly stimulated by digitonin and reached its maximum level (325 ± 11 nmol mg protein^{-1} min^{-1}) at 0.010% (w/v) digitonin. At higher levels of digitonin, the activity of this enzyme slowly declined (Figure 4.1). In ISO vesicles, the initial activity level of 1,3-β-D-glucan synthase in the absence of digitonin was four times higher (133 ± 4 nmol mg protein^{-1} min^{-1}) than that in RSO vesicles (32 ± 5 nmol mg protein^{-1} min^{-1}), but its activity increased to a lesser extent in

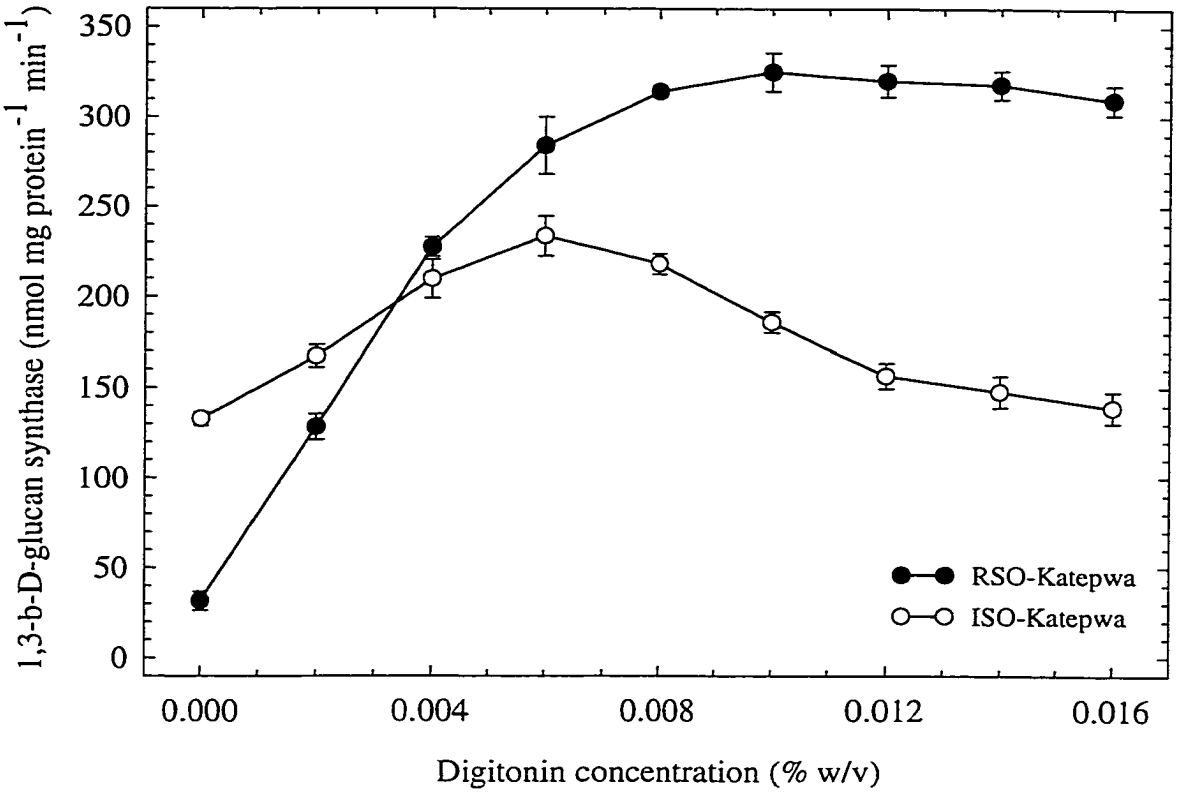

Figure 4.1. The effect of digitonin on the activity of 1,3-β-D-glucan synthase in RSO and ISO plasma membrane vesicles isolated from the roots of *Triticum aestivum* cv. Katepwa. Values for specific activity are means ± SE of three replicates.

the presence of digitonin. Maximum activity (234 ± 11 nmol mg protein^{-1} min^{-1}) was observed in the presence of 0.006% (w/v) digitonin (Figure 4.1).

An increase of the activity of 1,3-β-D-glucan synthase was also observed in the presence of A23187 (Figure 4.2), although not as much as in the presence of digitonin. The presence of A23187 (0-10 nmol mg protein^{-1}) increased the specific activity of 1,3-β-D-glucan synthase in RSO vesicles from 41 ± 2 to 185 ± 3 nmol mg protein^{-1} min^{-1}, but it had a lesser effect on the activity of 1,3-β-D-glucan synthase in ISO plasma membrane vesicles (from 121 ± 2 to 171 ± 2 nmol mg protein^{-1} min^{-1}) (Figure 4.2). Increasing the amount of ionophore in the assay medium above 10 nmol mg protein^{-1} reduced the specific activity of this enzyme in both RSO and ISO vesicles.

Activity of 1,3-β-D-glucan synthase was clearly Ca-dependent. Calcium stimulated the activity of 1,3-β-D-glucan synthase in both RSO and ISO plasma membrane vesicles above 24 ± 2 and 22 ± 6 nmol mg protein^{-1} min^{-1} observed in the minus Ca treatments (Figure 4.2). Although the presence of 2 mM $CaCl_2$ (in absence of digitonin and A23187) only slightly increased the specific activity of 1,3-β-D-glucan synthase in RSO orientation (from 24 ± 2 to 41 ± 2 nmol mg protein^{-1} min^{-1}), it had a dramatic effect on the activity of this enzyme in ISO vesicles (from 22 ± 6 to 121 ± 2 nmol mg protein^{-1} min^{-1}) (Figure 4.2). In the presence of either digitonin or A23187, calcium had a greater effect on activity in RSO vesicles. The activity of the enzyme increased from 41 ± 2 to 229 ± 6 nmol mg protein^{-1} min^{-1} or from 41 ± to 185 ± 3 nmol mg protein^{-1} min^{-1}, in the presence of digitonin and A23187 respectively. The smaller

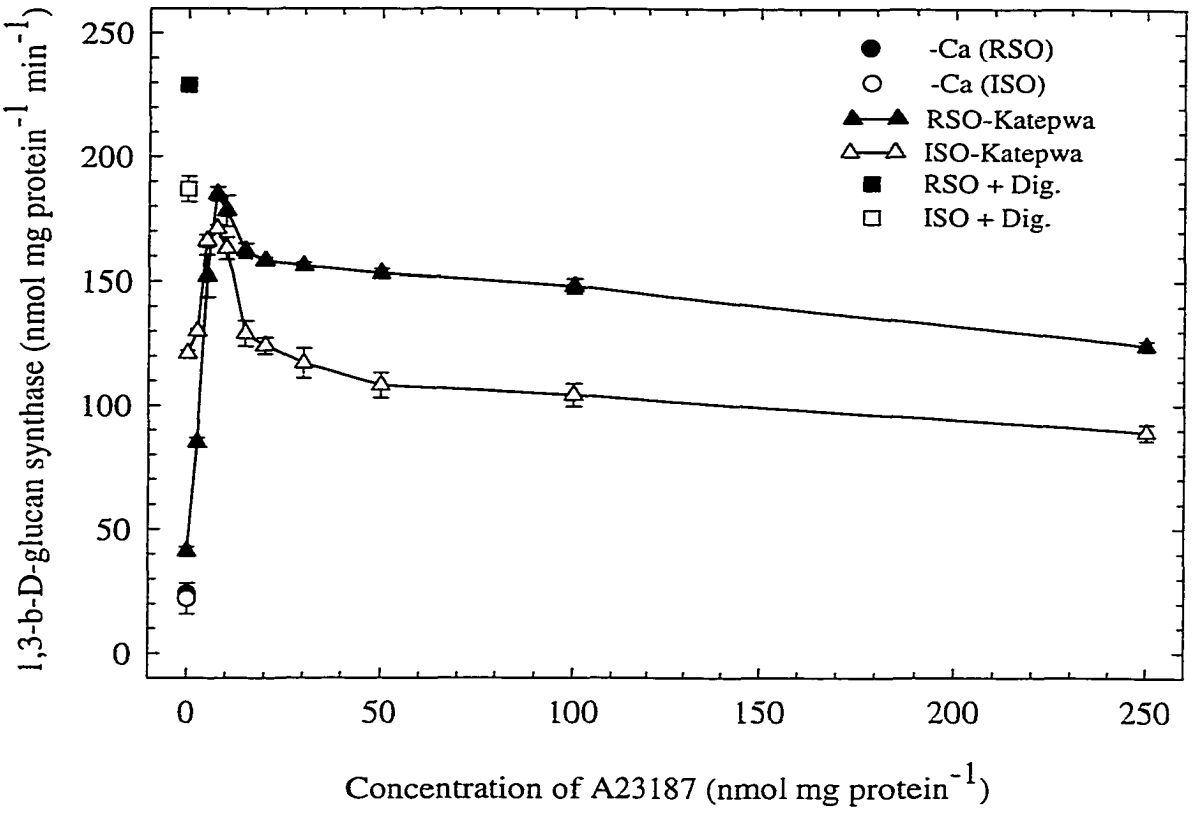

Figure 4.2. The effect of calcium ionophore A23187 on the activity of 1,3-β-D-glucan synthase in RSO and ISO plasma membrane vesicles isolated from roots of *Triticum aestivum* cv. Katepwa. Except where indicated, activity was measured in the presence of calcium and the absence of digitonin. Values for specific activity are means ± SE of three replicates.

increase in 1,3-β-D-glucan synthase activity in ISO vesicles in the presence of digitonin and A23187 was probably due to contamination with RSO vesicles (33%) in these experiments.

Once the optimal concentrations of A23187 and digitonin were found, the activity of 1,3-β-D-glucan synthase was examined in the presence of 0 - 100 μM $AlCl_3$. In the presence of Ca, but absence of both digitonin and A23187, the specific activity of 1,3-β-D-glucan synthase in both RSO and ISO vesicles was reduced by Al. Activity decreased by 47% (from 43 ± 2 to 23 ± 2 nmol mg protein^{-1} min^{-1}) in RSO vesicles and by 70% (from 249 ± 15 to 75 ± 12 nmol mg protein^{-1} min^{-1}) in ISO vesicles (Figure 4.3).

To determine if digitonin and/or A23187 had an effect on Al-induced inhibition of 1,3-β-D-glucan synthase, these two compounds were included in the assay medium either in isolation or in combination. In RSO vesicles (without digitonin and A23187), Al inhibited the activity of 1,3-β-D-glucan synthase by 47 ± 2.5% (from 32 ± 2 to 17 ± 2 nmol mg protein^{-1} min^{-1}) (Figure 4.4 and Table 4.1) and 50 ± 3.0% (from 40 ± 4 to 20 ± 3 nmol mg protein^{-1} min^{-1}) (Figure 4.5 and Table 4.1) in Alikat and Katepwa, respectively. An Al-induced reduction in the activity of 1,3-β-D-glucan synthase was still observed in the presence of digitonin or A23187. For Alikat and Katepwa (respectively), inhibitions of 40 ± 2.0% and 52 ± 2.8% were observed in the presence of digitonin and inhibitions of 47 ± 2.3% and 40 ± 3% were observed in the presence of A23187. In the presence of digitonin and A23187 in combination, Al still inhibited the activity of 1,3-β-D-glucan synthase by 28 ± 2.7% (from 331 ± 15 to 238 ± 13 nmol mg protein^{-1} min^{-1}; Figure 4.4

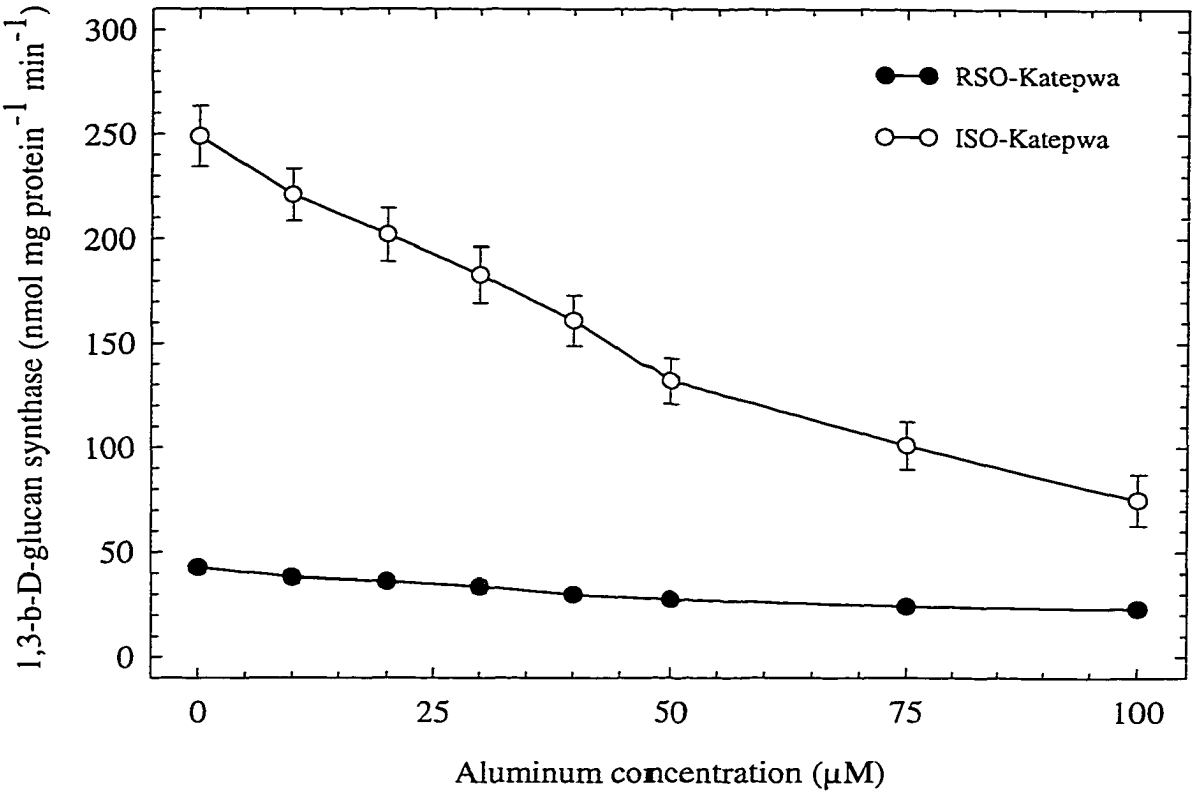

Figure 4.3. The effect of Al on the activity of 1,3-β-D-glucan synthase in RSO and ISO plasma membrane vesicles isolated from *Triticum aestivum* cv. Katepwa. The activity was measured in the absence of digitonin and A23187. Values for specific activity are means ± SE of three replicates

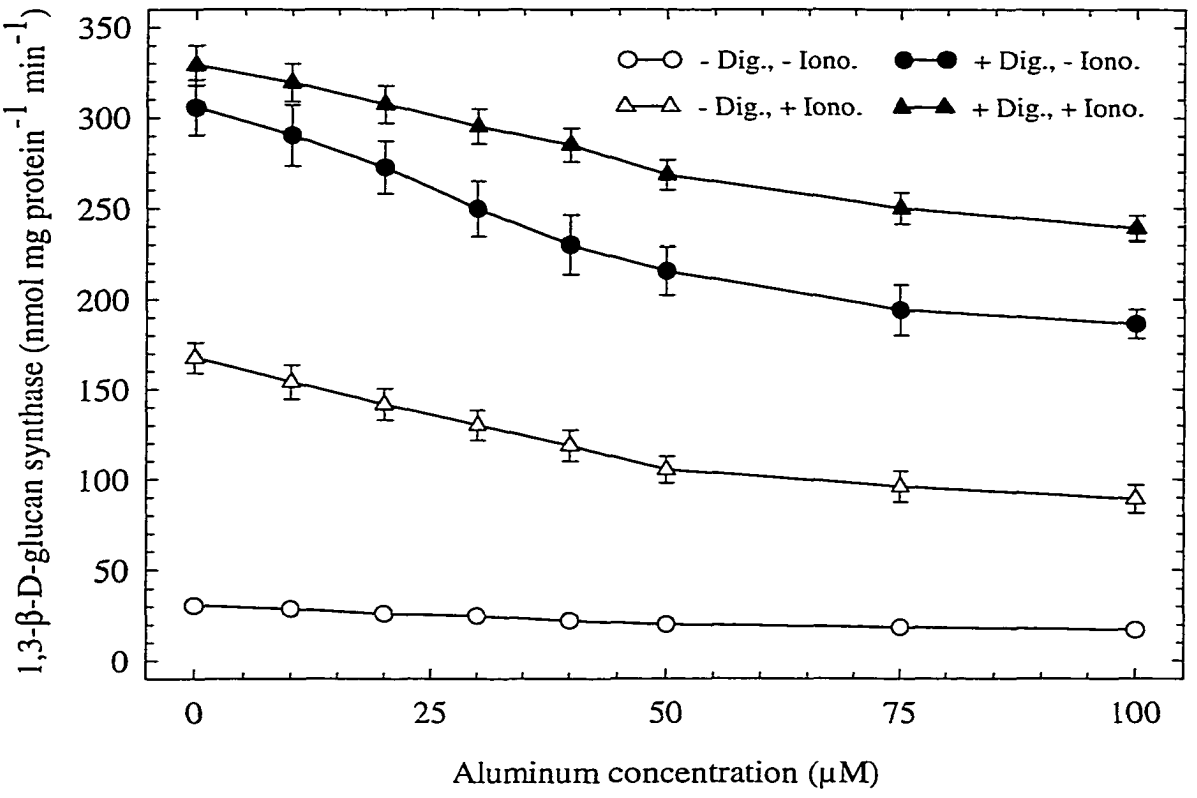

Figure 4.4. The effect of Al on the activity of 1,3-β-D-glucan synthase in RSO plasma membrane vesicles isolated from roots of *Triticum aestivum* cv. Alikat. Experiments were conducted in the presence or absence of digitonin and A23187. Values for specific activity are means ± SE of three replicates.

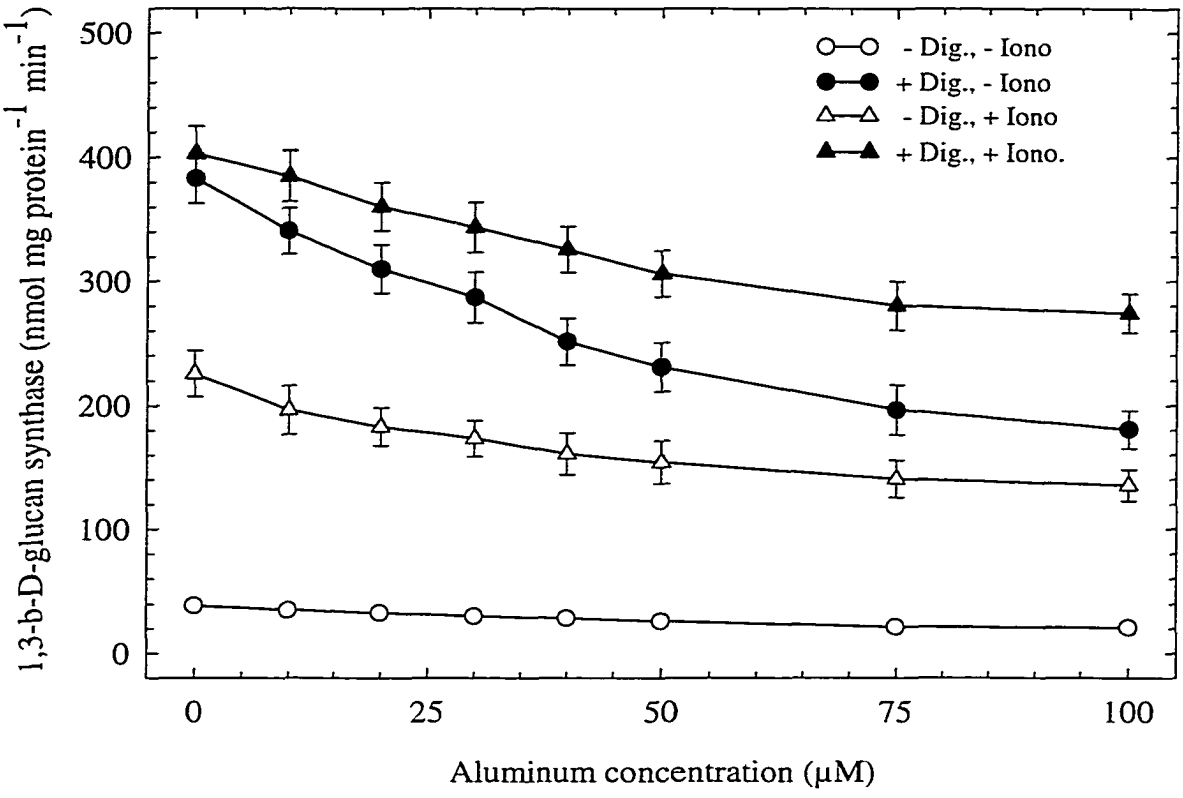

Figure 4.5. The effect of Al on the activity of 1,3-β-D-glucan synthase in RSO plasma membrane vesicles isolated from roots of *Triticum aestivum* cv. Katepwa. Experiments were conducted in the presence or absence of digitonin and A23187. Values for specific activity are means ± SE of three replicates.

Table 4.1. The percentage inhibition of Al (100 μM) on the activity of 1,3-β-D-glucan synthase in RSO and ISO plasma membrane vesicles isolated from roots of *Triticum aestivum* cv. Alikat and Katepwa. Values for the percentage are means ± SE of three replicates.

	RSO vesicles		ISO vesicles	
Treatments	Alikat	Katepwa	Alikat	Katepwa
- Dig. and - Iono.	47 ± 2.5	50 ± 3.0	70 ± 2.0	70 ± 2.3
+ Dig. and - Iono.	40 ± 2.0	52 ± 2.8	65 ± 3.0	54 ± 2.8
- Dig. and + Iono.	47 ± 2.3	40 ± 3.0	43 ± 3.4	55 ± 3.0
+ Dig. and + Iono.	28 ± 2.7	31 ± 2.5	56 ± 2.5	49 ± 2.8
Average	40 ± 2.0	42 ± 2.5	58 ± 2.7	57 ± 2.7

and Table 4.1) and 31 ± 2.5% (from 401 ± 22 to 275 ± 16 nmol mg protein^{-1} min^{-1}; Figure 4.5 and Table 4.1) in vesicles of Alikat and Katepwa respectively. This negative effect on 1,3-β-D-glucan synthase was less than observed with digitonin or A23187 in isolation.

The sensitivity of 1,3-β-D-glucan synthase to Al was greater in ISO than in RSO vesicles in all four treatments examined (Al by itself, Al in the presence of either digitonin or A23187 in isolation, or Al and both digitonin and A23187 together). Both Alikat and Katepwa showed a similar reduction in 1,3-β-D-glucan synthase activity. In the absence of digitonin and A23187, Al (0-100 μM) reduced the specific activity of 1,3-β-D-glucan synthase by 70% (from 154 ± 13 to 48 ± 9 nmol mg protein^{-1} min^{-1}; Figure 4.6) in Alikat and by 70% (from 182 ± 11 to 52 ± 11 nmol mg protein^{-1} min^{-1}; Figure 4.7) in Katepwa. In the presence of digitonin or A23187 in isolation, Al reduced the specific activity of 1,3-β-D-glucan synthase in ISO vesicles from Alikat by 65 and 43 % respectively (Figure 4.6 and Table 4.1). In the same treatments, the specific activity of 1,3-β-D-glucan synthase in ISO vesicles from Katepwa was reduced by 54 and 55% (Figure 4.7 and Table 4.1). In contrast to RSO vesicles, the presence of digitonin and A23187 together had little effect on the Al-induced inhibition of 1,3-β-D-glucan synthase activity in ISO vesicles. At 100 μM Al, the specific activity of 1,3-β-D-glucan synthase was still reduced by 56% (from 259 ± 11 to 116 ± 9 nmol mg protein^{-1} min^{-1}) in ISO vesicles from Alikat (Figure 4.6 and Table 4.1) and by 49% (from 287 ± 12 to 148 ± 10 nmol mg protein^{-1} min^{-1}) in ISO vesicles from Katepwa (Figure 4.7 and Table 4.1).

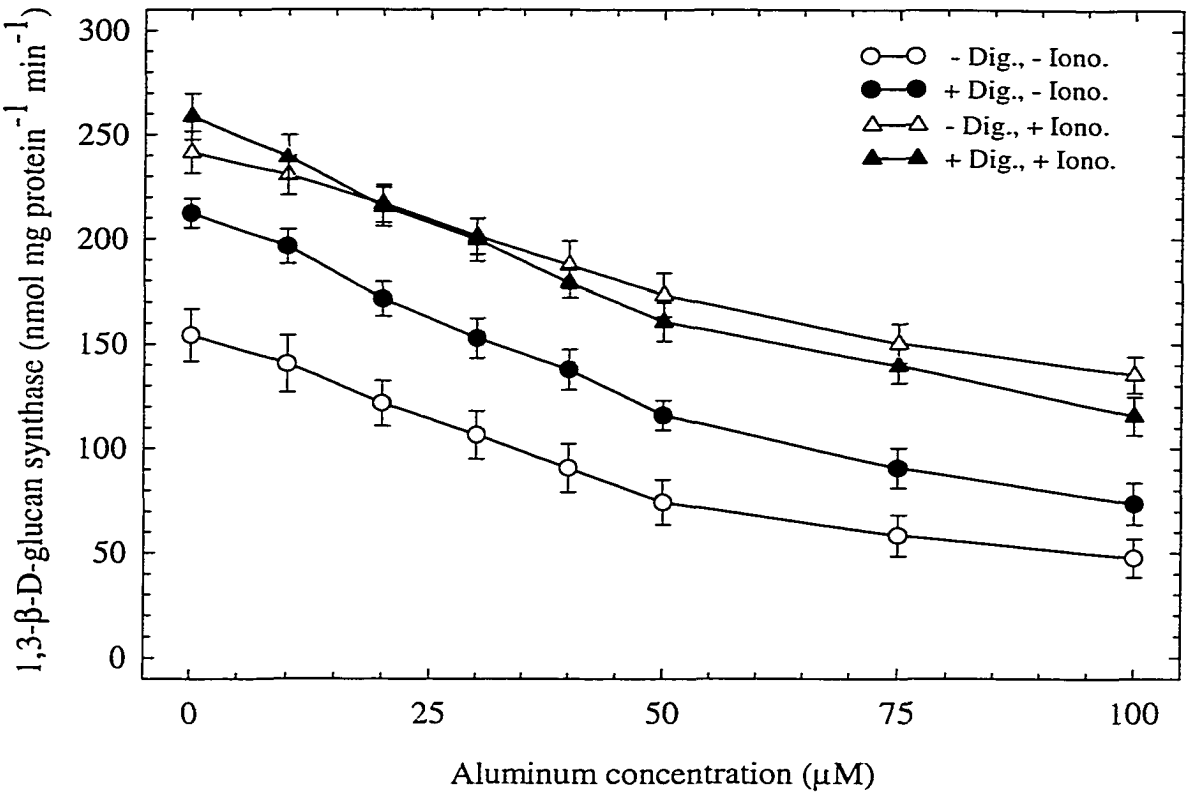

Figure 4.6. The effect of Al on the activity of 1,3-β-D-glucan synthase in ISO plasma membrane vesicles isolated from roots of *Triticum aestivum* cv. Alikat. Experiments were conducted in the presence or absence of digitonin and A23187. Values for specific activity are means ± SE of three replicates.

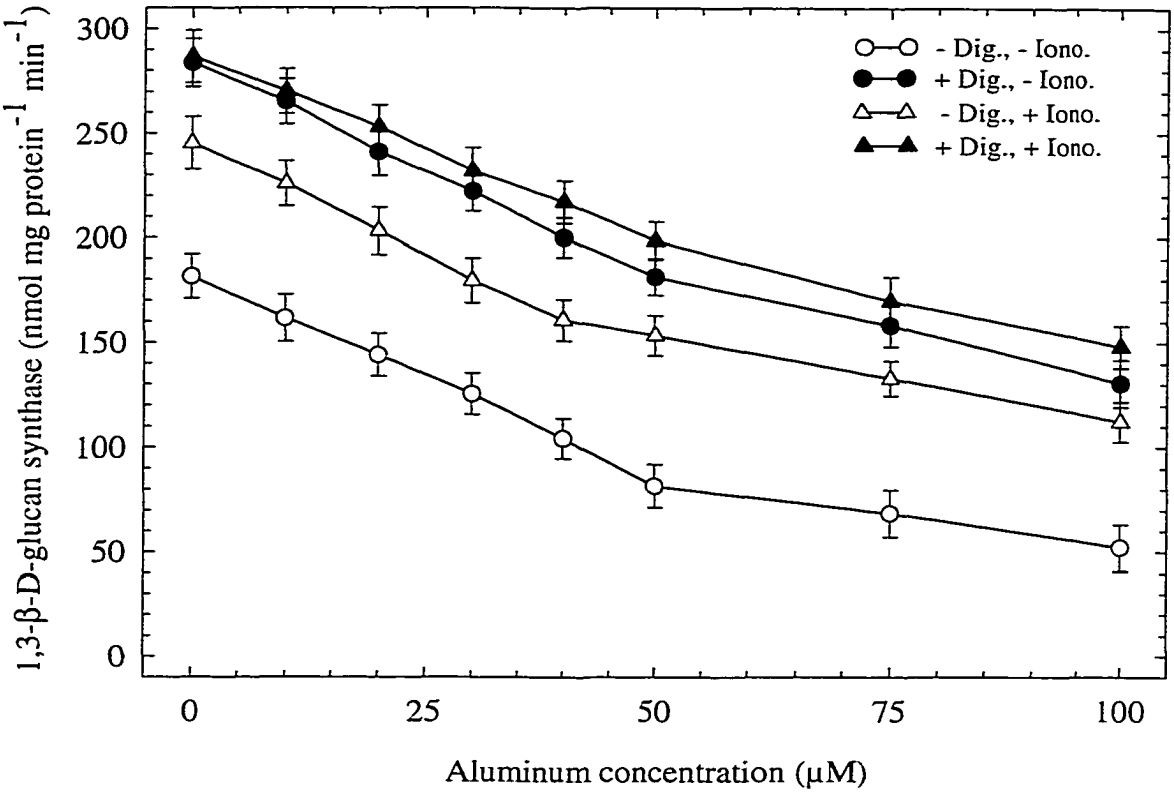

Figure 4.7. The effect of Al on the activity of 1,3-β-D-glucan synthase in ISO plasma membrane vesicles isolated from roots of *Triticum aestivum* cv. Katepwa. Experiments were conducted in the presence or absence of digitonin and A23187. Values for specific activity are means ± SE of three replicates.

4.4. Discussion

The optimum concentration of digitonin required to maximize specific activity, and the maximum specific activity of 1,3-β-D-glucan synthase in root PM isolated from *Triticum aestivum* was dependent upon sidedness of the vesicles. The activity of 1,3-β-D-glucan synthase increased by 176% to 234 ± 11 nmol mg protein^{-1} min^{-1} in ISO and by 1016% to 325 ± 11 nmol mg protein^{-1} min^{-1} in RSO PM vesicles at the presence of 0.006% and 0.010% digitonin (w/v), respectively (Figure 4.1). The increase in activity observed in the presence of digitonin was probably a result of increased membrane permeability, which would increase the availability of substrates and cofactors such as UDP-glucose and Ca^{2+} (Eiberger and Wasserman, 1987; Hayashi *et al.*, 1987; Read and Delmer, 1987; Sloan *et al.*, 1987; Dhugga and Ray, 1994; Kauss, 1996; Li *et al.*, 1997; Palmgren *et al.*, 1990; Johansson *et al.*, 1995), respectively. This would be especially true in RSO PM vesicles (present in both RSO preparations and as a contaminant in ISO preparations), due to the location of the active sites of 1,3-β-D-glucan synthase on the cytosolic face of the PM. In addition, digitonin has been found to interact directly with 1,3-β-D-glucan synthase and activate the enzyme (Kauss and Jeblick, 1986; Fredrikson and Larsson, 1989). In experiments using RSO and ISO PM vesicles isolated from leaves of sugar beet (*Beta vulgaris* L.), Fredrikson and Larsson (1989) demonstrated that maximum activity of 1,3-β-D-glucan synthase occurred at 0.006% digitonin in both vesicle types. In my experiments, activity declined at concentrations above the optimum in ISO vesicles, while the activity in RSO vesicles leveled off. Increased sensitivity of the ISO vesicles may be due to the direct contact of digitonin with the active sites of 1,3-β-

D-glucan synthase, which would face the outside in ISO vesicles (Larsson et al., 1984; Fredrikson, 1993).

The presence of A23187 also increased the activity of 1,3-β-D-glucan synthase, although its stimulating effect was not as high as that of digitonin (Figure 4.2). This is probably due to the specificity of A23187 for Ca influx, whereas digitonin would provide all components necessary to stimulate 1,3-β-D-glucan synthase activity. The optimum level of A23187 required to maximize activity of 1,3-β-D-glucan synthase in both RSO and ISO vesicles was 10 nmol mg protein^{-1}. The actual proportion of enzyme to A23187 may be important, since activity decreased at concentrations of A23187 both above and below 10 nmol mg protein^{-1} (Figure 4.2). The increase in enzyme activity observed as levels of A23187 increased from 0 to 10 nmol mg protein^{-1} was presumably a result of A23187 providing channels for Ca to cross the PM. Unfortunately this does not explain why A23187 inhibited the activity of 1,3-β-D-glucan synthase at higher levels. Increasing concentrations of A23187 above 10 nmol mg protein^{-1} may not only raise the level of $[Ca^{2+}]_{cyt}$ to the upper limit which may cause a negative feedback to the activity of 1,3-β-D-glucan synthase, but it may also disrupt the integrity of PM and 1,3-β-D-glucan synthase protein as well. High concentrations of A23187 may also have indirect effects on metabolic reactions such as callose formation as a consequence of changes in the intracellular ionic composition. Future studies are necessary to unfold this mystery.

The requirement for Ca^{2+} as a cofactor for 1,3-β-D-glucan synthase activity was supported by the observation that the presence of Ca itself increased the activity of 1,3-β-

D-glucan synthase in both the ISO and RSO PM vesicles (Figure 4.2). In fact, the activity of this enzyme in ISO vesicles increased 5.5 fold in the presence of 2mM $CaCl_2$ and no digitonin or A23187. The dependence of 1,3-β-D-glucan synthase on Ca has been well documented in the literature (Fink *et al.*, 1987; Hayashi *et al.*, 1987; Sloan and Wasserman, 1989; Read and Delmer, 1990; Wu and Wasserman, 1993; Kauss, 1996) and was confirmed in the present study.

To determine the effect that Al had on 1,3-β-D-glucan synthase activity, the assay was performed in the presence of Al (0 - 100 μM $AlCl_3$). When RSO vesicles from both cultivars (Alikat and Katepwa) were exposed to Al, the activity of 1,3-β-D-glucan synthase declined in proportion to the concentration of Al present in the assay. The Al-induced inhibition was strong in the absence of digitonin and A23187 (Figure 4.4, 4.5, Table 4.1). Similar results have also been found with 1,3-β-D-glucan synthase in PM fractions from roots of wheat (*Triticum aestivum*) and spruce (*Picea abies*) (Widell *et al.*, 1994). In the presence of 100 μM $AlCl_3$, the activity of 1,3-β-D-glucan synthase in RSO vesicles decreased by approximately 47% compared to vesicles incubated without $AlCl_3$. Aluminum may affect the activity of 1,3-β-D-glucan synthase in these membrane vesicles by displacement of Ca^{2+} from PM binding sites, which in turn could decrease Ca^{2+} uptake and availability to 1,3-β-D-glucan synthase (Kinraide and Parker, 1987; Rengel and Elliott, 1992). Aluminum may also bind directly to 1,3-β-D-glucan synthase inducing conformational changes to the enzyme which in turn may affect its activity (Haug, 1984).

When ISO vesicles were exposed to Al in the absence of digitonin and A23187, the activity of 1,3-β-D-glucan synthase was inhibited to a greater extent than that in RSO (Table 4.1). My experiments do not provide much insight into the cause of this effect. The greater degree of inhibition observed in the ISO vesicles may due to the Al having a better access to the active sites of the enzyme in this orientation. On the other hand, in the absence of digitonin and A23187, the inhibition of 1,3-β-D-glucan synthase by Al in RSO PM vesicles may caused by Al blocking Ca^{2+} influx into the vesicle interior.

If these were the only factors affecting 1,3-β-D-glucan synthase activity, then the degree of inhibition in RSO and ISO vesicles would be expected similar in the presence of digitonin. This was not the case. The presence of digitonin and A23187 had a large effect on the degree of inhibition observed in the presence of Al. However, when digitonin and A23187 were present together with Al in the assay medium, the degree of inhibition observed was still greater in ISO vesicles than in RSO vesicles (Table 4.1). Interestingly, in both RSO and ISO vesicles, the degree of inhibition was generally greater in the absence than in the presence of digitonin and A23187 (Table 4.1). Thus, the expectation that the presence of detergents would speed up the inhibitory effects of Al was not shown in these experiments. This may be due to contradictory roles of digitonin, allowing access of Al to inhibit the activity of 1,3-β-D-glucan synthase on one hand, and directly stimulating the activity of 1,3-β-D-glucan synthase, on the other hand.

The activity of 1,3-β-D-glucan synthase in RSO fractions in the absence of digitonin, appeared to reflect a contribution by the ISO contaminant (approximately

10%). However, this was not always the case. The presence of A23187 raised the activity of 1,3-β-D-glucan synthase in RSO fractions well above the 10% estimated by contaminant alone (Figure 4.4, 4.5). A similar phenomenon was shown in ISO fractions with 33% RSO contaminant, where the specific activity of 1,3-β-D-glucan synthase was below the estimated quantities (Figure 4.1, 4.2). I realize that the presence of 10% ISO contaminant in RSO fractions and 33% RSO contaminant in ISO fractions complicates any generalization that might be drawn from these experiments.

Few differences were found in the inhibitory effect of Al on 1,3-β-D-glucan synthase in the two wheat cultivars examined. This suggests that the Al binding affinity of 1,3-β-D-glucan synthase from Alikat is the same as that from Katepwa and that the PM in these two cultivars may have similar properties in terms of allowing movement of Al to cross the lipid bilayer. In other words, the properties of the PM in these two cultivars may not account for the differences in cultivar sensitivity towards Al. Further research is necessary to explore this assumption.

These *in vitro* experiments indicated that Al strongly inhibits the specific activity of 1,3-β-D-glucan synthase in both RSO and ISO PM vesicles isolated from roots of *Triticum aestivum*. Although the presence of digitonin and A23187 decreased the overall degree of inhibition found, Al was always inhibitory. These results were observed even though enzyme assays were performed at pH 7.25. This suggests that physiological pH does not render Al non-toxic, despite the effect that this pH would have on the overall speciation of Al in solution. No differences in the sensitivity of 1,3-β-D-glucan synthase

to Al toxicity were observed between these two *Triticum aestivum* cultivars despite their different resistance. This condition may indicate that in addition to the same direct-sensitivity of 1,3-β-D-glucan synthase to Al, the PM of Al-sensitive cv. Katepwa and Al-resistant cv. Alikat provide similar access for Al to bind the 1,3-β-D-glucan synthase protein.

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5. Characterizing the effect of aluminum on the activity of 1,3-β-D-glucan synthase *in vivo*

5.1 Introduction

Studies using secondary ion mass spectrometry (SIMS) have suggested that Al accumulates in the cytoplasm of root-apex cells of soybeans (*Glycine max*) after 30 min of exposure (Lazof *et al.*, 1994, 1996). Other experiments on giant internodal cells of *Chara corallina*, where cell wall contents were separated from the cell protoplasm by microsurgery, have also shown that Al influx across the PM occurs without delay (Taylor *et al.*, 1999). However, there is a lack of information on the binding of Al to biological ligands inside plant cells, and it is difficult to conceive of "significant" free Al^{3+} at the near-neutral pH of the cytoplasm (Foy, 1988). While the speciation of Al inside plant cells has yet to be determined, several lines of evidence suggest that submicromolar concentrations of Al can affect biochemical processes. For example, Shi *et al.*, (1993) using permeabilized neuroblastoma cells demonstrated that nmol range [nmol $(gfr.wt)^{-1}$] internal concentrations of Al were effective in disrupting the phosphatide inositol pathway. Similarly, Schofl *et al.* (1990) demonstrated that the affinity of Al for binding to inositol triphosphate (PIP_3) was approximately 10^6-fold higher than to ATP. Therefore, while little information on the speciation of Al within cells is available, results from various systems suggest that metabolic effects due to Al are possible, even within the cytoplasmic environment (Lazof *et al.*, 1997).

Callose synthesis, which is driven by the activity of 1,3-β-D-glucan synthase (EC 2.4.1.34), has been used as an indicator of Al injury and a selection criterion for Al sensitivity in *Glycine max* (Wissemeier *et al.*, 1987, 1992), *Picea abies* (Jorns *et al.*,

1991), *Triticum aestivum* (Schreiner *et al.*, 1994; Zhang *et al.*, 1994), and *Zea mays* (Llugany *et al.*, 1994). The activity of this enzyme requires calcium as a cofactor and uses uridine 5'diphosphate (UDP)-glucose as a substrate (Aist, 1993; Kauss *et al.*, 1983, 1985; Fredrikson and Larsson, 1989; Lin and Lee, 1991). At toxic concentrations normally found in soils (10-100 μM), Al is capable of blocking voltage-gated plasma membrane Ca^{2+} channels, disrupting inositol 1,4,5-triphosphate-mediated signal events (Jones and Kochian, 1995; Huang *et al.*, 1995), occupying Ca^{2+}-binding sites in Ca^{2+} requiring enzymes, or altering Ca^{2+}-mediated cytoskeletal dynamics (Haug, 1984; Taylor, 1988; Rengel, 1992b; Delhaize and Ryan, 1995). These effects may bring immediate changes in cell Ca^{2+} homeostasis. As the length of time of contact with Al increases, a reduction of net Ca^{2+} influx at the root tip may cause localized Ca deficiency, affecting both structure and function of membranes and cells (Rengel *et al.*, 1995).

The temporal lag between detection of Al effects and evidence of intracellular Al led to an assumption that Al-toxicity might be mediated either through signal transduction from the apoplasm (Kinraide *et al.*, 1992, 1994; Rengel, 1992a, b), or from specific cellular sites in the root apex (Bennet *et al.*, 1985a, b; Bennet and Breen, 1990). Aluminum has been reported to induce a rapid, transient increase in $[Ca^{2+}]_{cyt}$ in protoplasts isolated from roots of *Triticum aestivum* (Lindberg and Strid, 1997). However, restriction of root hair elongation occurred as much as 20 min before detectable changes $[Ca^{2+}]_{cyt}$ within root hairs, suggesting that disruption of free calcium concentrations was not required to initiate the process of Al toxicity (Jones *et al.*, 1998). Likewise, detection of Al effects on Ca^{2+} fluxes into root hairs using a vibrating Ca^{2+}-

selective microelectrode system have revealed that Al levels that inhibited root hair growth failed to block Ca^{2+} fluxes (Jones *et al.*, 1995). These results suggest that Al toxicity is not always preceded by an alteration in Ca^{2+} homeostasis.

Despite these contradictory lines of evidence, I have hypothesized that the stimulatory effect of Al on 1,3-β-D-glucan synthase activity, resulting from an Al-induced increase in $[Ca^{2+}]_{cyt}$, is greater than the direct inhibitory effect. If this hypothesis is correct, it should be possible to observe an Al-induced inhibition of 1,3-β-D-glucan synthase activity *in vivo* when the enzyme is initially stimulated by high $[Ca^{2+}]_{cyt}$ levels (induced by A23187 or detergents). The extent of inhibition may increase under conditions promoting free access of Al to the cytoplasm (induced by detergents).

5.2 Materials and methods

5.2.1 Plant material

Seeds of Al-tolerant (Alikat) and Al-sensitive (Katepwa) cultivars of *Triticum aestivum* were surface sterilized in 1.2% sodium hypochlorite for 20 min., and germinated overnight in solution of 0.005 gL^{-1} Vitavax to limit fungal growth. Seedlings were then grown for 5 days on nylon mesh suspended over 15 L of a full nutrient solution containing (mM) 3.30 NO_3^- -N, 0.30 NH_4^+ -N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S; and (μM) 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo (pH 4.4) in a growth chamber with 16 h of light (20 °C, 68% relative humidity) and 8 h of darkness (16 °C, 85% relative humidity). The photosynthetic photon flux (PPF) was 335 ± 12 μmol m^{-2} s^{-1} at plant base level.

5.2.2 Experimental design

Preliminary experiments were performed using Al-sensitive cv. Katepwa to find optimal concentrations of detergents (digitonin and CHAPS), A23187 and the general effects of Al on callose production at the root tips. In these experiments, working concentrations of digitonin varied from 0 - 75 μM, CHAPS varied from 0-80 μM, A23187 varied from 0 - 80 μM, and Al varied from 0 - 100 μM. After determining optimal concentrations, additional experiments were conducted to test the above hypothesis. Five-day-old plants from Al-resistant cv. Alikat and Al-sensitive cv. Katepwa were exposed to Al (0, 10, 20, 30, 40, 50, 75, and 100 μM) in the absence or the presence digitonin, CHAPS, A23187, and combinations of digitonin and A23187 or CHAPS and A23187 in a simple salt solutions (4 mM $CaCl_2$) for 24 h. Each treatment had three replicates and experiments were repeated three times independently. After 24-h exposure, root tips (2 cm) were harvested and stored in 1.5 mL microcentrifuge tubes containing 0.8 mL 95% ethanol.

5.2.3 Spectrofluorometric determination of callose

Callose determination was performed according to Zhang *et al.* (1994). Ethanol was decanted from root tips and 0.2 mL of 1 N NaOH was added to the tubes. Root tips were ground for 20 s in the tubes using a Teflon pestle (Mandel) mounted to an electric drill. The pestle was rinsed with 0.8 mL of 1 N NaOH after each grinding. Samples were placed in a water bath (80 °C) for 15 min to solubilize callose and centrifuged for 3 min at 15,000 x g in a Centra-M centrifuge (International Equipment Company). The reaction mixture contained 0.2 mL supernatant, 0.2 mL 1 N NaOH, 0.8 mL 0.1% aniline blue

(Polysciences), 0.42 mL 1 N HCl, and 1.18 mL glycine-NaOH buffer (pH 9.5). The reaction mixture was incubated for 20 min at 50 °C, and for 30 min at room temperature (22 °C). Callose content was determined using a spectrofluorometer (Aminco Bowman Series 2) with excitation at 398 nm and emission at 495 nm. Pachyman (Calbiochem) was used as a standard, and callose contents were expressed as microgram pachyman equivalent per gram fresh root mass (μg pachyman equivalents g^{-1}, FW).

5.3 Results

Production of 1,3-β-D-glucans (callose) by 1,3-β-D-glucan synthase in root tips of Al-resistant (Alikat) and Al-sensitive (Katepwa) cultivars increased with concentrations of Al in the growth media, although the extent of increase was different between cultivars. In the absence of Al, callose production in cv. Alikat (328 ± 16 μg g^{-1} FW) was similar to that in Katepwa (367 ± 18 μg g^{-1} FW), but as concentrations of Al increased, accumulation of callose was approximately two-fold higher in the sensitive cultivar than in the resistant cultivar. For example, at 100 μM Al, root tips of cv. Katepwa accumulated 2964 ± 230 μg g^{-1} FW of callose, while root tips of Alikat accumulated 1570 ± 147 μg g^{-1} FW (Figure 5.1).

Preliminary experiments were also performed to identify a concentration of A23187 that triggered the highest production of callose by engaging 1,3-β-D-glucan synthase activity through an increase in $[Ca^{2+}]_{cyt}$. Callose production in root tips of cv. Katepwa increased from 284 ± 17 to 598 ± 11 μg g^{-1} FW in the absence of Al as concentrations A23187 increased from 0 to 2.5 μM. In contrast, callose production

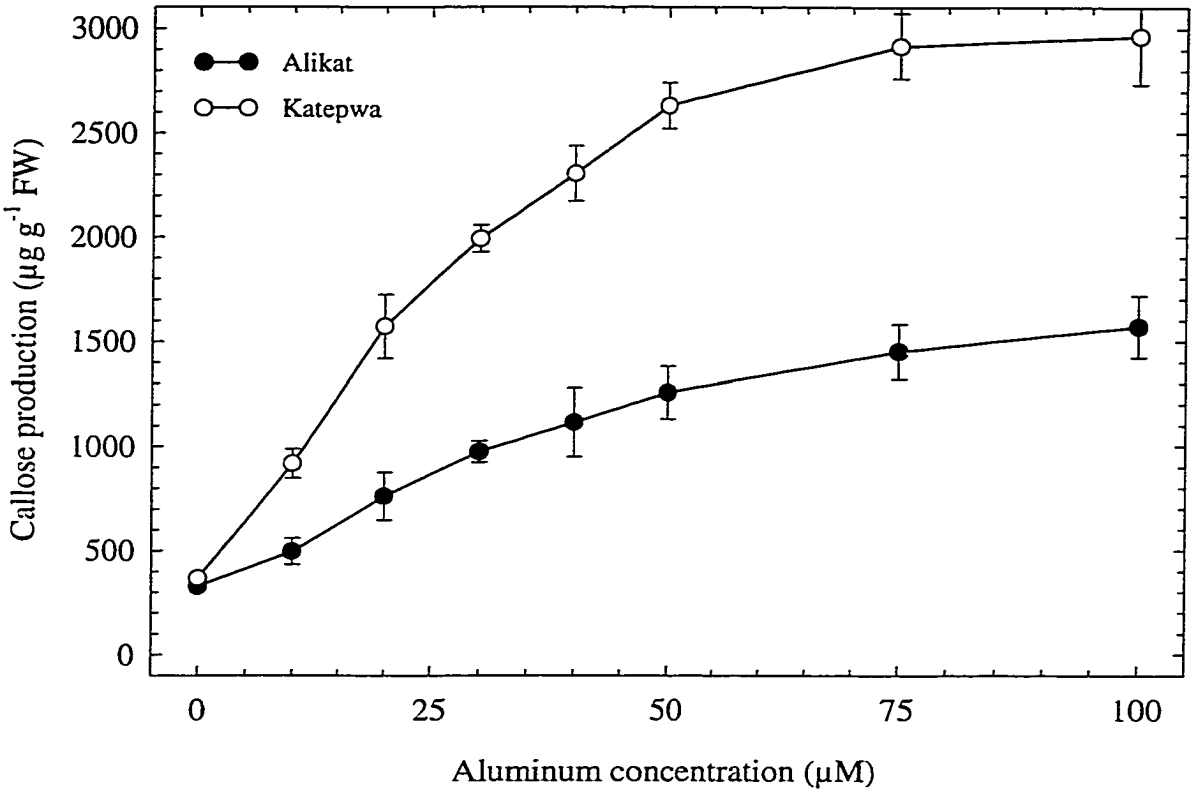

Figure 5.1. The effect of Al on callose production in root-tips of *Triticum aestivum* cv. Alikat and Katepwa. Values are means ± SE of three replicates.

reached a maximum level of 4226 ± 25 μg g^{-1} FW at 0.5 μM A23187 in the presence of 50 μM Al (Figure 5.2). Maximal callose accumulation in the presence of Al was approximately 7-fold higher than in the absence of Al, suggesting that treatment with A23187 alone was insufficient to fully engage callose synthase (1,3-β-D-glucan synthase) activity. Above each of these optimal concentrations (in the presence or absence of Al) synthesis of callose declined, although callose concentrations were still higher than in the absence of A23187. This was particularly true in the presence of 50 μM Al (Figure 5.2).

Similar experiments were performed in the presence of detergents (digitonin and CHAPS). Digitonin increased callose production in root tips of cv. Alikat (from 339 ± 14 to 742 ± 12 μg g^{-1} FW) and Katepwa (from 359 ± 16 to 1325 ± 22 μg g^{-1} FW), in the absence of Al. The extent of increase was greater in cv. Katepwa than in cv. Alikat (Figure 5.3). Similar results were observed with CHAPS. Callose production in the root tips of Katepwa increased from 347 ± 4 to 818 ± 3 μg g^{-1} FW, while callose production in Alikat increased from 316 ± 3 to 442 ± 3 μg g^{-1} FW (Figure 5.4). A different pattern was observed in the presence of 50 μM Al. At low concentrations of digitonin (2 - 8 μM), and CHAPS (2 - 14 μM) callose production decreased below that in the absence of detergents. Low concentrations of digitonin decreased callose levels from 993 ± 16 to 635 ± 17 μg g^{-1} FW (35%) in cv. Alikat and from 1823 ± 19 to 1273 ± 22 μg g^{-1} FW (42%) in cv. Katepwa (Figure 5.3). Low concentrations of CHAPS decreased callose levels from 1048 ± 6 to 621 ± 7 μg g^{-1} FW (32%) in cv. Alikat and from 1846 ± 12 to1290 ± 8 μg g^{-1} FW (42%) in cv. Katepwa (Figure 5.4). At higher concentrations (10 –

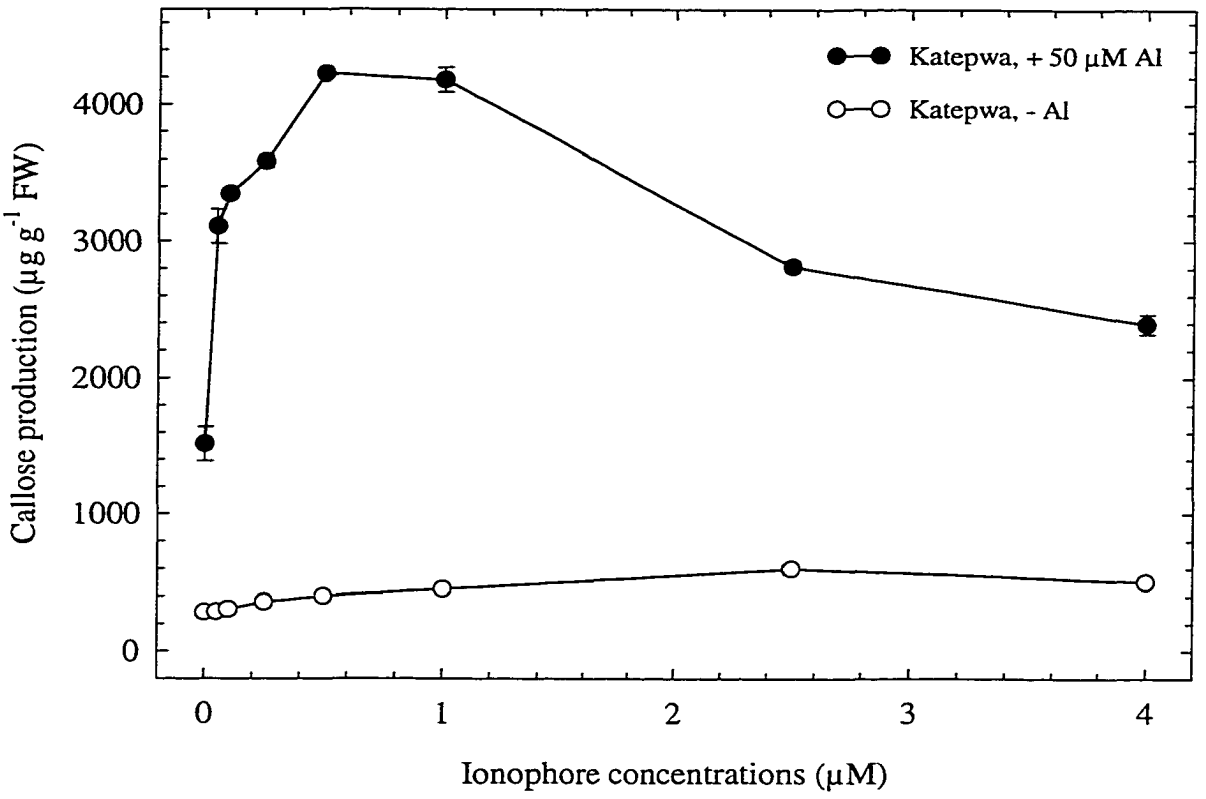

Figure 5.2. The effect of ionophore A23187 on callose production in root-tips of *Triticum aestivum* cv. Katepwa in the presence and absence of 50 µM Al. Values are means ± SE of three replicates.

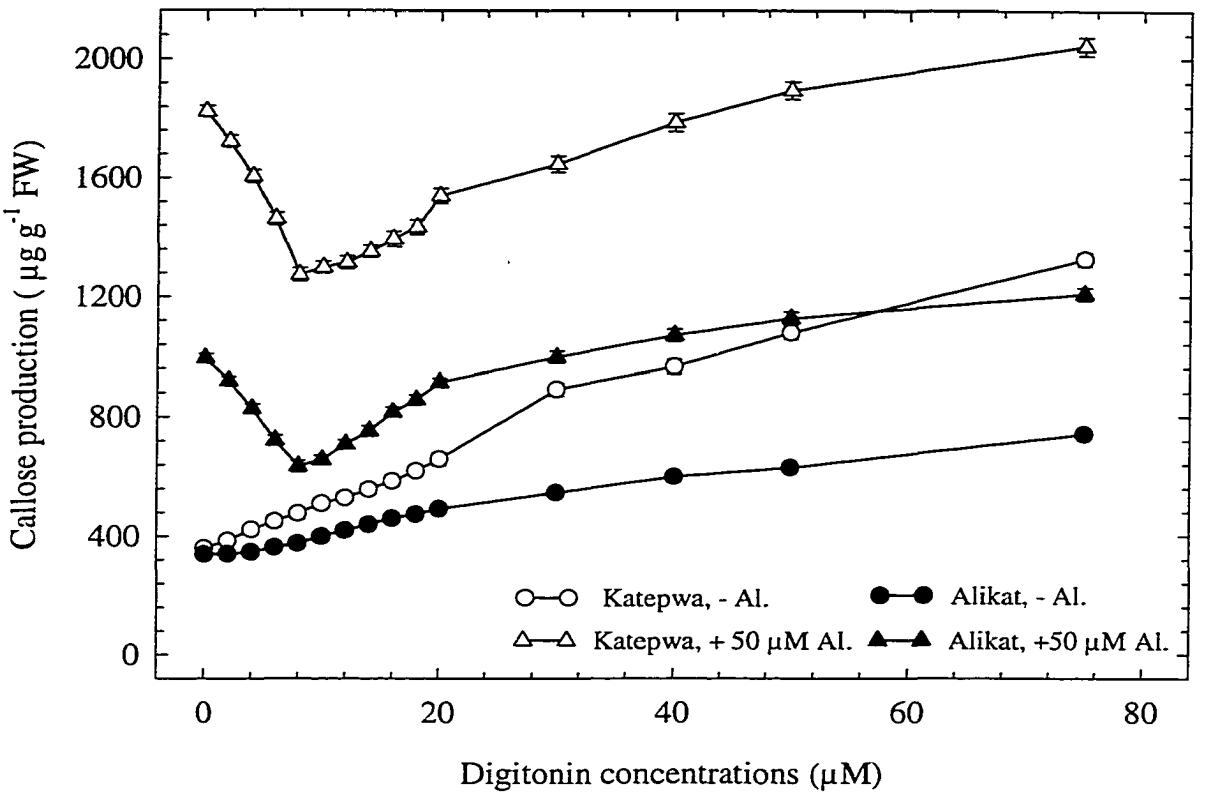

Figure 5.3. The effect of digitonin on callose production in root-tips of *Triticum aestivum* cv. Alikat and Katepwa in the presence and absence of 50 µM Al. Values are means ± SE of three replicates.

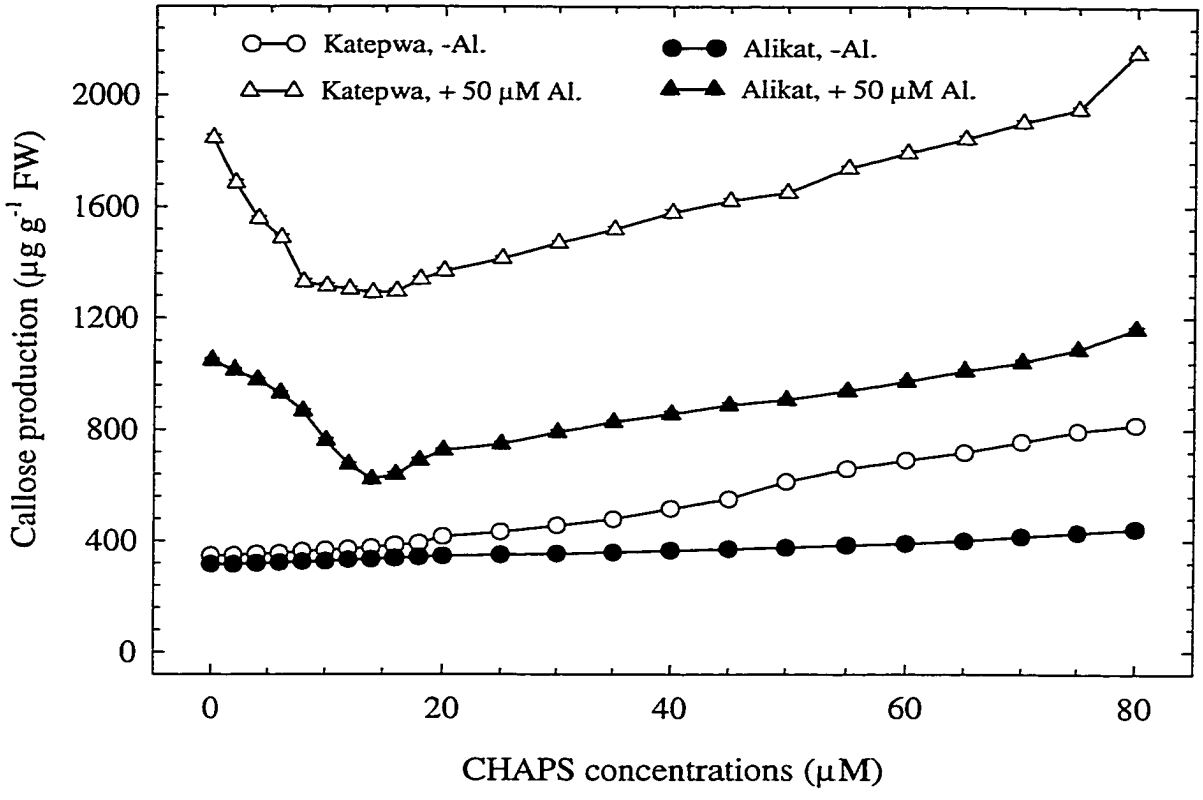

Figure 5.4. The effect of CHAPS on callose production in root-tips of *Triticum aestivum* cv. Alikat and Katepwa in the presence and absence of 50 μM Al. Values are means ± SE of three replicates.

75 μM), digitonin increased production of callose with maximal concentrations reaching 1207 ± 22 μg g^{-1} FW in Alikat and 2042 ± 30 μg g^{-1} FW in Katepwa (Figure 5.3). Similar to digitonin, higher concentrations (16 - 80 μM) CHAPS also triggered callose production to a maximal level of 1195 ± 31 μg g^{-1} FW in Alikat and 2203 ± 44 μg g^{-1} FW in Katepwa (Figure 5.4).

The effect of Al toxicity on callose production was further explored using the concentrations of A23187 (0.5 μM), where callose production was highly induced, and the concentrations of digitonin (8 μM) or CHAPS (14 μM), where callose production was highly reduced. Callose accumulation was higher when Al-resistant (Alikat) and Al-sensitive (Katepwa) cultivars were subjected to the combination of Al, CHAPS, and A23187 than when treated alone or a combination of two. For example, the combination of A23187 and CHAPS in the presence of 100 μM Al triggered callose production to 2506 ± 54 μg g^{-1} FW in Alikat and 4512 ± 37 μg g^{-1} FW in Katepwa (Figure 5.6), an increase of approximately 144% compared with the presence of Al alone (see Figure 5.1). Without A23187, the combination of Al and CHAPS decreased callose accumulation by 38% in Alikat and 46% in Katepwa compared with Al alone. In contrast, Al-resistant and Al-sensitive cultivars responded differently when they were subjected to a combination of Al, digitonin, and A23187. Callose accumulation increased by 35% when the Al-resistant cv. Alikat was subjected to the combination of Al, digitonin, and A23187. Without A23187 a combination of Al and digitonin decreased callose production by 38% compared with Al alone (Compare Figure 5.1 with Figure 5.5). While in Al-sensitive cv. Katepwa, the presence of digitonin in a combination with A23187 decreased callose

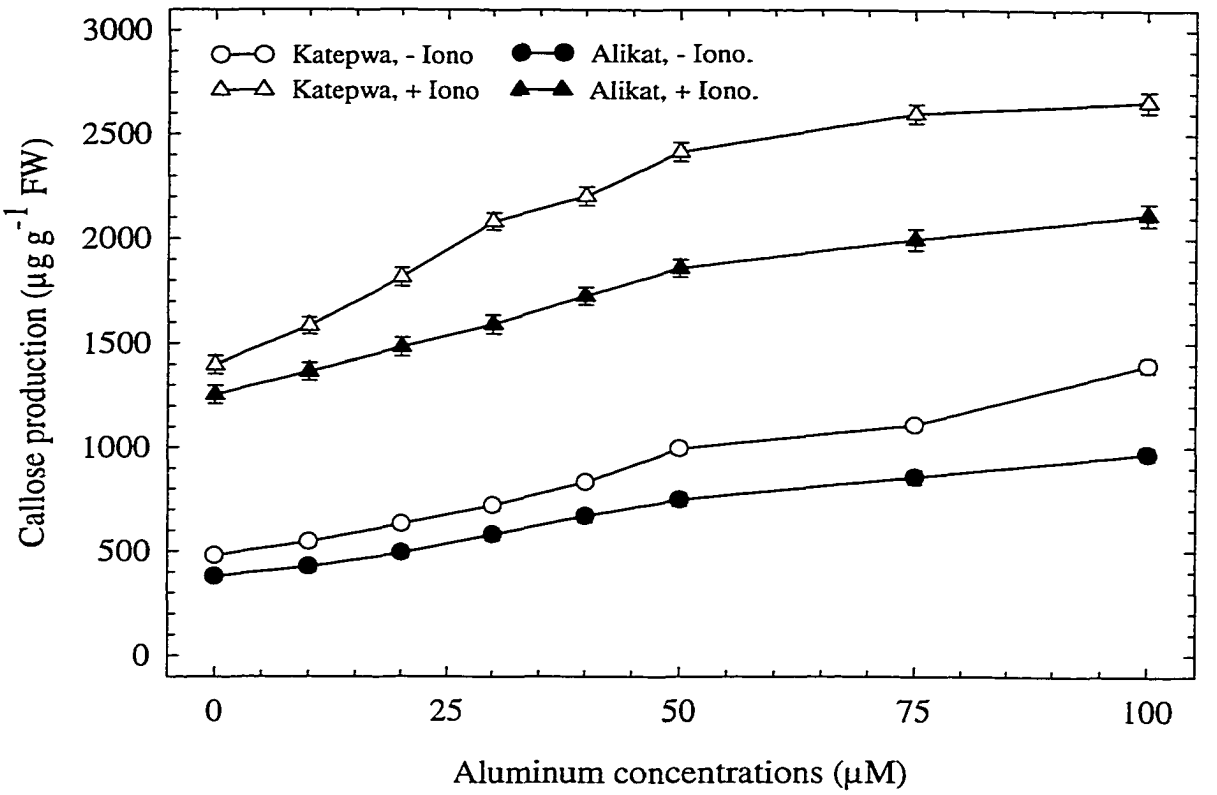

Figure 5.5. The effect of Al on callose production in root-tips of *Triticum aestivum* cv. Alikat and Katepwa in the presence of 8 μM digitonin and in the presence and absence of 0.5 μM ionophore. Values are means ± SE of three replicates.

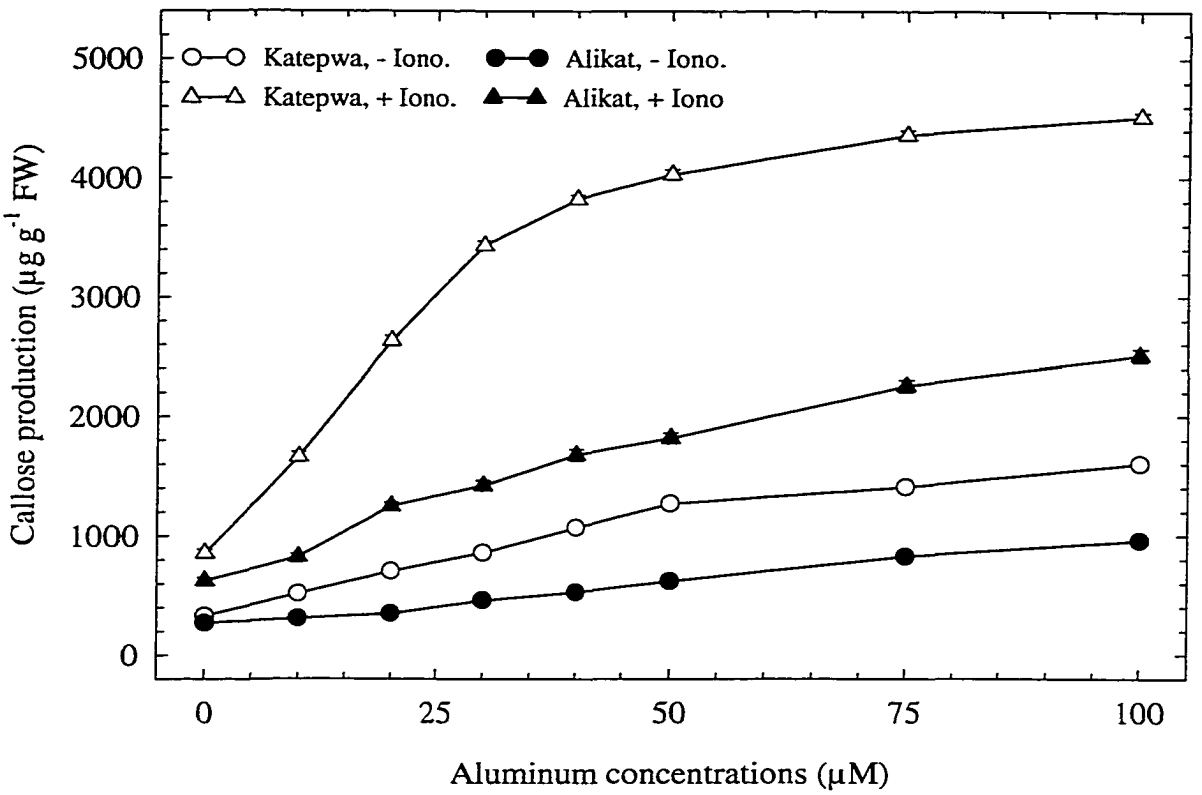

Figure 5.6. The effect of Al on callose production in root-tips of *Triticum aestivum* cv. Alikat and Katepwa in the presence of 14 μM CHAPS and in the presence and absence of 0.5 μM Ionophore. Values are means ± SE of three replicates.

production by 10.4%, and in a condition without A23187 the inhibition of callose production was stronger (53%; compared Figure 5.1 with Figure 5.5).

5.4 Discussion

In contrast to the inhibitory effect of Al on 1,3-β-D-glucan synthase activity *in vitro* (Chapter 4), the presence of Al in exposure solutions increased callose accumulation *in vivo* (Figure 5.1). The intensity of callose accumulation in *Triticum aestivum* was dependent upon genotypical differences in Al resistance, an observation that is consistent with previous reports by Zhang *et al.* (1994) and Nichol and Oliveira (1995). Aluminum-induced accumulation of callose in the Al-sensitive cv. Katepwa, was approximately two-fold higher than in the Al-resistant cv. Alikat (Figure 5.1).

I hypothesized that the stimulatory effects of Al-*in vivo* reflected an increase in $[Ca^{2+}]_{cyt}$. Thus, efforts were made to increase $[Ca^{2+}]_{cyt}$ and callose production using detergents and A23187. Treatments with increasing concentrations of detergents (digitonin or CHAPS) and A23187 alone (Fig, 5.2 – 5.4) increased callose accumulation, but were not sufficient to trigger callose production as high as in the presence of Al alone. The inability of these agents to induce high levels of callose production could reflect an inability to increase the supply of calcium in the cytoplasm (where the 1,3-β-D-glucan synthase active sites are located) to the same extent as Al. Interestingly, however, the effects of detergents and A23187 on callose accumulation were different in the presence of Al (Fig 5.2 – 5.4).

In the presence of Al, A23187 induced a larger increase in callose production (280%) at low concentrations (0-0.5 uM), followed by a progressive decline at higher concentrations (0.5-4 uM). The opposite effect was observed with increasing concentrations of digitonin and CHAPS. At low levels (≤ 8 μM digitonin or ≤ 14 μM CHAPS) of detergents, Al inhibited callose production in Alikat by 35% (in the presence of digitonin, Figure 5.3) and 32% (in the presence of CHAPS, Figure 5.4). With the same treatments, a stronger effect of Al on callose production was observed in the Al-sensitive cv. Katepwa, in which callose synthesis declined by 42% in the presence of digitonin (Figure 5.3) or CHAPS (Figure 5.4). As concentrations of detergents increased, the inhibitory effect was overshadowed by a stimulatory effect of Al on callose production. My data do not provide insight into the cause of these effects. The increase in callose production in the absence of Al and in the presence of Al at low concentrations of A23187 could reflect an increase in $[Ca^{2+}]_{cyt}$. Zhang *et al.* (1998) demonstrated that A23187 elicited a rapid increasing of $[Ca^{2+}]_{cyt}$ in the cells of intact wheat root. An Stimulation of callose accumulation by A23187 may due to the specific ability of A23187 to bind and then transport Ca from the external medium (Pfeiffer *et al.*, 1974; Deber *et al.*, 1985).

The detergent-induced increase in callose production in the absence of Al and in the presence of Al at high concentrations of digitonin and CHAPS levels (above 8 μM of digitonin, and 14 μM of CHAPS) could also reflect an increase in $[Ca^{2+}]_{cyt}$. Alternatively, the induction of callose production at high levels of detergent may also be due to the direct effects of detergents on 1,3-β-D-glucan synthase. Wu and Wasserman (1993)

indicated that detergents often stimulate callose synthesis. Even after solubilizing in digitonin or CHAPS, callose synthase (1,3-β-D-glucan synthase) was still active (Eiberger and Wasserman, 1987). The inhibition of callose accumulation by Al in the presence of low levels of digitonin or CHAPS in this *in vivo* system could reflect improved access of Al to the cytosolic face of 1,3-β-D-glucan synthase. At high levels, however, the stimulating effects of digitonin and CHAPS on 1,3-β-D-glucan synthase activity may overshadow the inhibitory effects of Al.

When CHAPS, A23187, and Al were present together, the ability of root tips (both Al-resistant cv. Alikat and Al-sensitive cv. Katepwa) to synthesize callose was stronger (144%) than Al alone (Figure 5.6). The response of plants to the combination of Al, digitonin, and A23187 reflected their degree of sensitivity. In this condition, the Al-resistant cv. Alikat accumulated more callose (38% higher than with Al alone), whereas the Al-sensitive cv. Katepwa reduced its capacity to accumulate callose by 10% (Figure 5.5). Without A23187, combinations of Al and digitonin or CHAPS always reduced callose accumulation in both cultivars, although the degree of inhibition was always higher in Katepwa. Although caution should be taken when comparing the results of different experiments conducted at different times, these observations are consistent with the hypothesis that the presence of detergents may increase the ability of Al to cross the PM and bind to 1,3-β-D-glucan synthase.

The prediction that callose production in an *in vivo* system would be inhibited by Al when 1,3-β-D-glucan synthase is initially stimulated by treatment with A23187 and

the prediction that extent of inhibition may increase under conditions promoting free access of Al to the cytoplasm (induced by detergents) was not fully supported through this series of experiments. In all treatments (except at low concentrations of digitonin or CHAPS and in the presence of Al), the inhibitory effects of Al were overshadowed by its stimulatory effects. The inability of digitonin, CHAPS, and A23187 alone to mimic the induction of callose synthesis by Al may indicate that digitonin, CHAPS, or A23187 when present alone are not able to trigger a sufficiently large increase in $[Ca^{2+}]_{cyt}$ to stimulate 1,3-β-D-glucan synthase. It is also possible that the response of 1,3-β-D-glucan synthase to Al may involve more complex processes, including not only an increase in $[Ca^{2+}]_{cyt}$, but also changes in the availability of substrates for 1,3-β-D-glucan synthase. This may contribute to the different trends of Al in *in vivo* and *in vitro* systems.

The present experiments showed that it may be possible to manipulate the concentration of detergents to exploit the inhibitory or the stimulatory effects of Al. Future studies exploring the possible effects of Al on $[Ca^{2+}]_{cyt}$, the availability of substrates for 1,3-β-D-glucan synthase, and its links to signal transduction are required.

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6. Characterizing the effects of aluminum on cytosolic calcium concentrations and accumulation of 1,3-β-D-glucans

6. Introduction

Higher plant cells, in common with those of other organisms, are capable of maintaining their cytoplasmic free calcium concentration ($[Ca^{2+}]_{cyt}$) at approximately 0.1 μM by the action of membrane calcium (Ca) transporters. These transporters act to remove Ca from the cytosol and expel it to the apoplast or deposit it in intracellular stores. Transport occurs against a steep electrochemical potential gradient favoring the influx of Ca into the cytoplasm (Evans *et al.*, 1991). The maintenance of low $[Ca^{2+}]_{cyt}$ and the mechanisms responsible for generation (and subsequent abolition) of transient rises in $[Ca^{2+}]_{cyt}$ are essential for the operation of a Ca-based signaling system. It has been proposed that transient increases in $[Ca^{2+}]_{cyt}$ may serve to modulate the activity of key regulatory enzymes such as protein kinases in response to a variety of stimuli including hormone binding (Poovaiah and Reddy, 1987), blue light interception (Gallagher *et al*, 1988), and metal toxicity (Gandolfi *et al.*, 1998).

Schofield *et al.* (1998) documented the distribution Al and Ca in Al-intoxicated roots of *Allium cepa*. They suggested that not enough Al was present in the apoplasm to have replaced, atom-for-atom, more than a minor fraction of Ca. Furthermore, an inverse relationship between variations in Al and Ca concentrations was not observed for pairs of adjacent 30-μm-diameter regions. Therefore, their experimental results did not support the hypothesis that Al substantially reduces the quantity of bound Ca by competing with

Ca for binding sites in the apoplasm. Instead they suggested that reductions in Ca content were a non-local and indirect consequence of Al-intoxication.

Among cell organelles, the plasma membrane (PM) plays a key role in the overall regulation of $[Ca^{2+}]_{cyt}$. Rengel *et al.* (1995) demonstrated that Ca^{2+} channels isolated from root PM of *Triticum aestivum* and incorporated in planar lipid bilayers remained mainly closed at membrane potentials more negative than -130 mV. When the membrane was depolarized down to -110 or -100mV, the channels were open about 90% of the recording time. A small decrease in membrane potential could therefore increase the probability of Ca^{2+} channels being open, resulting in an increased inward Ca^{2+} flux from the large apoplastic pool. Intracellular pools and their membranes may also be involved in regulation, but their finite capacity may limit their involvement to short-term, local regulation. For example, the endoplasmic reticulum (ER) may play a role in Ca homeostasis (Hepler and Wayne, 1985) by functioning in short-term buffering of $[Ca^{2+}]_{cyt}$ and as a source of Ca for transient fluxes. Although it is difficult to establish the proportional contribution of external and internal Ca^{2+} stores in stimulus-evoked elevation of $[Ca^{2+}]_{cyt}$, the dominant Ca^{2+} source in higher plants appears to be the central vacuole (the principal intracellular Ca^{2+} store in most mature plant cells; Evan *et al.*, 1991), rather than the external (apoplastic) medium (Johannes *et al.*, 1991).

Published reports on the effects of aluminum (Al) on Ca flux have been contradictory. For example, Al has been shown to reduce both membrane potential (Akeson *et al.*, 1989; Kinraide *et al.*, 1992) and net Ca^{2+} flux (Huang *et al.*, 1992b; 1993).

A possible explanation for these observations is that Al binding not only caused membrane depolarization, but also blockage of Ca^{2+} channels, causing direct inhibition of channel function (Rengel *et al.*, 1995). An alternative hypothesis may be that Al binding to other proteins and/or phospholipids in the PM reduces surface negativity (Kinraide *et al.*, 1992), thus affecting membrane function, including regulation of Ca^{2+} flux. Akeson *et al.* (1989) indicated that the probability of Al ions binding to phosphatidylcholine vesicles was 560-fold higher than Ca^{2+}, causing neutralization of the surface charge and a corresponding shift of a membrane surface potential (from -30 to + 11 mV).

The effects of Al on $[Ca^{2+}]_{cyt}$ have also been contradictory. Lindberg and Strid (1997) demonstrated that the presence of Al increased $[Ca^{2+}]_{cyt}$ in protoplasts isolated from Al-resistant and Al-sensitive wheat cultivars. In contrast, Pineros and Tester (1995) indicated that addition of Al decreased $[Ca^{2+}]_{cyt}$ in PM vesicles of wheat roots probably by acting as a Ca-channel blocker in the PM. Transient elevations of Ca (sometimes called spikes) may last anywhere from a few seconds to many minutes in plant cells, depending on the characteristics of the stimulating signal. Thus, different signals may induce Ca waves of different shape, form, and kinetics (Trewavas, 1999).

Since 1,3-β-D-glucan synthase operates as a Ca-dependent enzyme, I hypothesized that changes in $[Ca^{2+}]_{cyt}$ at the active site of 1,3-β-D-glucan synthase might explain the different effects of Al *in vivo* and *in vitro*. However, the expectation that digitonin and ionophore would permeabilize the PM to Ca, and hence increase callose accumulation to the extent that the inhibitory effects of Al on callose production would

be observed, was not fulfilled in my previous experiments (Chapter 5). While it is possible that my hypothesis is invalid, it is also possible that these chemicals did not induce an increase in $[Ca^{2+}]_{cyt}$ and hence, trigger callose production to the same extent as Al. To test this hypothesis, the effects of Al, digitonin, and A23187 on $[Ca^{2+}]_{cyt}$ and their relationship with callose synthesis in intact root tips of Al-sensitive cv. Katepwa were examined using confocal laser scanning microscopy (CLSM).

6.2 Materials and methods

6.2.1 Plant material

Seeds of an Al-sensitive (Katepwa) cultivar of *Triticum aestivum* were surface sterilized in 1.2% sodium hypochlorite for 20 min., and germinated overnight in a solution of 0.005 gL^{-1} Vitavax to limit fungal growth. Seedlings were then grown for 5 days on nylon mesh suspended over 15 L of a full nutrient solution containing (mM) 3.30 NO_3^--N, 0.30 NH_4^+-N, 0.10 P, 0.80 K, 1.00 Ca, 0.30 Mg, 0.10 S; and (μM) 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, and 0.1 Mo (pH 4.4) in a growth chamber with 16 h of light (20 °C, 68% relative humidity) and 8 h of darkness (16 °C, 85% relative humidity). The photosynthetic photon flux (PPF) was 335 ± 12 μmol m^{-2} s^{-1} at plant base level.

6.2.2 Experimental design for callose and cytosolic calcium imaging

Preliminary experiments were set up to identify an exposure time, where Al-induced callose accumulation was detectable using CLSM. Five-day-old seedlings were

subjected to 100 μM Al in a simple solution containing 0.4 mM $CaCl_2$. After 0, 0.5, 1, 2, and 3h of exposure, 1 cm root tips were labeled with Aniline Blue Fluorochrome (specific for 1,3-β-D-glucans, callose; see below). Based on the intensity of callose accumulation from each treatment, a three-hour-exposure time was selected for further experiments. Experiments were then performed by exposing five-day-old plants to Al (0, 20, 40, 60, 80, and 100 μM) for 3h in a simple salt solution (0.4 mM $CaCl_2$). Similar experiments were also conducted to measure callose accumulation in intact root tips after exposing the plants to digitonin (0, 2, 4, 6, 8, and 10 μM) and A23187 (0, 0.25, 0.5, 1, 2, 3 μM) for 3h. Each treatment included three independent replicates and the primary root tips were selected for callose determination.

Parallel experiments were conducted to measure the effects of Al, digitonin, and A23187 on $[Ca^{2+}]_{cyt}$. Plants were exposed to the calcium probe Fluo-4-AM for 3h to load the probe into the cytoplasm before treatments were imposed. This was based on preliminary experiments that demonstrated a three-hour-loading time was sufficient to detect increases in $[Ca^{2+}]_{cyt}$. Once again each treatment included three independent replicates and primary root tips were chosen to detect $[Ca^{2+}]_{cyt}$.

6.2.3 Callose imaging

After washing with 25 mM phosphate buffer (pH 8.2), callose in primary root tips (1 cm) was labeled with 0.1 μM Aniline Blue Fluorochrome (specific for 1,3-β-D-glucans; Biosupplies, Parkville, Australia) in a medium containing 25 mM phosphate buffer (pH 8.2) for 20 minutes (Kudlicka and Brown, 1997). Root tips were then washed

with phosphate buffer without Aniline Blue. Callose accumulation was determined using a Molecular Dynamics Multiprobe 200 CLSM (Image Space II, Operating System: Iris 5.2) with a fluorescein isothiocyanate filter and excitation and emission wavelengths of 480 and 570 nm, respectively. Callose accumulation was determined based on relative fluorescence intensity (pixel number).

6.2.4 Cytosolic free-calcium imaging

Five-day-old plants were incubated for 3h in a buffer containing 100 mM KCl, 10 mM MOPS (pH 4.5) in the presence of 1.3 μM Fluo-4-AM (Molecular Probes, Eugene, USA) and 0.05% Pluronic F127 (Sigma, St. Louis, USA; to avoid precipitation of the fluorescent calcium probe). Stock solutions of Fluo-4-AM and Pluronic F127 were prepared by dissolving 50 μg and 500 μg in 700μl and 5 ml of dimethylsulfoxide (DMSO), respectively (final concentration of DMSO in the buffer solution was 2.5%). Preliminary experiments (data not shown) indicated that a 3-h incubation was optimal for loading Fluo-4-AM into the cells. After incubation, plants were washed 3x with buffer without Fluo-4-AM. Selected plants were then treated with 0-100 μM Al, 0-10 μM digitonin, or 0-3 μM A23187 in a simple salt solutions (0.4 mM $CaCl_2$) for another 3h. After washing with buffer, cytosolic free calcium concentrations ($[Ca^{2+}]_{cyt}$) were determined using CLSM at excitation and emission wavelengths 494 and 516nm, respectively. Observations were focused on the primary root tips of plants in each treatment. Cytosolic free calcium concentrations were determined based on relative fluorescence intensity (pixel number).

6.3 Results

The relatively small effects of digitonin and A23187 on callose production in previous experiments (Chapter 5) led me to question the assumption that these chemicals induced a large enough increase in $[Ca^{2+}]_{cyt}$ to trigger callose production to the same extent as Al. To explore this question, separate experiments testing the effects of Al, digitonin, and A23187 on $[Ca^{2+}]_{cyt}$ and its relationship with callose accumulation were performed using CLSM. The effects of Al, digitonin, and A23187 were similar to previous experiments (Chapter 5), in which Al induced the greatest callose production compared with digitonin or A23187. After exposure for 3h, callose accumulation in intact root tips of *Triticum aestivum* cv. Katepwa increased from 5 ± 3 to 134 ± 10 relative fluorescence intensity (pixels) in the presence of 0-100 μM Al (Figure 6.1a, 6.2), from 4 ± 3 to 47 ± 7 pixels in the presence of 0-10 μM digitonin (Figure 6.1b, 6.3), and from 4 ± 3 to 45 ± 5 pixels in the presence of 0-3 μM A23187 (Figure 6.1c, 6.4). Since working concentrations of digitonin and A23187 in the previous experiments were 8 μM and 0.5 μM respectively, the current experiments confirm that previous digitonin and A23187 treatments were not as effective as Al in eliciting callose production.

Different effects of Al, digitonin, and A23187 on root tips of Al-sensitive cv. Katepwa were not only detected in fluorescence intensity, but also in the distribution of callose accumulation (Figure 6.2 - 6.4). Callose accumulation was evident throughout the field of view (450 μm) in the presence of Al, with cells of the root cap, epidermis, and cortex showing intensive fluorescence. In contrast, digitonin and A23187 increased

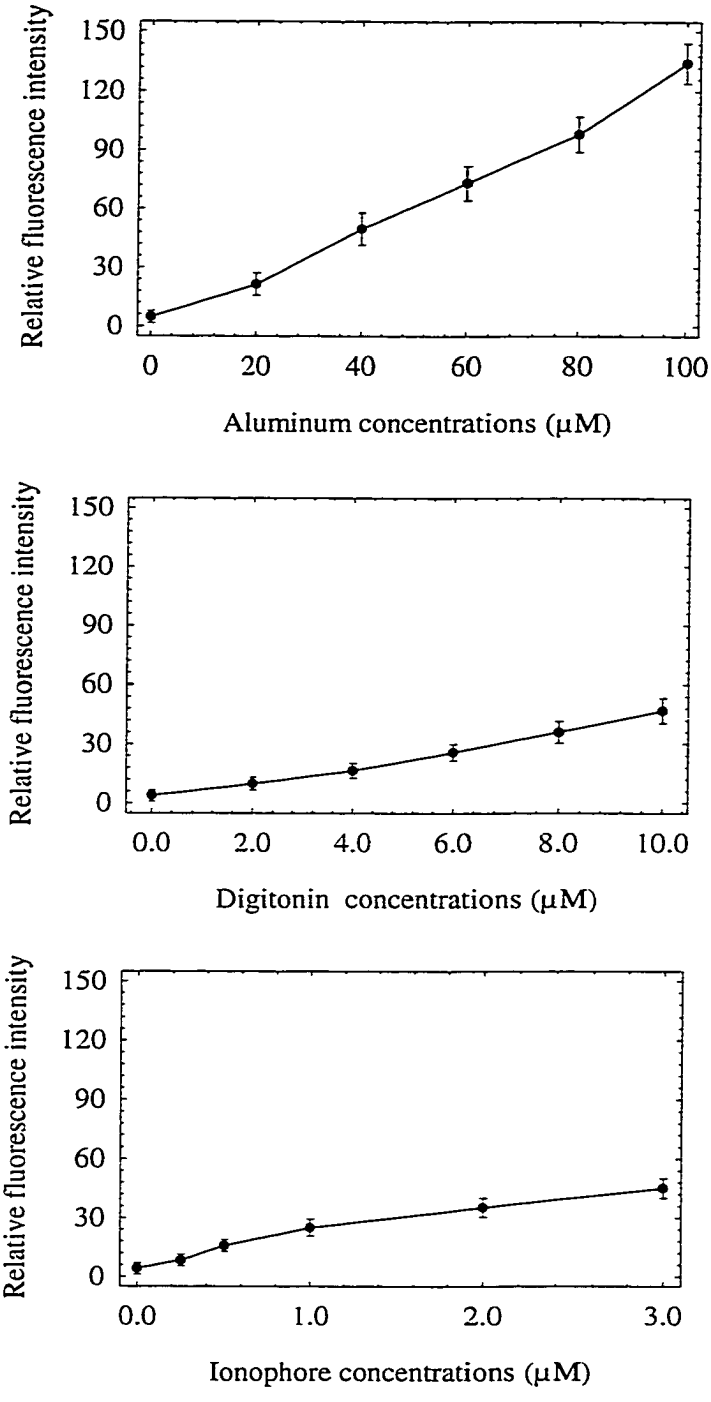


Figure 6.1. The effects of Al, digitonin, and A23187 on callose accumulation in *Triticum aestivum* cv. Katepwa (based on relative fluorescence intensity). Values are means ± SE of three replicates.

Figure 6.2. The effect of Al on relative fluorescence intensity of callose accumulation in intact root tips of *Triticum aestivum* cv. Katepwa. Values are means ± SE of three replicates. A. In the absence of Al. B. In 20 μM Al. C. In 40 μM Al. D. In 60 μM Al. E. In 80 μM Al. F. In 100 μM Al.

Figure 6.3 The effect of digitonin on relative fluorescence intensity of callose accumulation in intact root tips of *Triticum aestivum* cv. Katepwa. Values are means ± SE of three replicates. A. In the absence of digitonin. B. In 2 μM Digitonin. C. In 4 μ M digitonin. D. In 6 μM digitonin. E. In 8 μM digitonin. F. In 10 μM digitonin.

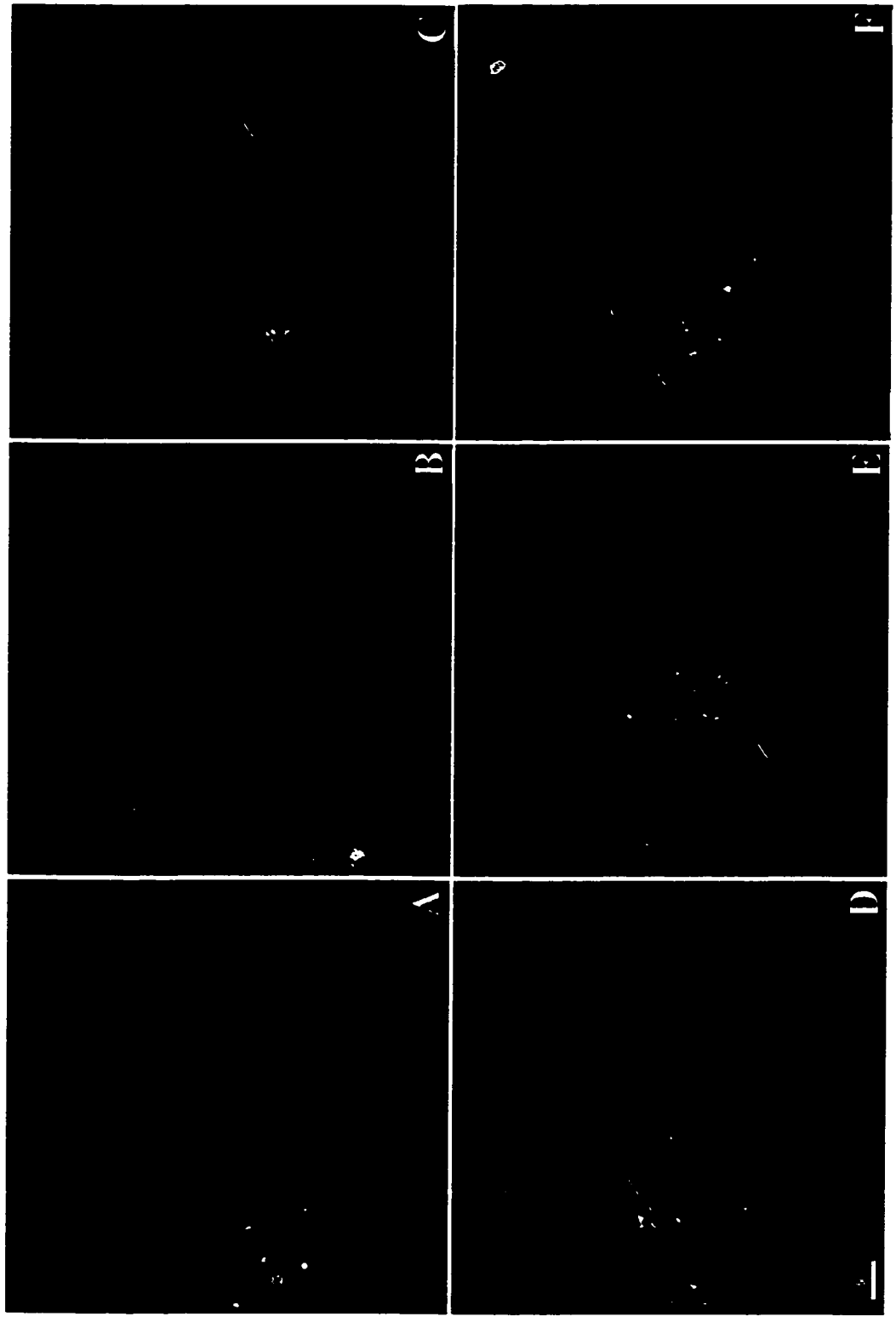
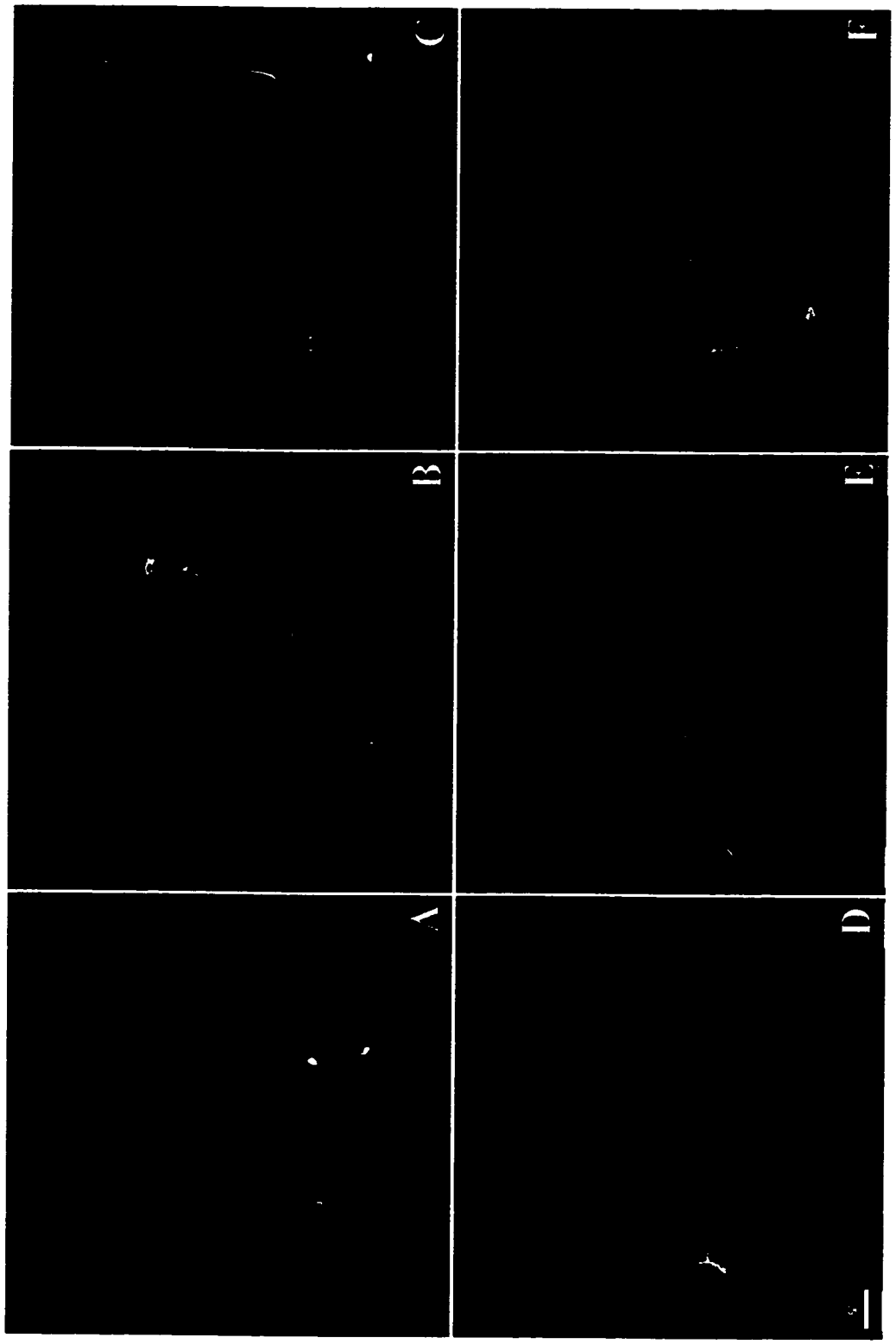


Figure 6.4. The effect of A23187 on relative fluorescence intensity of callose accumulation in intact root tips of *Triticum aestivum* cv. Katepwa. Values are means ± SE of three replicates. A. In the absence of A23187. B. In 0.25 μM A23187. C. In 0.5 μM A23187. D. In 1 μM A23187. E. In 2 μM A23187. F. In 3 μM A23187.



callose accumulation in regions distant from the root cap.

Although A23187 triggered the least callose accumulation, it had the strongest effects on $[Ca^{2+}]_{cyt}$ compared with the Al and digitonin treatments. In the absence of Al, digitonin, or A23187, the relative fluorescence intensity of $[Ca^{2+}]_{cyt}$ in root tips of Al-sensitive cv. Katepwa was approximately 26 ± 8 pixels after 3 h exposure. As concentrations of A23187 increased from 0 to 3 μM, relative fluorescence intensity of $[Ca^{2+}]_{cyt}$ increased 6.6-fold higher than the controls to 174 ± 15 pixels (Figure 6.5c, 6.8). The relative fluorescence intensity of $[Ca^{2+}]_{cyt}$ appeared to reach a saturation level at 2-3 μM A23187 (Figure 6.5c). In contrast, the effects of Al (0-100 μM) and digitonin (0-10 μM) increased relative fluorescence intensity by 4.7-fold (Figure 6.5a, 6.6) and 4.4-fold (Figure 6.5b, 6.7), respectively, with no sign of saturation.

Similar to the effect of Al on callose accumulation, Al triggered increases in $[Ca^{2+}]_{cyt}$ throughout the field of view (450 μm from the root cap) although cells of the root cap showed the highest fluorescence (Figure 6.6). In contrast, the pattern of increasing $[Ca^{2+}]_{cyt}$ due to the effect of digitonin was only evident in more distant regions of the root, with cells of epidermis and cortex showing high fluorescent intensity (Figure 6.7). Although the effect of A23187 induced a different pattern of callose accumulation to that of Al, both of these agents triggered similar patterns of increasing $[Ca^{2+}]_{cyt}$ along the root tip cells of Al-sensitive cv. Katepwa, with cells of root cap showing the highest fluorescent intensity (Figure 6.8).

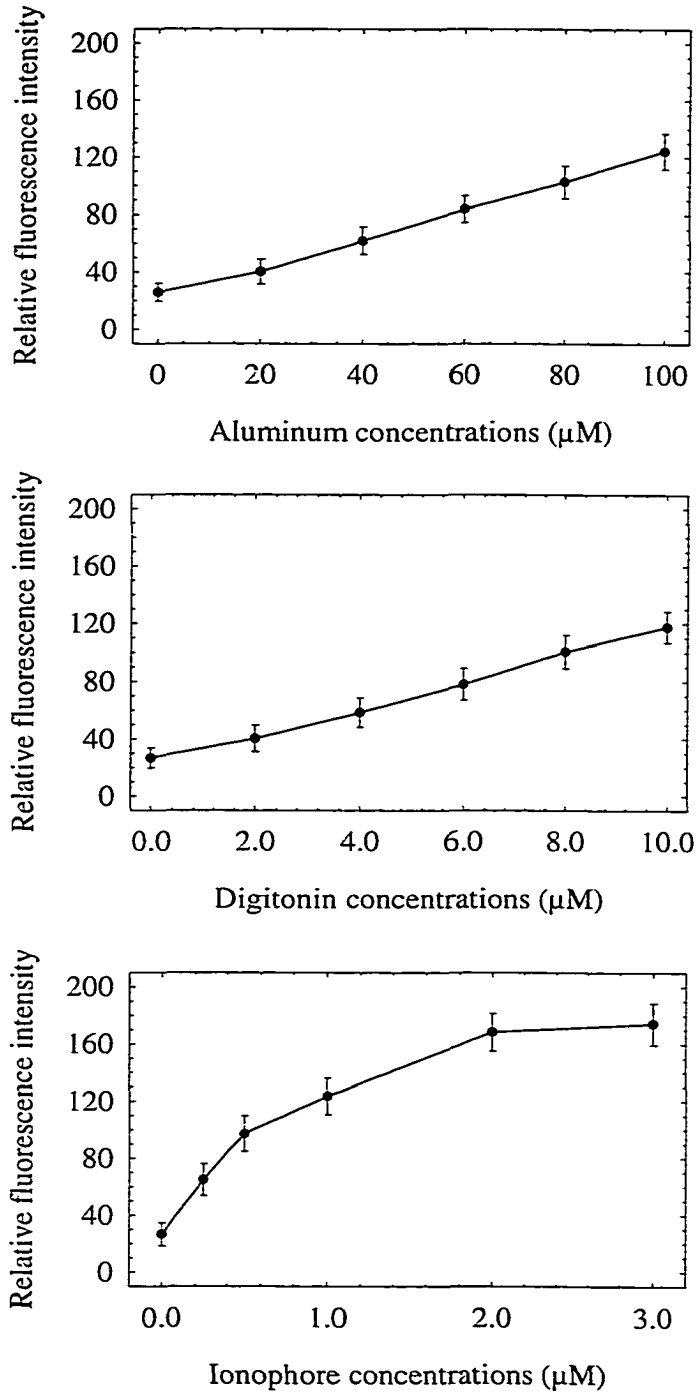


Figure 6.5. The effects of Al, digitonin, and A23187 on $[Ca^{2+}]_{cyt}$ in *Triticum aestivum* cv. Katepwa (based on relative fluorescence intensity). Values are means ± SE of three replicates.

Figure 6.6. The effect of Al on relative fluorescence intensity of $[Ca^{2+}]_{cyt}$ in intact root tips of *Triticum aestivum* cv. Katepwa. Values are means ± SE of three replicates. A. In the absence of Al. B. In 20 μM Al. C. In 40 μM Al. D. In 60 μM Al. E. In 80 μM Al. F. In 100 μM Al.

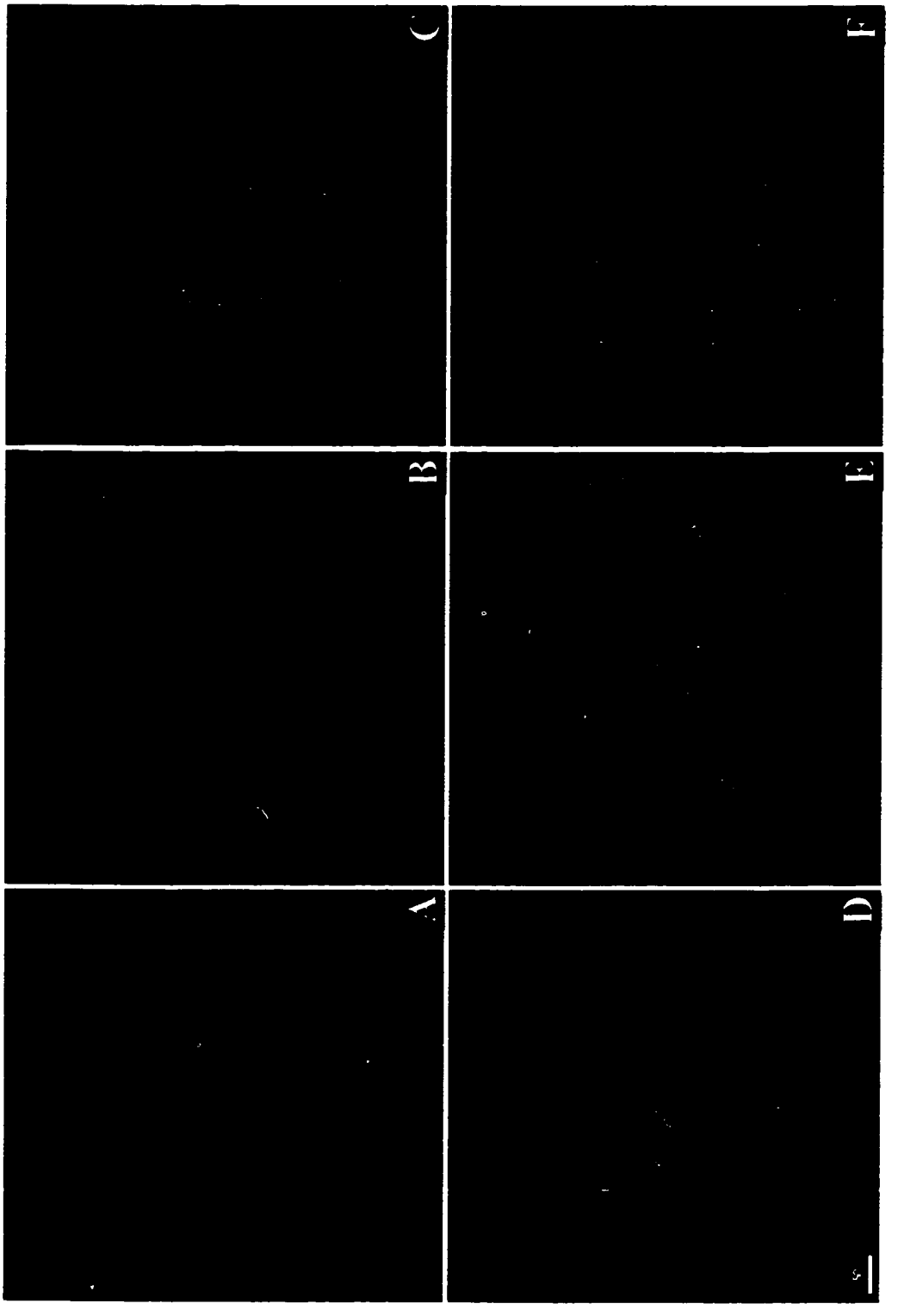


Figure 6.7. The effect of digitonin on relative fluorescence intensity of $[Ca^{2+}]_{cyt}$ in intact root tips of *Triticum aestivum* cv. Katepwa. Values are means ± SE of three replicates. A. In the absence of digitonin. B. In 2 μM Digitonin. C. In 4 μ M digitonin. D. In 6 μM digitonin. E. In 8 μM digitonin. F. In 10 μM digitonin.

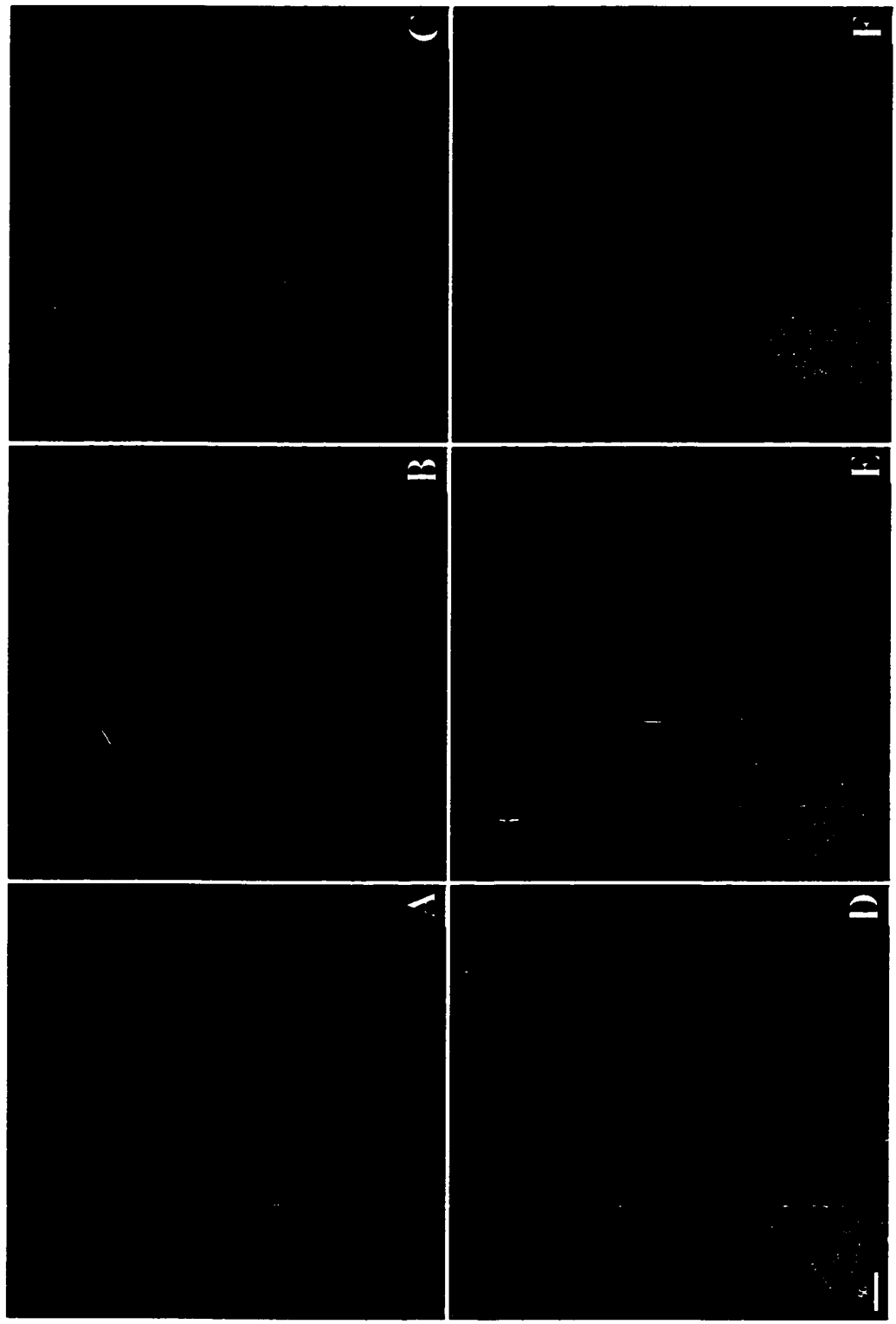
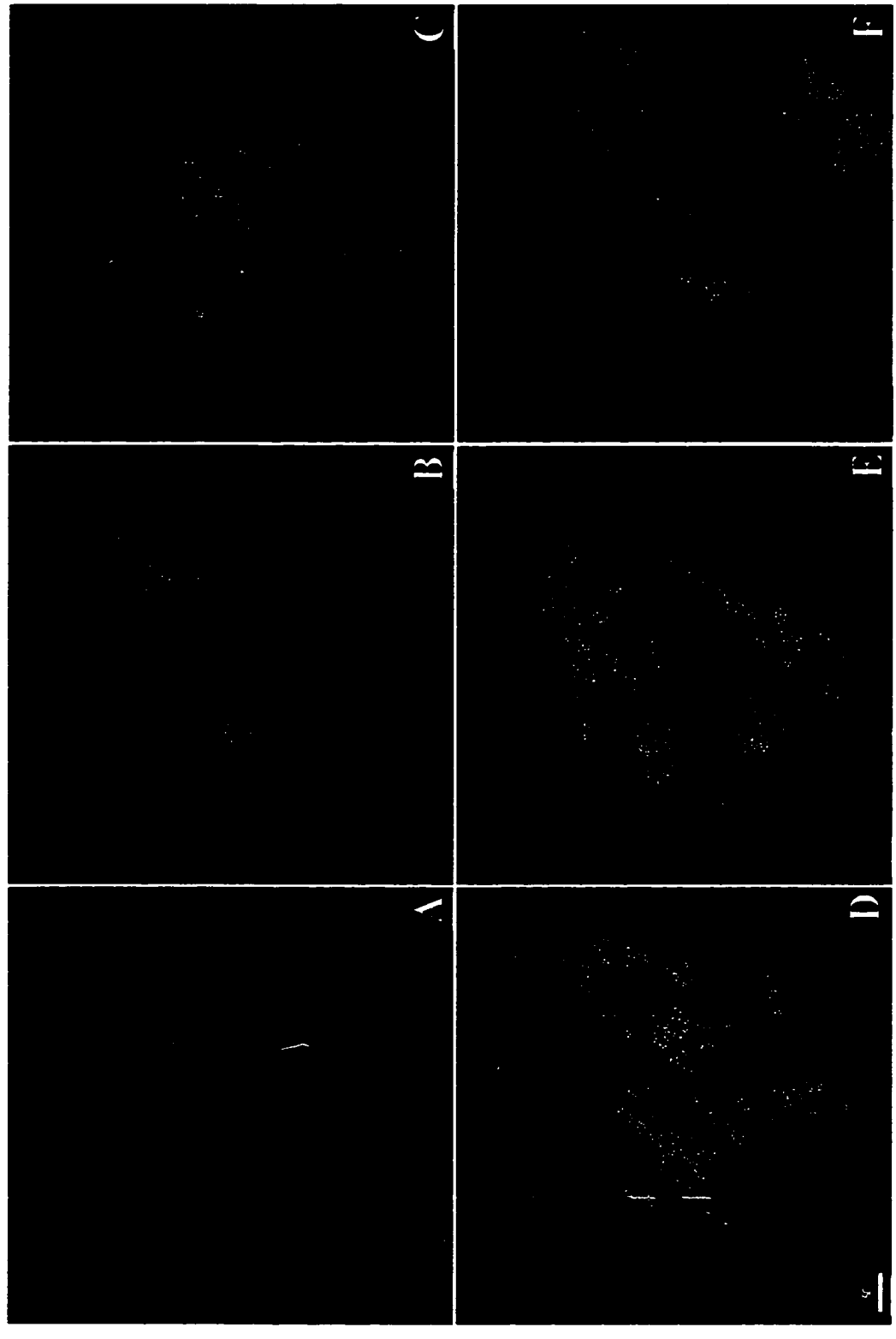


Figure 6.8. The effect of A23187 on relative fluorescence intensity of $[Ca^{2+}]_{cyt}$ in intact root tips of *Triticum aestivum* cv. Katepwa. Values are means ± SE of three replicates. A. In the absence of A23187. B. In 0.25 μM A23187. C. In 0.5 μM A23187. D. In 1 μM A23187. E. In 2 μM A23187. F. In 3 μM A23187.



6.4 Discussion

Using the calcium probe, Fluo-4-AM, to detect $[Ca^{2+}]_{cyt}$ *in situ* and Aniline Blue fluorochrome to detect callose deposition in the root tips of *Triticum aestivum* cv. Katepwa, the present study failed to demonstrate a clear relationship between Al-induced callose production (Figure 6.1 - 6.4) and an Al-induced increase in $[Ca^{2+}]_{cyt}$ (Figure 6.5 - 6.8). Aluminum triggered the highest callose accumulation (2.9-fold higher than digitonin and 3-fold higher than A23187), while the capacity of Al to increase $[Ca^{2+}]_{cyt}$ was only 71% compared with the strength of A23187. The lack of positive relationship between callose accumulation and $[Ca^{2+}]_{cyt}$ suggested that induction of high levels of $[Ca^{2+}]_{cyt}$ was not sufficient to engage callose production to maximal levels.

The ability of digitonin to permeabilize the PM (Palmgren *et al.*, 1990; Johansson *et al.*, 1995; Kauss, 1996) and the capacity of A23187 to stimulate Ca^{2+} influx across the PM have been confirmed by the increase of $[Ca^{2+}]_{cyt}$ (Figure 6.5, 6.7 and 6.8). However, while A23187 induced higher levels of $[Ca^{2+}]_{cyt}$ than Al or digitonin, it had a similar effect on the intensity of callose accumulation as digitonin. Thus, the failure of these agents to stimulate high levels of callose accumulation can not simply reflect the availability of calcium as an enzyme cofactor. For example, the ability of A23187 to trigger a large increase in $[Ca^{2+}]_{cyt}$ without an accompanying increase in callose production may indicate that Al and A23187 have different sites of action. This possibility is supported by the observation that Al triggered accumulation of callose in a different zone of the root compared with the effect A23187, even though both agents had similar effects on the spatial distribution of increasing $[Ca^{2+}]_{cyt}$. Another possibility is that

the local distribution of $[Ca^{2+}]_{cyt}$ may be different in the presence of Al and A23187. Increasing concentrations of $[Ca^{2+}]_{cyt}$ in the Al treatments likely originated from endostorage pools (endoplasmic reticulum or vacuole), while increasing concentrations of $[Ca^{2+}]_{cyt}$ in the A23187 and digitonin treatments likely originated from the external medium. These different sources could provide quantitative differences in the distribution of Ca in the cytoplasm or the availability of Ca to 1,3-β-D-glucan synthase. Regardless, differences in the distribution of callose accumulation and increasing $[Ca^{2+}]_{cyt}$ indicate that digitonin and ionophore are not good mimics of Al.

Although the capacity of Al to trigger an increase in $[Ca^{2+}]_{cyt}$ was less than that of A23187, the results of the present study supported the work of Lindberg and Strid (1997), who demonstrated that Al increases $[Ca^{2+}]_{cyt}$ protoplasts of *Triticum aestivum*. Since Al has been shown to block Ca channels in the PM and reduce the influx of Ca to the cytoplasm (Lindberg, 1990; Huang *et al.*, 1992a,b; Rengel, 1992a,b,c; Rengel and Elliot, 1992), this suggests that Al may invoke a stress signal by binding to a receptor at the PM or by interfering with a signal metabolite (Bennet and Breen, 1991; Haug *et al.*, 1994; Delhaize and Ryan, 1995; Kochian, 1995). This could activate a signal cascade that induces an increase in $[Ca^{2+}]_{cyt}$. The effect of Al on this putative signal transduction pathway may cause release of both Ca^{2+} and β-furfuryl (a precursor of β-furfuryl-β-glucoside) from the vacuole (Ohana *et al.*, 1992) or other cell compartments to activate 1,3-β-D-glucan synthase. In other words, complex conditions within the *in vivo* system provide alternative factors affecting the expression of 1,3-β-D-glucan synthase activity due to Al toxicity. The lack of cell compartments such as vacuoles and endoplasmic

reticulum (ER) in the *in vitro* system may preclude Al-activation of these factors and provides a better chance for Al to bind to and inhibit the activity of 1,3-β-D-glucan synthase.

The hypothesis that digitonin and A23187 alone may not be able to trigger a similar increase in $[Ca^{2+}]_{cyt}$ as that induced by Al was not supported by the present experiments. These experiments demonstrated that although Al induced the highest production of callose, it triggered less $[Ca^{2+}]_{cyt}$ than A23187 and nearly similar to the effect of digitonin. This indicates that increasing availability of $[Ca^{2+}]_{cyt}$ is not the only factor modulating increased in callose production in the *in vivo* system, or the differences in behavior of *in vitro* and *in vivo* systems. The involvement of multiple effects of Al to activate a signal cascade that increases the supply of Ca and substrate from internal sources may explain the high intensity of callose production.

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7. General discussion and conclusion

7.1 General

Potential mechanisms that regulate the synthesis and activity of 1,3-β-D-glucan synthase under conditions of aluminum (Al) stress have not been fully resolved. Quantitative studies conducted by Zhang *et al.* (1994) showed that callose synthesis in roots (*in vivo*) of Al-sensitive cultivars of *Triticum aestivum* increased 38-fold after 48 hours exposure to 75 μM Al. In contrast, studies with purified plasma membrane (PM; *in vitro*) isolated from *Picea abies* (Widell *at al.* 1994) have shown that Al is a powerful inhibitor of 1,3-β-D-glucan synthase activity. I hypothesized that Al inhibits the activity of 1,3-β-D-glucan synthase, but this effect may not be detected *in vivo*. Aluminum may be able to cross the PM to gain access to the cytosolic face of 1,3-β-D-glucan synthase, but its activity may be too low to have a powerful inhibitory effect. Consequently the stimulatory effect of Al resulting from an Al-induced increased in cytosolic calcium ($[Ca^{2+}]_{cyt}$) is greater than the direct inhibitory effect on 1,3-β-D-glucan synthase activity. In contrast, inhibition of 1,3-β-D-glucan synthase activity *in vitro* may reflect better access of this metal to the cytosolic side of PM and the lack of internal sources of Ca such as the endoplasmic reticulum, mitochondria and vacuoles. To verify this hypothesis, a series of experiments was performed and the results are discussed below.

7.2 1,3-β-D-glucan synthase as a plasma membrane marker

I began my work by developing techniques to purify the PM and isolate vesicles of known sidedness. The two-phase partitioning method (Larsson *et al.*, 1994) provided a

simple and effective tool for membrane fractionation. Approximately 82% of total 1,3-β-D-glucan synthase activity and less than 2.0% of the activities of each of negative marker enzyme activities (cytochrome c-reductase, cytochrome c-oxidase, nitrate-sensitive ATPase, azide-sensitive ATPase) were recovered from PM-enriched fractions. These data, plus the enrichment of vanadate-sensitive ATPase activities, indicated that vesicles isolated using this technique are highly enriched in the PM (Chapter 2; Fredrikson, 1993; Palmgren *et al.*, 1990; Larsson *et al.*, 1994).

Most (90.6%) PM vesicles formed upon aqueous two-phase partitioning were in the RSO orientation. However, some PM enzymes, such as 1,3-β-D-glucan synthase and H^+-ATPase (Fredrikson, 1993; Johansson *et al.*, 1995), expose their active sites towards the cytoplasm. Thus, the use of detergents such as digitonin or techniques to invert vesicles to the ISO orientation is necessary for optimal activity and subsequent experiments designed to explore the characteristics of these enzymes more precisely. Changes in the latency of H^+-ATPase activity indicated that nearly 100% of PM vesicles could be converted to the ISO orientation by adding the detergent Brij-58 into the assay medium (Chapter 2; Johansson *et al.*, 1995). Unfortunately, the activity of 1,3-β-D-glucan synthase was strongly inhibited (77.1%) by Brij-58. While, I did not investigate the possible mechanism of this inhibition, changes in protein-lipid interactions or membrane fluidity might explain how Brij-58 reduces 1,3-β-D-glucan synthase activity without affecting H^+-ATPase activity. Regardless of the cause, the inhibitory effect of Brij-58, made it unsuitable for my experiments. Thus, an alternative technique, freeze-thaw cycles was used to alter vesicle sidedness and counter current distribution (CCD)

procedure was used to select vesicles of known sidedness. These techniques were not as effective as Brij-58, providing enrichment of vesicles with the ISO orientation up to 67% (33% RSO as contaminant). The presence of ISO vesicles (approximately 10%) in the RSO fraction and the presence of RSO vesicles (approximately 33%) in the ISO fraction must be taken into consideration when interpreting experimental data.

7.3 Comparing the effects of aluminum on the activity of 1,3-β-D-glucan synthase in *in vitro* and *in vivo* systems

Previous experiments that showed Al inhibited the activity 1,3-β-D-glucan synthase in isolated PM of *Picea abies* (Widell *et al.*, 1994) guided me to predict that Al may bind to this enzyme *in vivo* and *in vitro*. Testing the hypothesis directly is problematic, given the lack of a suitable radioisotope. Thus, I made use of metal chelate affinity chromatography to test for Al binding *in vitro*. In working with Al-activated columns, 96% of total activity of solubilized (digitonin) 1,3-β-D-glucan synthase was detected in 20 mM EDTA and 100 mM EDTA fractions. In contrast, 99.7% of total activity of 1,3-β-D-glucan synthase was detected from chelex-H_2O and K-phthalate fractions. These data clearly indicate that 1,3-β-D-glucan synthase interacts directly with Al in this *in vitro* system. Another interesting result from these experiments was a large reduction (81.7%) in the total activity of 1,3-β-D-glucan synthase eluted from the Al-activated column. Several factors could lead to a decrease in the activity of 1,3-β-D-glucan synthase, such as binding of 1,3-β-D-glucan synthase with Al, EDTA, or Al-EDTA. These interactions could change the conformation and functional integrity of the enzyme (Haug, 1984).

The prediction that Al would inhibit the activity of 1,3-β-D-glucan synthase *in vitro* was strongly supported by experiments using RSO and ISO PM vesicles isolated from roots of Al-sensitive (Katepwa) and Al-resistant (Alikat) cultivars. Although the presence of digitonin and A23187 decreased the overall degree of inhibition found, Al always inhibited the activity of 1,3-β-D-glucan synthase. This was particularly true in ISO vesicles, where the degree of inhibition was stronger than that in the RSO vesicles. There are two possible ways that Al might inhibit the activity of 1,3-β-D-glucan synthase in RSO vesicles. First, Al might block the influx of Ca (Jones *et al.*, 1998; Rengel and Elliot, 1992) from the external medium (the only source of Ca *in vitro*) to the cytosolic side of the PM, where the active site of this enzyme is located. Second, Al might directly alter both the physical and functional integrity of this Ca^{2+}-requiring enzyme (Haug, 1984; Taylor, 1991; Rengel, 1992; Delhaize and Ryan, 1995). The higher sensitivity of 1,3-β-D-glucan synthase in ISO PM vesicles might reflect a direct access of Al to the active site of 1,3-β-D-glucan synthase.

In the *in vitro* system, few differences were found in the inhibitory effect of Al on 1,3-β-D-glucan synthase in the two *Triticum aestivum* cultivars examined. This suggests that the binding affinity of Al for 1,3-β-D-glucan synthase isolated from Alikat is the same as that isolated from Katepwa. Similarly, the PM in these two cultivars may provide a similar barrier for movement of Al across the lipid bilayer. Differences in the sensitivity of 1,3-β-D-glucan synthase to Al between Al-sensitive cv. Katepwa and Al-resistant cv. Alikat *in vivo* could reflect the operation of specific resistance mechanisms in Alikat, which act to protect the plant from Al-induced injury. For example, the Al-resistant cv.

Alikat could have a higher capacity to release organic anions, such as citrate or malate, thereby excluding Al from the root apex (Miyasaka *et al.*, 1991; Delhaize *et al.*, 1993; Basu *et al.*, 1994; Pellet *et al.*, 1995). The lack of this capacity in Al-sensitive cv. Katepwa would make it more vulnerable to Al toxicity. As a result, this sensitive cultivar would synthesize more 1,3-β-D-glucan synthase, a well-known marker for Al-injury (Taylor, 1991; Zhang *et al.*, 1994).

The activity of 1,3-β-D-glucan synthase requires uridine 5'diphosphate (UDP)-glucose as a substrate and uses calcium as a cofactor (Aist, 1983; Fredrikson and Larsson, 1989; Kauss *et al.*, 1983; Kauss and Jeblick, 1985; Lin and Lee, 1991). The presence of A23187 *in vivo* was expected to stimulate an increase in cytosolic free calcium concentrations ($[Ca^{2+}]_{cyt}$), while the presence of digitonin was expected to promote free access of Ca and Al to the cytoplasm. I had hoped that this increase would fully engage Ca-stimulated 1,3-β-D-glucan synthase activity, thus, allowing me to see the direct inhibitory effect of Al. Unfortunately, treatments with A23187 or detergents (digitonin or CHAPS) alone were not sufficient to trigger *in vivo* callose production as high as in the presence of Al alone. I hypothesized that the inability of digitonin, CHAPS, and A23187 to induce high levels of callose accumulation may reflect the inability of these agents to increase the supply of Ca in the cytoplasm where the active site of 1,3-β-D-glucan synthase is located. However, this hypothesis was not supported by measurements of $[Ca^{2+}]_{cyt}$ in intact root tips of Al-sensitive cv. Katepwa using confocal laser scanning microscopy (CLSM). In these experiments, A23187 triggered the highest level of $[Ca^{2+}]_{cyt}$, while digitonin increased $[Ca^{2+}]_{cyt}$ to a similar level as Al.

This result suggests that my hypothesis may have been simplistic. The capability of A23187 to induce the highest level of $[Ca^{2+}]_{cyt}$, but only trigger intermediate levels of callose compared with Al leads me to suggest that a change in $[Ca^{2+}]_{cyt}$ levels is not the only factor accounting for the observed Al-induced increase in callose production *in vivo*. There are a number of factors that might account for this complexity.

One possibility is that the presence of Al may directly or indirectly alter the conformation of cellulase synthase, the enzyme catalyzing the synthesis of 1,4-β-D-glucans. It has been postulated that a disorganized PM cellulose synthase may be capable of catalyzing synthesis of both cellulose and callose. According to this hypothesis, a variety of stresses may be capable of shifting the activity of cellulose synthase from synthesis of 1,4-β-D-glucans (cellulose) to 1,3-β-D-glucans (callose; Gibeaut and Carpita, 1994; Delmer, 1999). The process of isolating plasma membrane vesicles may force this conformational change. If this were true, the results of *in vitro* studies would reflect 1,3-β-D-glucan synthase activity alone, which is inhibited by Al. In *in vivo* systems, synthesis of 1,4-β-D-glucans normally predominates, but treatment with Al may promote the change from 1,4-β-D-glucan synthase activity to 1,3-β-D-glucan synthase activity. This Al-induced change in the synthase complex would result in a stimulation of 1,3-β-D-glucan synthase activity, which could overshadow the inhibitory effects of Al. Indirect evidence indicates that inhibition of root function occurs within 1 hr after treatment with Al (Lance and Pearson (1969), which may suggest that the action of Al more likely reflects blockage of cellulose polymerization reactions than inhibition of enzyme synthesis.

A second possible explanation for the stimulatory effect of Al on *in vivo* callose accumulation is the potential involvement of a putative 1,3-β-D-glucan synthase from the Golgi apparatus (Gibeaut and Carpita, 1994). Isolation of purified plasma membrane vesicles should limit the extent to which the putative Golgi 1,3-β-D-glucan synthase contributes to 1,3-β-D-glucan synthase activity in *in vitro* systems. Thus, the inhibitory effect of Al in plasma membrane-enriched vesicles should reflect the effect of Al (inhibition) on the plasma membrane 1,3-β-D-glucan synthase. In *in vivo* systems, the activity of these two glucan synthases cannot be separated. Thus, it is possible that the inhibitory effect of Al on plasma membrane 1,3-β-D-glucan synthase activity might be overshadowed by a stimulation of the 1,3-β-D-glucan synthase from the Golgi.

Another possibility is that Al may cause the plants not only to exploit alternative sources of Ca (endostorage), but also to activate the supply of substrate (UDP-glucose) and/or other activators (β-furfuryl-β-glucoside) of 1,3-β-D-glucan synthase activity. There are two possible ways that Al could increase UDP-glucose in the cytoplasm. First, the presence of Al in the cytoplasm may directly suppress the activity of an antiporter responsible for transporting β-furfuryl-β-glucoside into the vacuoles (Ohana *et al.*, 1992). Under normal conditions, UDP-glucose: β-furfuryl-β-glucoside glycosyltransferase catalyses the reaction of UDP-glucose in the cytoplasm with β-furfuryl, to produce β-furfuryl-β-glucoside. An antiporter transport system then transports β-furfuryl-β-glucoside to the vacuole, the site where secondary metabolites are synthesized and sequestrated (Werner and Matile, 1985; Ohana *et al.*, 1992; Wagner, 1982). Inhibition of

this transport system by Al could lead to end-product inhibition of UDP-glucose: β-furfuryl-β-glucoside glycosyltransferase. This would increase the concentration of UDP-glucose and β-furfuryl-β-glucoside in the cytoplasm, providing both substrate and activators for 1,3-β-D-glucan synthase. Aluminum could also trigger a signal transduction cascade (Haug *et al.*, 1994) that causes a decrease in cytoplasmic pH. This might also inhibit the antiport system for transporting β-furfuryl-β-glucoside into the vacuoles (Ohana *et al.*, 1992). Further research is required to disclose these puzzles.

Although the presence of detergents alone was not sufficient to fully induce callose accumulation, the assumption that detergents may increase the access of Al to the cytoplasm was supported through this series of experiments. This was true, especially in absence of A23187. Digitonin or CHAPS reduced the extent of Al-induced callose accumulation in both cultivars, although the degree of inhibition was always higher in cv. Katepwa than in cv. Alikat. Interestingly, these two detergents also caused different responses in Al-resistant and Al-sensitive cultivars. Combinations of digitonin, A23187, and Al induced more callose accumulation (38% higher than with Al alone) in Al-resistant cv. Alikat, whereas it reduced callose accumulation in Al-sensitive cv. Katepwa by 10%. In contrast, combinations of CHAPS, A23187 and Al increased callose production of both cultivars approximately 144% higher than Al alone. The factors giving rise to these differences are not clear. Furthermore, regardless of the inhibition of *in vivo* callose accumulation by detergents, the inhibitory effects of Al were always overshadowed by its stimulation in all treatments. Once again, access of Ca and Al to the

cytosolic face of the 1,3-β-D-glucan synthase can not be the only factors affecting the *in vivo* response of the protein to Al.

7.4 1,3-β-D-glucan synthase and cytosolic calcium concentrations

In chapter 6, I postulated that the inability of digitonin and A23187 alone to trigger callose accumulation as high as Al might reflect the inability of these agents to increase $[Ca^{2+}]_{cyt}$ as strong as Al. Measurements of callose production and $[Ca^{2+}]_{cyt}$ in intact root tips of Al-sensitive cv. Katepwa using CLSM were inconsistent with this hypothesis. Aluminum induced the highest level of callose accumulation compared with digitonin and A23187. Nevertheless, Al triggered an increase in $[Ca^{2+}]_{cyt}$ that was only 71% of that induced by A23187 and similar to that induced by digitonin. The lack of quantitative relationship between callose accumulation and increases in $[Ca^{2+}]_{cyt}$ further emphasizes that the level of calcium availability in the cytoplasm is not the only factor modulating increased Al-induced callose production in the *in vivo* system, or the differences in behavior of *in vitro* and *in vivo* systems.

The different effects of Al, digitonin, and A23187 on the distribution of callose accumulation and $[Ca^{2+}]_{cyt}$ along root tips may evoke another possibility that might help to piece together this complicated puzzle. Each of these chemicals may have a different site of effect. Digitonin and A23187 may accelerate influx of Ca from the external medium to the cytoplasm by increasing the permeability of the PM and acting as Ca^{2+}-carrier. In contrast, Al blocks Ca^{2+}-influx from the external medium, while its interaction with signal transduction cascade may allow plant cells to respond by recruiting Ca^{2+} from

the internal storage pools. Release of Ca^{2+} from internal pools may overcome the need for Ca, which can not be obtained from the external medium, to activate 1,3-β-D-glucan synthase. However, it is possible that differences in the cellular localization of Ca recruited from these different pools may affect the extent of enzyme activation.

7.5 Future studies

Investigation of several other aspects of the effects of Al on 1,3-β-D-glucan synthase would be helpful to further characterize the potential mechanisms that regulate the synthesis of 1,3-β-D-glucan synthase and its product, 1,3-β-D-glucans, under conditions of Al stress. The involvement of Golgi apparatus to produce callose under Al stress is also an important avenue to be explored. Kinetic studies to characterize whether Al inhibits competitively or non-competitively the activity of 1,3-β-D-glucan synthase would be an interesting alternative approach. As a competitive inhibitor, Al would have to bind to the active site of 1,3-β-D-glucan synthase in order to reduce 1,3-β-D-glucan synthase activity. If this is the case for Al, plants should also be able to maintain the activity of 1,3-β-D-glucan synthase by supplying more substrate. In addition, the lack of a quantitative relationship between callose accumulation and the increase of $[Ca^{2+}]_{cyt}$ suggest the need for further studies to trace the involvement of Al in modulating $[Ca^{2+}]_{cyt}$. Related to this, experiments designed to identify the source of increasing $[Ca^{2+}]_{cyt}$, whether it is from the symplast or apoplast would be intriguing. Knowing the involvement of symplastic Ca^{2+} will open an avenue to explore the possible relationship between Al and Ca^{2+} internal pools, such as mitochondria, vacuoles and endoplasmic

reticulum. In this case, use of protoplasts could eliminate the potential influence of cell wall on Al and Ca uptake. Another avenue to explore, particularly in *in vivo* systems, is the effect of Al on the uptake of β-furfuryl-β-glucoside from the cytoplasm to the vacuoles or its interaction with signal transduction pathway to decrease cytoplasmic pH. Both of these factors could lead to the increase supply of UDP-glucose for 1,3-β-D-glucan synthase.

7.6 Conclusion

Several potential mechanisms that regulate the activity of 1,3-β-D-glucan synthase under Al stress have been demonstrated through this research. First, using Al-activated metal affinity column chromatography, I have shown that 1,3-β-D-glucan synthase binds to Al *in vitro*. This supports the assumption that the binding of Al to 1,3-β-D-glucan synthase might cause an inhibitory effect on the activity of this enzyme. Second, the hypothesis that Al inhibits the activity of 1,3-β-D-glucan synthase was strongly demonstrated in my *in vitro* experiments using RSO and ISO PM vesicles isolated from Al-sensitive (cv. Katepwa) and Al-resistant (cv. Alikat). Regardless of the presence of detergents and ionophores, the effect of Al was always inhibitory. In contrast to *in vitro* experiments, *in vivo* experiments showed that the inhibitory effect of Al is overshadowed by its stimulatory effect on 1,3-β-D-glucan synthase. To some extent, this appears to reflect an Al-induced increase in $[Ca^{2+}]_{cyt}$ and limited access of Al to the cytoplasmic face of 1,3-β-D-glucan synthase. However, this series of experiments demonstrates that other factors must also be mediating the increase in callose production in the *in vivo* system, or

the differences in behavior of *in vitro* and *in vivo* systems. While questions still remain, the present experiments have improved our understanding of the stimulatory and inhibitory effects of Al on 1,3-β-D-glucan synthase.

7.7 Literature cited

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