Identification of molecular markers for *Thinopyrum distichum* chromosomes contributing to salt tolerance

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

ABSTRACT

The detrimental effect of soil salinity on crop production is a growing problem worldwide (Tanji, 1990b). The degree to which plants can tolerate high concentrations of salt in their rooting medium is under genetic control with different genetic and physiological mechanisms contributing to salt tolerance at different developmental stages (Epstein & Rains, 1987). Only limited variation exists for salt tolerance in the cultivated cereals. This has prompted attempts to select tolerant progeny following hybridisation of cultivated species and wild, salt-tolerant species. *Thinopyrum distichum*, an indigenous wheatgrass that is naturally adapted to saline environments (McGuire & Dvorak, 1981), was crossed with triticale (x *Triticosecale*) in an attempt to transfer its salt tolerance and other hardiness characteristics (Marais & Marais, 1998). The aims of this study were to (i) identify *Thinopyrum* chromosomes carrying genes for salt tolerance and to identify molecular markers for these chromosomes, (ii) identify a number of diverse monosomic and disomic addition plants.

Bulked segregant analysis (BSA), in combination with AFLP, RAPD and DAF marker analysis was implemented to screen for polymorphisms associated with salt tolerance. Five putative AFLP markers and two RAPD markers were detected using bulks composed of salt tolerant plants and bulks composed of salt sensitive plants. The distribution of the markers in these bulks suggests that more than one *Thinopyrum* chromosome carry genes for salt tolerance.

Salt tolerant monosomic and disomic addition plants were characterised for AFLP, RAPD and DAF polymorphisms in an attempt to find markers associated with the chromosome(s) conditioning salt tolerance. One salt tolerant monosomic and one disomic addition plant was identified. One AFLP and two RAPD markers were identified for the *Thinopyrum* chromosome(s) present in the monosomic addition plant, while three AFLP and three RAPD markers were identified for the disomic addition plant.

An attempt was also made to identify diverse chromosome addition plants having complete or near complete triticale genomes plus an additional random *Thinopyrum* chromosome. Plants with 2n = 43 / 44 were identified and characterised for molecular markers (AFLP and RAPD). Cluster analysis was used to group the putative monosomic or disomic addition plants according to the specific *Thinopyrum* chromosomes they retained. Seventeen AFLP and RAPD markers could be used to group the 24 putative addition plants into six broadly similar groups with different additional *Thinopyrum* chromosomes. While the members of each group are likely to carry the

same additional *Thinopyrum* chromosomes, this may not necessarily be the case as the interpretation of the marker results is complicated by heterogeneity among plants with regard to the triticale background chromosomes they possess. It is also likely that chromosome translocations occurred during backcrossing which may further complicate data. Nonetheless, it is now possible to select disomic addition plants from each group that are likely to represent different *Thinopyrum* chromosomes. The data will also be useful in future attempts to find further addition plants carrying the remaining *Thinopyrum* chromosomes.

OPSOMMING

Die skadelike effek van grond versouting op gewasproduksie neem wêreldwyd toe (Tanji, 1990b). Die mate waartoe plante hoë konsentrasies sout in die wortelstelsel kan hanteer is onder genetiese beheer en verskillende genetiese en fisiologiese meganismes dra by tot die soutverdraagsaamheid tydens verskillende ontwikkelingstadia (Epstein & Rains, 1987). Slegs beperkte variasie bestaan vir soutverdraagsaamheid in verboude grane. Dit het aanleiding gegee tot pogings om soutverdraagsame nageslag te selekteer na hibridisasie van verboude spesies en wilde, soutverdraagsame spesies. *Thinopyrum distichum*, 'n inheemse koringgras, wat aangepas is by brak omgewings (McGuire & Dvorak, 1981), is met korog (x *Triticosecale*) gekruis in 'n poging om die gene vir soutverdraagsaamheid en ander gehardheidseienskappe oor te dra (Marais & Marais, 1998). Die oogmerke van hierdie studie was om (i) *Thinopyrum* chromosome te identifiseer wat gene bevat vir soutverdraagsaamheid en molekulêre merkers te vind vir hierdie chromosome, (ii) 'n aantal diverse monosomiese en disomiese addisieplante te identifiseer.

Bulksegregaatanalise (BSA), gekombineer met AFLP-, RAPD- en DAF-merkeranalise, is gebruik om polimorfismes geassosieerd met soutverdraagsaamheid op te spoor. Vyf moontlike AFLP-merkers en twee RAPD-merkers is geïdentifiseer met gebruik van bulks bestaande uit soutverdraagsame plante en bulks bestaande uit soutgevoelige plante. Die verspreiding van die merkers in soutverdraagsame bulks dui daarop dat meer as een *Thinopyrum* chromosoom bydra tot soutverdraagsaamheid.

Soutverdraagsame, monosomiese en disomiese addisieplante is gekarakteriseer vir AFLP- en RAPD-polimorfismes in 'n verdere poging om merkers te vind vir chromosome betrokke by soutverdraagsaamheid. Een soutverdraagsame monosomiese en een disomiese addisieplant is geïdentifiseer. Een AFLP- en twee RAPD-merkers is geïdentifiseer vir die *Thinopyrum* chromosoom(e) teenwoordig in die monosomiese addisieplant, terwyl drie AFLP- en drie RAPD-merkers geïdentifiseer is vir die disomiese addisieplant.

'n Poging is ook gemaak om diverse addisieplante te identifiseer met 'n volledige koroggenoom plus 'n addisionele *Thinopyrum* chromosoom. Plante met 2n = 43 / 44 is geïdentifiseer en gekarakteriseer met molekulêre merkers (AFLP en RAPD). Tros-analise is gebruik om die vermoedelik monosomiese of disomiese addisieplante te groepeer volgens die spesifieke *Thinopyrum* chromosome wat hulle behou het. Sewentien AFLP- en RAPD-merkers is gebruik om die 24 vermoedelike addisieplante in 6 groepe met verskillende *Thinopyrum* chromosome te

groepeer. Alhoewel dit voorkom of die verskillende plante in 'n groep dieselfde addisionele *Thinopyrum* chromosoom het, is dit nie noodwendig die geval nie aangesien die interpretasie van die merkers bemoeilik word deur die heterogeniteit tussen die plante wat betref die agtergrond korogchromosome wat hulle besit. Dit is ook moontlik dat chromosoom herrangskikkings plaasgevind het gedurende die terugkruisings, wat die data verder kan bemoeilik. Nietemin, dit is nou moontlik om disomiese addisies te selekteer uit elke groep wat moontlik verskillende *Thinopyrum* chromosome bevat. Die data kan ook gebruik word om in die toekoms verdere addisieplante te identifiseer wat die oorblywende *Thinopyrum* chromosome bevat.

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ABBREVIATIONS USED

α	Alpha
°C	Degree centigrade
µg	Microgram
µl	Microlitre
µM	Micromolar
AFLP	Amplified fragment length polymorphism
ATP	Adenosine triphosphate
bp	Basepair
Ca	Calcium
DAF	DNA amplification fingerprinting
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
dSm^{-1}	Decisiemens per meter
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
g	Gram
HCl	Hydrochloric acid
kb	Kilobase
λ	Litre
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute(s)
ml	Millilitre
mm	Millimetre
mM	Millimolar
N	Nitrate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
pmol	Picomol
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RE	Restriction enzyme
rpm	Revolutions per minute
s	Seconds
SDS	Sodium dodecyl sulphate
Taq DNA pol	*Thermus aquaticus* DNA polymerase
TBE buffer	Tris borate EDTA buffer
Th.	*Thinopyrum*
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
UV	Ultra violet

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CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Cultivation of plants started as long ago as 10 000 BC. Throughout the ages, selection of superior plants for future reproduction and, more recently, breeding plants in a systematic way have been the main tools for crop improvement. Breeding has accounted for an estimated three percent gain per year in yield during the past 30 to 40 years for corn, wheat, soybeans, cotton and sorghum (Wittington, 1982; Christensen & Lewis, 1982). Saline soils, however, remain an obstacle to crop improvement. Crops were originally not selected for their tolerance to salinity and therefore cultivars today are more sensitive to saline environments than their wild counterparts.

Saline soils are known to occur in many parts of the world, particularly in arid and semi-arid regions. Under conditions of limited rainfall, salt is not leached out of the soil, contributing to increased soil salinity. The majority of saline soils appear to have been affected by excess soluble salts for thousands of years, but a large area has become saline within the period of recorded history. Evidence suggests that these areas of secondary salinity result from human activities. It is believed that around 2400 BC, increasing soil salinity played an important part in the break-up of the ancient Sumerian civilization (Jacobsen & Adams, 1958).

The impact of salinity on crop production is becoming increasingly important (Tanji, 1990a; Flowers & Yeo, 1995). The extent of salt affected soil is estimated at 950 x 10^6 ha worldwide, which account for approximately six percent of the world's land surface (Tanji, 1990b). In addition, secondary salt-affected soils, which have decreased crop productivity, are increasing due to extensive irrigation. Irrigation water pumped onto the land contains dissolved salts, the water evaporates, leaving the salt to accumulate in the soil until it is not feasible to grow cereal crops such as wheat (Batie & Healy, 1983; Boyer, 1982).

The degree to which plants can tolerate high concentrations of salt in their rooting medium is under genetic control (Epstein & Jefferies, 1964; Epstein, 1985; Shannon 1990). The most effective way to overcome limitations to crop productivity in salinized areas may be the development of salt tolerant varieties. Therefore, an understanding of the genetics of salt tolerance in cereals is essential to establish an efficient breeding strategy for tolerance. At the genetic level, salinity tolerance has proved to be a quantitative trait (Foolad & Jones, 1993) and has not responded well to selection. Plant yield and productivity are also quantitatively determined and the introduction of adaptational

characteristics to cope with saline soils may lower the potential yield under normal conditions. It would thus be necessary to balance productivity with salinity or drought tolerance. Since the combination of productivity and tolerance has remained elusive, it seems necessary to first catalogue the genes that govern tolerance or resistance in a species. RFLP-mapping, RAPD-mapping and QTL-mapping are advanced techniques with which to obtain meaningful data about the gene complement governing the tolerance phenotype (Wing *et al.*, 1994).

All plants contained the machinery to respond to salinity stress (Bressan *et al.*, 1998), but most have lost it during evolution since they left the seas and colonised the land 400 million years ago. Naturally tolerant plants contain the fully developed and functional mechanism for salt-stress tolerance to make these species productive under stress. Tolerance seems to be a whole-pathway metabolic program rather than the expression of just one gene in any one of several pathways. It will be easier to transfer the pathway genes / enzymes from salt tolerant species into the germplasm of a sensitive species rather than tamper with the resident genes.

While productivity is paramount in the developed countries, justified by production costs, the problem is very different in areas where subsistence farming provides most of the income and livelihood. If plants could be protected from turgor loss or photo-inhibition only a few days longer, chances for continued growth and earlier harvest would be improved, time between irrigations could be longer and salinity content of the irrigated soil would be less critical. In those areas, yield, even at lower than optimum level, does make a difference (Bohnert & Jensen, 1996).

1.2 BIOLOGICAL STRESS

A body, A, exerting a force on body B, experiences an equal and opposite force from body B. The two forces are called action and reaction and are parts of an inseparable whole known as stress. When subjected to a stress, a body is in a state of strain. Biological stress can be defined as any environmental factor capable of inducing a potentially injurious strain in living organisms. Unlike mechanical systems, plants are able to erect barriers between its living matter and the environmental stress. The stress is therefore measured in units of energy. According to Levitt (1980), biological stress is not necessarily a force and the biological strain is not necessarily a change in dimensions, but can be a physical (e.g. cessation) or a chemical strain (a shift in metabolism). If the strain is sufficiently severe, the organism may suffer permanent injury or death. A specific organism will experience a specific strain when subjected to a specific stress. It therefore has its own resistance to physical or chemical change.

1.3 WATER AND ION TRANSPORT

More than 80 percent of most plant tissue comprise of water which is required as a physiological solvent, a transport medium, an evaporative coolant and a pressure source to support form and function and drive the growth process (Yeo, 1998). Plants obtain nearly all the water they need from the soil solution via the roots, but have little control over the water and ion concentrations in the soil. The concentration of solutes fluctuates due to changes in water source, drainage, evapotranspiration, solute availability, hydrostatic pressures and more. Water and selected solutes move from the soil and through the plant due to the difference in osmotic potential existing between the soil, roots, leaves and atmosphere. Nearly all the water taken up is evaporated at the leaf surface and only a small fraction becomes part of the permanent plant structure. A plant can change its leaf water as much as ten times in a day (Yeo *et al.*, 1997).

As water passes through the plant in response to its potential gradient, salts are filtered through the biological membranes where they are exposed to a variety of active and passive transport mechanisms (Volkmar *et al.*, 1997). Some ions are accumulated as useful nutrients, while others may be excluded or sequestered as wastes. The ionic concentrations at which normal cellular processes occur are very limited, although the concentration of the ions in the soil water may differ widely. The ability of plant roots to absorb water (and thus salts) from the soil decreases as the water content of the soil decreases or the concentration of ions like Na^+, Cl^- and SO_4^{2-} in the soil increases. Plants have to adapt to maintain the inflow of water and ions.

1.3.1 ENERGY USED IN TRANSPORT

Plants must maintain a state of inequilibrium with its environment in order to survive. If growing in normal soil containing the usual low concentrations of nutrient ions, it must increase these concentrations and the ion balance in its protoplasm to a level suitable for the normal functioning of the cell. Maintenance of this unbalanced, steady state requires the continuous expenditure of energy. If growing in saline soil, the plant must also concentrate and balance these ions. The plant must decrease the concentration of the sodium salt or ions in its protoplasm below that in the soil, in order to maintain ionic concentrations and balances that support normal functioning of the cell. The maintenance of a favourable ion gradient for ion compartmentation, as well as the synthesis of organic solutes to balance the osmotic potential of the salt-enriched vacuole, uses energy that could have been used to power biosynthetic processes (Volkmar *et al.*, 1997).

1.3.2 WATER TRANSPORT PROTEINS

The flux of ions across the plasma membrane and tonoplast occurs via transport proteins (Binzel & Dunlop, 1995; Ballesteros *et al.,* 1996). Ion transporters and their regulatory systems perform several physiological roles including maintenance of the intracellular ion concentrations within the optimal range for cellular systems and generating electrochemical gradients. Ion flux across membranes is dependent on the electrical gradient or membrane potential and the chemical gradient across the membrane (Nobel, 1991). Transport proteins that mediate ion flux can be categorised as pumps, carriers and channels. Pumps directly utilise metabolic energy for vectorial transport, while carriers couple the uphill transport of one solute to the downhill movement of another. Channels mediate passive transport, i.e. movement down a free energy gradient.

Regulation of cytoplasmic ion concentrations is achieved through regulation at the plasma membrane and tonoplast. This includes the selectivity and gating of channels, active transport processes and passive or unregulated leakage through the membranes. How all the membrane transport processes are co-ordinated to maintain the needed conditions is not yet fully understood (Yeo, 1998).

Potassium is an essential nutrient to growth and development, thus needed by plants in large amounts. There is limited knowledge of how channels / transporters regulate intracellular potassium levels (Smart *et al.*, 1996). Low-affinity uptake of potassium involves channels allowing potassium to enter along an electrochemical gradient. The Na^+ electrochemical gradient dictates that Na^+ influx across the plasma membrane is passive and efflux is active. Na^+ acts as competitor of K^+ uptake (Watad *et al.*, 1991; Schroeder *et al.,* 1994) suggesting that the uptake mechanisms for both cations are similar. Several cation channels have been shown to have poor selectivity between K^+ and Na^+, (Amtmann *et al.*, 1997; Tyerman *et al.*, 1997), accounting for the influx of sodium under saline conditions. When NaCl concentrations increases in the surrounding environment, these channels mediate low-affinity uptake of sodium, blocking potassium-uptake. The presence of high concentrations of Na^+ thus has the potential of swamping the mechanisms whereby K^+ is absorbed and utilised by plants. It is therefore of great importance for plants to have selective absorption and transport of K^+ in relation to Na^+ in saline environments (Rains & Epstein, 1967; Subbarao *et al.* 1990). Such a K^+ : Na^+ discriminating factor was, for example, mapped to chromosome arm 4DL in hexaploid wheat (AABBDD genome) (Gorham *et al.*, 1987; Dubcovsky *et al.*, 1996).

1.4 SALT DAMAGE

Salt may damage plants in two major ways. Secondary salt injury is due to physiological drought caused by a decrease in soil water potential. This leads to an osmotic dehydration, which is the cause of salt injury (Levitt, 1980). This only lasts temporarily, for the plant takes up salt into its tissues or manufactures organic solutes to decrease the water potential in its leaves and maintain a gradient of water potential along the transpirational pathway from the soil, through the plant and into the atmosphere. The accumulation of solutes within the plant in response to saline conditions is called osmotic adjustment.

Primary salt damage is caused by the toxicity of the salt entering the plant. Damage is caused by the high concentrations of ions, but also by the suppression of uptake of essential nutrients such as potassium. High sodium concentrations relative to other salts can disrupt root permeability to ions by displacing calcium in the plasma membrane. Upsetting calcium metabolism and nutrition within the cell may cause additional effects (Lauchli & Epstein, 1990). The toxicity of salts within the leaves also depends on the sensitivity of cellular processes to high salt concentrations and on the distribution of salts between and within leaves and cellular compartments. Both primary and secondary salt damage may be involved simultaneously.

To distinguish between secondary osmotic and primary salt injury it is necessary to compare the effects of isotonic solutions of a salt with those of organic substances. If the salt injury is simply osmotic in nature, all solutes should produce the same injury at the same osmotic potential. $CaCl_2$ inhibits the growth and yield of wheat slightly more than does NaCl at the same osmotic potential (-7.3 bars, Aceves-N *et al.*, 1975a), however, NaCl was more detrimental to germination of wheat and berseem seeds than was KCl (Malik, 1975). This indicates that the damage is not osmotic in nature. The relative toxicity of specific salts are not constant for all plants under all conditions (Maas, 1990).

The specific toxic effects of salts are evident in the following (Longenecker, 1974): (a) Organic solutes are not injurious even at higher osmolalities than the threshold concentration where salts become harmful. (b) Different salts have different threshold osmotic concentrations for injury. (c) The presence of some organic solutes, though increasing the total osmotic concentration, may increase the threshold salt concentration for injury. (d) Ca^{2+} antagonises the injurious salt effects.

1.4.1 CALCIUM AND CHLORIDE SALT STRESS

Calcium salts present in nature are of low solubility, but the activity of the Ca^{2+} in the soil is much higher than that of ions responsible for ion stresses. Calcareous soils may injure, kill or simply inhibit the growth of some plants. Species, as well as ecotypes of a species, differ in their ability to grow on calcareous soils (Ingestad, 1973). The stress produced in the plant is usually not due to the primary Ca^{2+} stress, but is due to the secondary stresses induced by the Ca^{2+} (Kinzel, 1963), e.g. pH and mineral deficiency. In the presence of excess calcium, plants unable to grow and develop on calcareous soils (*calcifuge*), absorb Ca^{2+} at the expense of other ions (e.g. iron) and would therefore suffer from a deficiency of iron. Large quantities of Ca^{2+} can be taken up by the plant and precipitated in the cell sap as oxalates or carbonates, or neutralised in the soluble form in the cell sap as malates or citrates.

Little is known about the *in vivo* targets for chloride, but is it may interfere with ionic sites involved in binding RNA and anionic metabolites such as bicarbonates, carboxylates and sugar-phosphate (Serrano *et al.*, 1999).

1.4.2 SODIUM SALT STRESS

Most of the salt stresses in nature are due to sodium salts, particularly NaCl. Plants able to grow and proliferate on saline soil are termed halophytes, some completing their life cycles in salt concentrations of up to 500 mol m^{-3} (Ungar, 1991). Because of the wide range of tolerance found in halophytes, they have been subdivided to range from the extremely tolerant euhalophytes to the moderately tolerant oligohalophytes (Takada, 1954). Many halophytes are able to grow perfectly normally in low or nonsaline environments (Ungar *et al.*, 1969) and are therefore facultative halophytes. Others cannot and are therefore obligate halophytes (Weissenbock, 1969). Plants that cannot grow in the presence of high concentrations of sodium salts are called glycophytes ("sweet" plants). The major crops of the world are not tolerant to saline environments. Bean yield, for instance, is almost totally inhibited at NaCl concentration of 50 mol m^{-3} (Volkmar *et al.*, 1997). The causes of sodium toxicity remain controversial (Davenport *et al.*, 1997). The specific symptoms of sodium toxicity include high tissue sodium concentrations and low K^+ : Na^+ ratios, inhibition of root elongation and shoot calcium deficiency (Maas & Grieve, 1987). Growth is thus inhibited by sodium through affecting uptake of other ions and a direct toxicity in the cytoplasm.

1.4.3 ION ANTAGONISM

A single salt solution of Na^+ salts is injurious to most plants at almost any concentration, but a two-salt solution consisting of NaCl and $CaCl_2$ in a ratio of about 10:1 is "balanced" and non-toxic (Hyder & Greenway, 1965). This phenomenon is known as ion antagonism. Calcium decreased cell permeability that was increased by Na^+ under saline conditions. Calcium-rich water decreased the uptake of Na^+, but not K^+. In wheat, Ca^{2+} improved the phosphor intake in the presence of a high degree of salination and improved the K^+ supply at the expense of Na^+ (Azizbekova & Dadasheva, 1972). Ca^{2+} was shown to ameliorate NaCl stress by preserving K^+ / Na^+ selectivity of the membrane transport proteins (Zhong & Lauchli, 1994).

1.5 VARIATION IN THE NATURE OF SALT STRESS

Salt tolerance is a complex phenomenon, not only because different plants respond to saline conditions in fundamentally different ways, but also because of the great variation in the stress itself. In different locations, the conductivity, chemical composition and pH of the saline soil may vary. Salt in soil is very heterogeneous, both spatially (different depths, time and space) and in relation to plant development. Not only may different plants be more or less susceptible at different stages in their growth cycles, but the intensity of the stress may also change over time. The patchy nature of salt in soils is further complicated by the plant's ability to extract water from the least saline areas explored by its roots. Two plants next to each other may experience significantly different levels of stress. The effect of increased salinity on plants is determined not only by the absolute quantity of the ions in excess, but also by the relative amounts of certain other ions, especially SO_4^{2-} (Strogonov, 1964).

Salinity is often combined with other stresses such as drought, waterlogging, alkalinity and nutrient deficiency. The soil type also influences soil salinity. Sandy, light textured soils, for example, do not accumulate salts around the root zone. At higher sodium to calcium ratios, soil structure, tilth and soil permeability to water may be reduced (Shainberg & Singer, 1990).
Agricultural management practices and climate may reduce or increase the salinity effects on plants. For instance, not providing for adequate leaching of the soil water can lead to an increase in salt concentration and thus lower yields. Use of adequate irrigation systems and providing windbreaks and shading can lead to higher yields. These technological approaches are not always economical or practical and provide only temporary solutions (Ashraf, 1994).

1.6 PHYSIOLOGICAL AND MORPHOLOGICAL RESPONSE OF PLANTS TO SALINE ENVIRONMENTS

1.6.1 PHYSIOLOGICAL RESPONSES

Fundamental plant metabolic processes such as photosynthesis and respiration are sensitive to salts (Greenway & Osmond, 1972). Photosynthesis was initially inhibited in wheat and barley under saline stress, but in time returned to normal levels (Udovenko *et al.*, 1974). Sodium salts decreased the respiration rate of most crop plants, such as wheat and gram (*Cicer arietinum*) (Bhardwaj & Rao, 1960; Sarin, 1961), but an increase was noted in the more resistant barley and wheat species (Udovenko *et al.,* 1972). The net effect of salt on respiration rate may depend on opposite effects on the respiratory components. The salt-induced stimulation can be explained by an activation of the ion transport system, particularly the Na^+, K^+-ATPase of the plasmalemma, as well as a direct effect of Na^+ on the respiratory chain (Gordon & Bichurina, 1973).

Protein synthesis is decreased and protein hydrolysis increased by sodium salts in most crop plants. A stimulation of protein synthesis has, however, been found in certain crops such as barley (Helal *et al.,* 1975), wheat (increased seed protein content) (Singh & Vijayakumar, 1974) and desert fodder plants (increased N content) (Abd El-Rahman *et al.*, 1974). Hormonal imbalances are caused by salinity, which in turn also influence systems where they are active (O'Leary, 1970; Prisco & O'Leary, 1972). The levels of the hormones kinetin and abscisic acid (ABA), which have an effect on stomatal control, were respectively reduced and increased when the plants were exposed to salt stress (Polkajoff & Gale, 1975).

High salt concentrations inhibit most enzymes due to the perturbation of the hydrophobic-electrostatic balance between the forces maintaining protein structure (Pollard & Wyn Jones, 1979). In some cases an increase in activity occurs at low concentrations of salt and a decrease at high concentrations. Enzymes differ in their sensitivity to salt stress, e.g. LDH is more sensitive than ADH (Akthar *et al.*, 1998). In general, enzymes in halophytes are not less sensitive to high salt concentrations than those of glycophytes. The difference in metabolic rates between halophytes and glycophytes is rather the ability of halophytes to prevent the influence of the ions in the cytoplasm by, for example, compartmentation of salts in vacuoles (Flowers *et al.*, 1977).

Nucleic acid metabolism is also disturbed. At high NaCl levels, RNA and DNA decrease due to both inhibition of synthesis and an increased breakdown (Tsenov *et al.*, 1973). Both the decreases

in protein and nucleic acid contents may be due to an inhibition of synthesis or an increase in hydrolysis, or both. The enzyme RNase is commonly stimulated due to the salt-induced secondary osmotic stress. Due to disturbance of metabolic processes, substances such as diamines, alkaloids and even amino acids can accumulate and cause necrosis.

Photosynthesis, respiration, protein metabolism and nucleic acid metabolism are therefore all influenced by salination. Low salt levels often stimulate the processes while high concentrations inhibit them. The inhibition, if slight, may sometimes be temporary, being followed by a return to normal or a reversal. The metabolic processes are commonly stimulated or unaffected in halophytes by concentrations high enough to inhibit them in glycophytes. One salt may inhibit a specific process while another salt stimulates it at the same concentration. The metabolic change is the net effect of a stimulation of some processes and an inhibition of others. Any one of the metabolic disturbances can lead to an inhibition of the growth and development of the plant or cause plant death.

1.6.2 MORPHOLOGICAL RESPONSES

The growing of crops under saline conditions imposes many restrictions on growth and development. These restrictions lead to low productivity and low economical viability in the agricultural system. Since most agricultural crops are glycophytes, they are more suited to non-saline conditions and the physiological adjustments and morphological changes that are possible, are limited (Polkajoff-Mayber & Gale, 1975).

Plant response to excess salts in the root zone is quantitatively dependent upon the salt concentration, time of exposure and salt composition. Salt sensitivity varies greatly with growth stage, species, variety and ecotype (Munns & Termaat, 1986). Plants are often salt tolerant during germination, become more sensitive during the emergence and young seedling stage and are more tolerant through the reproductive stage with the exception of anthesis. Salt tolerance at germination and at the seedling stage are important because the initial plant stand affects the final production. A plant's sensitivity to salinity at these stages has therefore been used as a means for selecting for enhanced salinity tolerance (Ashraf *et al.,* 1986).

Salinity generally decreases plant growth at low concentrations and is lethal at high concentrations. Salt-affected plants appear darker green, are stunted and have fewer, shorter internodes. Shoots are more restricted by salinity than root growth, thereby decreasing the shoot : root ratio (Lauchli & Epstein, 1990). Leaves are the first to react on high salt concentrations, witnessed as,

for example, colour changes, tip burn, marginal necrosis and succulence. The response of leaf blades to NaCl salinity depends on their age and position on the main stem (Colmer *et al.*, 1995). New leaf growth is supported through the export of carbon from mature leaves. Excess salts are stored in the vacuoles of the older leaves. As the capacity of older leaves to sustain new leaf growth diminishes due to salt-induced leaf necrosis, the ability of new growth to handle the continuous export of salt from the root decreases. Plant death occurs because the rate of leaf death overtakes rate of new leaf production. According to Munns (1994), differences in salt tolerance among genotypes are related to the difference in the time that it takes for salt to reach its maximum concentration in the leaf vacuoles. Thus, salt-sensitive plants are unable to compartmentalise salts in their leaves as effectively or to as high concentrations as can tolerant plants.

The impact of salt on cell division in whole plants is not well understood and conflicting results are reported (Munns *et al.*, 1988 and Bernstein *et al.*, 1993). Root zone salinization affects plant ontogeny, e.g. a shorter time of flowering (Romero & Maranon, 1994) and delayed tiller development (Maas & Grieve, 1990). Grain yield of spring wheat is reduced by salinity through the reduction of the number of fertile tillers per plant (Hollington & Wyn Jones, 1990; Silberbush & Lipps, 1991). The magnitude of the reduction is directly related to increased salinity (Maas *et al.*, 1994; Hu, 1996). The effect is more apparent when spring wheat plants are subjected to salt stress early in their development (Grieve *et al.*, 1993; Francois *et al.*, 1994). Salinity also affects the number of kernels. The kernel mass is, however, less influenced than the number of tillers (Grieve *et al.*, 1993). Maas & Grieve (1990) found that the wheat grain mass per spike on the main stems remained nearly constant in saline medium because the decrease in kernel numbers per spike was compensated for by an increase in kernel mass. It appears that fewer seeds were produced, but they were of higher quality. These conclusions should, however, not be generalised for all species. For example, Aloy (1992) found that in barley, grains per spike and spikes per unit area were quite insensitive to field-applied salinity, while 1000-seed weight was most strongly affected, accounting for most of the decrease in grain yield.

1.7 PHYSIOLOGICAL AND MORPHOLOGICAL ADAPTATIONS OF PLANTS TO SALT STRESS

Salt tolerance of a plant (or genotype) can be considered as the ability to germinate, maintain growth and reproduce under persistent or interrupted salt stress (Jana, 1993). Naturally evolved salt tolerance is a quantitative and polygenic trait associated with numerous morphological, physiological and biochemical mechanisms to ensure survival under saline conditions (Epstein & Rains, 1987; Cushman *et al.*, 1989). Different species have evolved tolerance through different courses of evolution. The level of genetic complexity is evident by the multigenic inheritance, e.g. variable heritabilities of salt tolerance traits in taxa such as wheat (Schachtman & Munns, 1992) and various grass species (Ashraf *et al.*, 1986). It is therefore unwise to offer simple generalisations about mechanisms of tolerance in all plants based on results obtained with a specific variety or ecotype. This results in contradictory evidences concerning mechanisms of tolerance that complicates understanding.

1.7.1 PHYSIOLOGICAL ADAPTATIONS

Halophytes and glycophytes differ only quantitatively rather than qualitatively and are extreme examples lying on a continuum. Tolerance responses to excess salinity involve potentially numerous, extremely complex and highly integrated responses at the subcellular, cellular and interorgan level (Claes *et al.*, 1990; Adams *et al.*, 1992). Physiological attributes suggested as important components of a salt tolerant phenotype include:

1.7.1.1 Control over sodium and potassium transport

Ion transport and its regulation play key roles in the salt tolerance phenotype (Kramer, 1984; Flowers & Yeo, 1986). In general, halophytes compartmentalise both Na^+ and Cl^- into the vacuole and regulate ion transport at both the plasma membrane and tonoplast (Flowers *et al.*, 1977).

Plants accumulate fewer ions from the soil by lowering the passive and active uptake of ions. This is achieved by better regulation and by increased selectivity of ion transport proteins (Rubio *et al.*, 1995). Increased salt tolerance is associated with more effective exclusion of Na^+ and Cl^- and enhanced uptake of K^+ as well as retranslocation of K^+. Tolerant plants tend to have lower Na^+ and higher K^+ concentrations in the flag leaf (Gorham *et al.*, 1986). Salt adapted cells have been shown to have a higher K^+ / Na^+ selectivity at the plasma membrane (Watad *et al.*, 1991).

A gene(s) has been identified on the long arm of chromosome 4D which controls the ratio of K^+ to Na^+ accumulated in the shoots (Shah *et al.*, 1987; Gorham *et al.*, 1987). Other control mechanisms include an increased efflux of Na^+ at the plasma membrane and / or restricted transport of Na^+ and Cl^-, especially to the shoot (Jeschke, 1984) and the absorbing of excessive amounts of K^+ through K^+-selective membrane transport proteins. The K^+ also assists in osmoregulation (Albert & Kinzel, 1974).

1.7.1.2 Sodium partitioning to vacuoles in leaves.

It is vital for plants to prevent salts absorbed through the roots to accumulate in the cytoplasm where it may have an adverse effect on the enzymatic processes (Barkla *et al.*, 1995). This must be achieved without affecting the water balance between the water potential of the soil solution and that of the plant responsible for the inflow of water into the plant. Even for plants that survive in saline soils by minimising salt ion entry, solute concentration may still exceed the root system's capacity to entirely exclude the ion at which point salt ions will begin to accumulate in the shoot. Once saline solutes reach the leaf, there is only one plausible way to exclude it from the cytoplasm and that is to partition it into the cell vacuole. The vacuole comprises the bulk of the total cell volume and is therefore well suited for solute compartmentation. Salt ions cross the plasma membrane into the cytoplasm from where it is compartmentalised into the vacuole. The movement of salt ions into the vacuole must match the rate of export of salt from the root to the leaves to prevent the accumulation of salt ions in the cytoplasm. Plants adapted to high salt concentrations are able to compartmentalise salt in their vacuoles at a similar rate as that at which the salt ions are delivered from the xylem to the leaf. Salt-sensitive plants are unable to compartmentalise salts in their leaves as effectively, or to as high concentrations as can tolerant plants, and this leads to cell and plant death. Energy is used to maintain a favourable ion gradient for compartmentalisation as well as for the production of organic solutes in the cytoplasm to balance the osmotic potential of the salt-enriched vacuole. The accumulation of inorganic ions in the vacuoles is used by halophytes to balance the osmotic potential of the salt outside the plant (Flowers *et al.*, 1986).

1.7.1.3 Osmolytes

The accumulation of salt in the vacuole creates a strong osmotic gradient across the vacuolar membrane. The synthesis and accumulation of solute molecules (osmolytes) in the cytoplasm balance this gradient. The process is called osmotic adjustment (Wyn Jones & Gorham, 1983; McCue & Hanson, 1990). Osmoregulation is the main physiological adjustment in halophytes.

The accumulation of osmolytes in the cytoplasm helps maintain turgor and cell volume, as well as stabilising the active conformation of cytoplasmic enzymes, thereby protecting them against inactivation by inorganic ions (Pollard & Wyn Jones, 1979; Galinski, 1993). These organic solutes have properties such as low polar charge, high solubility and large hydration shells. Compounds performing as osmolytes are e.g. proline, glycine-betaine and other related quaternary ammonium compounds such as pinitol, mannitol and sorbitol. The enhancement of salt tolerance conferred by these osmo-protectant solutes has been demonstrated in barley and maize (Grumet & Hanson, 1986) and *Th. bessarabicum* (Gorham *et al.*, 1985). The production of osmolytes is, however, metabolically expensive, consuming significant quantities of energy and carbon that could otherwise be used for growth. A more energy efficient manner of osmotic adjustment is by using inorganic ions absorbed from the external medium, but high concentrations of these ions interfere with normal biochemical activities in the cell (Wyn Jones 1981; Yeo 1983).

1.7.1.4 Radical scavengers

A wide range of cellular processes is negatively affected by oxidating free radicals, emerging in response to the salt stress (Lewis *et al.*, 1997). Certain osmolytes act as radical scavengers e.g. mannitol, thereby protecting against oxidative damage (Shen *et al.*, 1997).

1.7.1.5 Other adaptations

Other adaptations enabling survival in saline soil are the mechanisms that override stress-induced increases in abscisic acid (ABA) levels in immature plants, (but this leads to accelerated ontogeny) and the expression of proteins playing an important part in water retention or protection e.g. dehydrins (Bray, 1993).

Salinity tolerant plants may be able to metabolise toxins produced due to the disruption of metabolic pathways. An enzyme tolerance or even requirement (in obligate halophytes) of high salt concentrations can explain tolerance of metabolic disturbances. Plants that possess C_4 photosynthesis commonly seem to require NaCl (Brownell & Crossland, 1972). NaCl can also switch the plant from C_3 to C_4 photosynthesis (Shomar-Ilan & Waisel, 1973). Since the salt shock is probably injurious by causing membrane damage, tolerance is also associated with more robust membrane composition (Blits & Gallagher, 1990).

1.7.2 MORPHOLOGICAL ADAPTATIONS

Several structural changes in stressed plants can be ascribed to salinity. The capacity of plant leaves to accommodate the export of salt from the root is closely linked to growth rate. New, expanding cells provide a continually replenishing storage reservoir for the vacuolar compartmentalisation of salt from the root. In this sense, growth itself represents a means by which the plant can regulate the concentration of salts in the cytoplasm (Volkmar *et al.*, 1997). This "dilution" of cell sap due to growth has been found in some moderately salt-resistant nonhalophytes, e.g. barley.

Inhibition of shoot growth with continued root growth has been considered as a morphological adaptation to salt stress. This decreases the shoot : root ratio, compensating for the decrease in water uptake because of the higher soil osmotic potential.

The presence of salt excreting glands can reduce the concentration of salt in the plant, such as occur in species of *Frankenia*, *Cressa* and *Glaux*. In the case of extrusion, active salt-extruding pumps are responsible for removing the salts from the plant. Plants that avoid salt stress through exclusion also implement extrusion, because ions may leak into the cell when exceeding a threshold concentration (Hale & Orcutt, 1975).

Other morphological adaptations include succulence (this leads to dilution of intracellular salt), development of small leaves, water storage hairs and aerenchyma; changes in number and size of stomata; thickening of the cuticle; extensive development of tyloses; earlier occurrence of lignification; inhibition of differentiation; changes in diameter and number of xylem vessels, etc.

1.8 MEASUREMENT OF SALT TOLERANCE

A large number of traits or indices have been used to measure the salt tolerance of plants. Characters that indicate growth, development and reproductive capacity are natural candidates (Ramage, 1980; Rana, 1986). Absolute and relative biomass yields have also been used. Although grain yield and its stability are two major goals in breeding for salt tolerance, selection for grain yield is inefficient because of its low heritability, especially under stressful field conditions (Fischer & Mauer, 1978; Blum, 1988) and itself being a complex trait. Selection is further complicated by variability in soil salinity. Evaluation should thus be conducted over a large number of replications over several years.

The United States Salinity Laboratory has conducted research on the salt tolerance of agricultural plant species and compiled a salt tolerance list for the different species (U.S. Salinity Laboratory Staff, 1954). The tolerance level to salinity is based on the electrical conductivity of saturated soil extracts for which the symbol is EC_e and the units are decisiemens per meter. The EC_e is related to the number of electricity conducting ions in the solution. An EC_e of 1.0 dSm^{-1} corresponds to a total salt concentration of approximately 640 parts per million.

Physiological criteria used in breeding for salt tolerance are osmotic adjustment, K^+ / Na^+ selectivity, Na^+ exclusion from leaves, cytokinin concentrations, photosynthesis and transpiration rate (Kuiper *et al.,* 1990; Karim *et al.,* 1992 and Morant-Avice *et al.,* 1999). Shannon (1985) emphasised the role of 'ontogenetic drift' in plant response to salt stress. Since a plant's salt injury depends on its ontogenetic stage as well as on the nature and severity of previous stresses, it is desirable to measure responses to salt stress at different growth stages. Such data would provide a more reliable overall measure of salt stress (Srivastava & Jana, 1984).

It is difficult to compare the reported limits of salt stress survival for several reasons (Levitt, 1980). (a) There is no standard method of measuring salt stress and at least seven different units of measurement are to be found in the literature. (b) The limits vary with the environmental conditions, and with the stage of development. Seedlings, for instance, are much more sensitive to salinity than later growth (c) Irrigation methods may lower the salt stress and therefore raise the limits of survival. With adequate leaching and drainage, a plant can tolerate far higher concentrations of salt in the soil water. (d) The length of time exposed to salt has not been standardised, and if not too long, growth may be inhibited without cell injury.

For evaluating a large number of lines, it is convenient to use biochemical and morphological markers for salt tolerance. Kahler & Wehrhann (1986) and Stuber *et al.* (1987) have shown associations of isozyme markers with complex quantitative traits such as grain yield and yield components. Esterase 1 has been shown to be a useful marker in screening for cold tolerance in rice. Biochemical or genetic markers may be very useful for screening large numbers of germplasm lines for salt tolerance, thereby reducing the number of lines for more rigorous agronomical and physiological studies. Marker-assisted selection can increase the efficiency and accuracy of selection, especially for traits that are difficult to phenotype (Zhang *et al.,* 1999).

1.9 CLIMATIC INFLUENCE ON SALT TOLERANCE

Various factors in the atmospheric environment affect a plant's ability to tolerate high salt concentrations in the soil. High atmospheric humidity increases tolerance by reducing transpiration rates (Hoffman *et al.*, 1971; Hoffman & Rawlins, 1971). Plants are more tolerant at moderate temperatures than at high temperatures (Francois & Goodin, 1972). Tolerance is reduced by high light intensity (Nieman & Poulsen, 1971) and low oxygen concentrations (Aceves *et al.*, 1975b). Salt stress has been shown to increase resistance to ozone damage, possibly because of the closure of the stomata (Hoffman *et al.*, 1975).

1.10 CHROMOSOMES AND GENES CONFERRING SALT TOLERANCE IN THE TRITICEAE

Plants show a complex response to salinity, which is controlled by a large number of genes (Epstein, 1985; Blum, 1988; Shannon & Nobel, 1990). A clear understanding of the genetic mechanism underlying the inheritance of salt tolerance is still lacking. The development of salt tolerant crops through genetic manipulation appears to require the introduction of many genes combined with the appropriate promoters, targeting sequences and control genes. Salt uptake and compartmentation characteristics are, for instance, quantitative in nature and do not show simple Mendelian inheritance.

Genetic analysis of quantitative traits often indicates manageable numbers of chromosome areas having major effects on complex physiological traits (Prioul *et al.*, 1997). Though salt tolerance is a polygenic trait, the genes having a major influence have been linked to specific chromosomes. Gene clusters involved in phenomena such as stress tolerance were reported in higher plants (Forster, 1994). It was found that chromosomes 3 and 5 of five different Triticeae species consistently conferred tolerance to sudden salt stress and gradually imposed salt stress (Zhong & Dvorak, 1995). There may thus be a similar basis for tolerance in the species of the tribe Triticeae (Forster, 1992). This offers more potential for practical improvement than if a large number of scattered genes, each with a small phenotypic effect, were responsible. This means that the most feasible approach to gene transfer may be genome shuffling following wide hybridisation or addition of whole chromosomes. By studying wheat disomic substitution lines of individual chromosomes from related species, chromosomes, chromosome arms and ultimately chromosome

regions controlling salt-stress tolerance in wheat and the related species can be identified (Omielan *et al.,* 1991; Forster *et al.*, 1988a; Mahmood & Quarrie, 1993).

Although a chromosome may carry a gene(s) conferring salt tolerance, it also carries other genes that may be agronomically deleterious. It is therefore better to introduce a small segment of the chromosome that carries the genes conferring tolerance, but which lacks the agronomically deleterious genes. The transfer of a segment of an alien chromosome into wheat can be achieved through homoeologous recombination between alien and wheat chromosomes at meiosis. Cytogenetic approaches have been instrumental in the identification of genes for salinity tolerance for transfer to bread wheat (McGuire & Dvorak, 1981; Dvorak *et al.*, 1985). Wheat, however, carries a gene, *Ph1*, which suppresses homoeologous recombination during meiosis (Kihara, 1936; Lacadena & Ramos, 1968), thus necessitating its removal before the chromosomal segment can be transferred.

An amphiploid between the bread wheat cv. Chinese Spring and *Th. elongatum* (Host) Löve outperformed Chinese Spring in grain yield and biomass production under salt stress. When various addition lines of *Th. elongatum* chromosomes in wheat were examined, it was found that several chromosomes contributed to the salt tolerance of the full amphiploid (Dvorak *et al.*, 1988). The most dramatic increase resulted from the presence of chromosome 3J (Omielan *et al.*, 1991) while the group two chromosomes decreased salt tolerance. In the case of *Th. bessarabicum*, chromosome 5J was found to confer salt tolerance in addition lines with Chinese Spring (Forster *et al.*, 1988b; Mahmood & Quarrie, 1993). The effect of chromosome 5J has been related to the amphiploid's ability to exclude sodium and chloride ions from young leaves and maintenance of the accumulation of K^+ in young tissues (Gorham *et al.*, 1985). The function of the gene(s) on chromosome 5J may thus be to restrict the influx of ions into the developing tissues.

A cross between Chinese Spring and *Th. bessarabicum* produced amphiploids able to survive on NaCl concentrations of 250 mol m^{-3} and to set seed. The first-backcross plants with 42 wheat and 7 single *Thinopyrum* chromosomes were substantially more tolerant to salt than wheat, but were not as tolerant as the amphiploid with 42 wheat and seven pairs of *Thinopyrum* chromosomes. This indicates that the full expression of salt tolerance results from a double dose of genes from *Th. bessarabicum* (Forster *et al.*, 1988).

A large number of genes in several functional groups (ion transport, oxidative stress defences, carbon metabolism and energy production / photosynthesis, cell wall / membrane structural

components, etc) have been identified that are activated during salt stress. Analysis of stress-induced gene expression has revealed the presence of multiple signal transduction pathways between the perception of the stress and gene expression (Winicov, 1998). Transformation of plants with structural or regulatory sequences that make sodium exclusion possible may enable them to counteract ultracellular sodium toxicity (Rios *et al.*, 1997). A series of cDNA clones of genes whose expression is enhanced by saline stress have been isolated, which can provide a powerful tool in the investigation of physiological responses of wheat and related species (Nemoto *et al.*, 1999). These cDNA's can be used as probes in *in situ* hybridisation to determine the chromosomal location of the genes, as well as the tissue and cell in which they are expressed. A small group of transcription factors have also been identified to bind to promoter regulatory elements in genes regulated by salt / drought stress (Shinozaki & Yamaguchi-Shinozaki, 1997).

1.11 GENE TRANSFER

In small grain cereals, alien gene transfer as a means to broaden the gene pool available to breeders has been well exploited. Gene transfer based on wide hybridisation of wheat and triticale with their wild relatives basically involves the following steps,

(a) the identification of related (alien) genotypes which carry potentially useful genes,
(b) hybridisation and identification of the target chromosomes and,
(c) the reconstitution of the recipient genotype with the retention of only the beneficial alien genes.

In order to carry out these steps it is necessary to produce three genotypes, not in themselves economically important, but invaluable in pin-pointing the chromosomal location of useful genes and simplifying the eventual transfer (Gale & Miller, 1987), namely;

(a) An amphiploid: the chromosomally doubled fertile product of a sterile hybrid between the target species and alien donor species.
(b) Wheat (triticale) – alien addition line: a complete wheat (triticale) genome with an additional chromosome pair from the alien species.
(c) Wheat (triticale) – alien substitution line: the target genome has a chromosome pair replaced by a homoeologous pair of alien chromosomes.

1.11.1.2 Additions

One way to reduce the negative effects of the complete *Thinopyrum* genome on the wheat genotype, is to produce monosomic or disomic addition lines with different *Thinopyrum* chromosomes in a wheat background (Sears, 1981). Addition lines may be cytologically unstable and less fertile than normal wheat. They are, however, valuable for genetic analysis of the *Thinopyrum* genome and give an indication of the expression of the target genes in a wheat genetic background.

1.11.1.2 Chromosome substitutions

Spontaneous, balanced substitution lines in which a pair of wheat chromosomes were substituted by *Thinopyrum* homoeologues were sporadically found in backcrosses of primary amphiploids (wheat x *Th. intermedium* or *Th. ponticum*). Controlled substitution of each wheat chromosome by a *Thinopyrum* homoeologous chromosome was made possible by employing the monosomic and telosomic lines developed in hexaploid wheat by Sears (1954, 1958) and Sears & Sears (1978). Substitution lines are generally more fertile and stable than addition lines. Certain *Th. elongatum* and *Th. ponticum* chromosomes compensate well for the replaced wheat chromosomes.

1.11.1.3 *Triticum / Thinopyrum* translocations

According to Cauderon (1979), substitutions of whole *Thinopyrum* chromosomes are usually accompanied with negative effects due the introduction of genes encoding wild characteristics. Faith (1983) identified only one line out of several wheat-*Thinopyrum* progeny tested that had a yield comparable with the best wheat cultivar. It is thus important to reduce the size of the segment transferred from *Thinopyrum* to wheat to as small as possible. Spontaneous crossing over between wheat and homoeologous, foreign chromosomes occur at a low frequency during meiosis. A locus on chromosome 5B was found to exert a major effect on the pairing behaviour of diploid wheat chromosomes during meiosis I (Okamoto, 1957; Riley & Chapman, 1958). Absence of this gene promotes homoeologous pairing, enabling the transfer of short chromosome segments through meiotic crossover. X-rays can also be used to induce chromosome breaks and translocations.

1.12 BREEDING FOR SALT TOLERANCE

Various strategies have been suggested to improve the salinity tolerance of crops. However, only a handful of cultivars have been reported which are tolerant to saline soil. The following breeding strategies are employed.

1.12.1 WIDE HYBRIDISATION METHODS

These efforts focus on the selection of tolerant progeny following hybridisation of cultivated species and related, wild, salt-tolerant species (Dvorak *et al.*, 1985; Forster *et al.*, 1988; Littlejohn, 1988). Several halophytic wild species of the Triticeae are naturally adapted to saline environments (McGuire & Dvorak, 1981). According to Dewey (1984), the most noteworthy of the perennial Triticeae, are the *elongata* and *juncea* complexes of the genus *Thinopyrum* (Shannon, 1978; McGuire & Dvorak, 1981).

Some amphiploids between salt tolerant Triticeae species and cultivated hexaploid bread wheat have been found to be much more salt tolerant than commercial cultivars of bread wheat (Forster *et al.*, 1987; Gorham *et al.*, 1986; Dvorak & Ross, 1986). Unfortunately the amphiploids are inferior to wheat with respect to grain yield and quality. Very little success has been achieved in developing amphiploids or partial amphiploids that are agronomically viable or have the potential to develop into a new crop (Flowers & Yeo, 1995). Desirable *Thinopyrum* characteristics were only partially expressed or sometimes not at all. One way of minimising the amount of undesired *Thinopyrum* chromatin is to add only the chromosomes that carry salt tolerance genes. Cytogenetics offer the possibility of introgressing whole chromosomes or parts of chromosomes from foreign species into crop species through additions, substitutions or translocations of single alien chromosomes or chromosome segments carrying useful genes.

There is an interest in the use of multiple hybrids, combining germplasm from several different species, in cereal breeding research programs (Jauhar, 1992). These hybrids offer the opportunity for transfer of a range of different characters into a crop, and because of their diverse origin, the characters may prove both robust and durable under field conditions.

King *et al.* (1997) are developing a novel salt tolerant crop, tritipyrum, a hybrid between wheat and *Thinopyrum* species. Tritipyrums differed depending on the wheat parent species used. It may therefore be possible to select specific wheat genotypes that interact positively with the *Thinopyrum* genome.

1.12.2 SCREENING AND SELECTION FOR VARIATION THAT EXISTS WITHIN A CROP.

Salt tolerance varies widely between species, varieties and ecotypes. By screening and selecting within existing germplasm of a crop, lines can be identified that have better tolerance to salinity (Norlyn, 1980). Due to the quantitative nature and low heritability of salt tolerance, selection has to be continued for a number of years to ensure that progress is made.

Cell tissue culture selection is also being used to develop salt tolerant lines. Advanced tissue culture techniques provide the means for the regeneration of salt-adapted cell lines. This method provides some advantages over conventional breeding, but many difficulties exist (Stavarek *et al.*, 1980; Dracup, 1991) and results achieved still differ (Dracup, 1993; Hasegawa *et al.*, 1994). Only a few of the salt tolerant variants have been successfully regenerated as whole plants, which has limited their usefulness for associating specific genetic changes with a salt tolerant phenotype. Regenerated plants also often lack expression of the salt tolerance trait.

1.12.3 TRANSGENIC PLANTS

Molecular genetics have led to the identification of transcriptional gene products and proteins associated with salt stress, creating the possibility to develop transgenic plants (Ingram & Bartels, 1996; Shinozaki & Yamaguchi-Shinozaki, 1997). Most attention has been given to structural genes (transport proteins, ion channels, enzymes of solute synthesis) (Yeo, 1998). A limited number of these genes have been tested in transgenic plants by overexpressing a gene encoding a single function. This approach may, however, not be sufficient to lead to optimal adaptation to saline environments (Flowers *et al.*, 1997). With the recognition that enhanced expression of a number of functionally related genes may be required for significant improvement in salt tolerance, molecular engineering has been expanded to include proposals for multiple gene transfers to enhance salt tolerance (Bohnert & Jensen, 1996).

1.12.4 DEVELOPING HALOPHYTES AS ALTERNATIVE CROPS.

The domestication of wild halophytic species has been proposed to exploit their genetic potential for high salt tolerance (Gallagher, 1985; O'Leary *et al.*, 1985). It may prove more successful and cheaper than to breed a conventional crop for salt tolerance (Evans, 1993; O'Leary, 1994). Glen *et al.* (1998) proposed the domestication of a wild, salt tolerant halophyte and watering it with sea water in arid regions.

1.12.5 BREEDING ONLY FOR YIELD POTENTIAL.

Richards (1993) suggests breeding not for salt tolerance, but rather for yield potential. Because of the patchiness of salt-affected soil, he proposes to plant the highest yielding cultivar, take the yield from the areas with lower salt concentrations and accept the losses in potential yield that might be associated with areas of too high salt concentration.

1.13 *THINOPYRUM*

The use of wild relatives of wheat for its improvement has been emphasised by Cauderon (1979), Comecau *et al.* (1985) and Fedak (1985). Of all perennial Triticeae, the species of *Thinopyrum* have been used the most widely by wheat breeders (Dewey, 1984). Tsitsin, Vakar and Verushkine made the first hybrids between wheat and *Thinopyrum* in the early 1930's, later followed by several others (Armstrong, 1936; Vinall & Hein, 1937; White, 1940). *Thinopyrum intermedium* and *Thinopyrum ponticum* were used in most cases. Numerous crosses have been made since to transfer genes for disease resistance (e.g. leaf and stem rust), drought and salt tolerance and other characteristics from *Thinopyrum* to wheat.

Several *Thinopyrum* species have been shown to be salt tolerant, or at least to be able to survive for prolonged periods under salt stress (Gorham *et al.*, 1985; McGuire & Dvorak, 1981). Full or partial hybrids between these species and hexaploid wheat are morphologically similar to wheat and many show enhanced salt tolerance (Dvorak & Ross, 1986; Forster *et al.*, 1987; Gorham *et al.*, 1986).

1.13.1 TAXONOMY

Perennial grasses with one spikelet per node were taxonomically classified in the genus *Agropyron* P.Beauvois (Cauderon, 1966, 1979). However, genome analysis and cytogenetic studies led to newer classifications based on genomic relationships (Löve; 1982, 1984 and Dewey; 1984). Former *Agropyron* species were now classified according to the genomes they posses, e.g. *Critesion* (H), *Psathyrostachys* (N) and *Thinopyrum* (J). Löve (1980) assigned six species of the former *Agropyron junceum* (L.) B.P. complex to *Thinopyrum*, while Dewey (1984) also transferred some of the species of *Lophopyrum* (sensu Löve) and *Elytrigia* (sensu Löve) to *Thinopyrum*. *Thinopyrum* thus consists of 25 species in three specific complexes, each with sectional status.

The section *Thinopyrum* includes the species of the *Th. junceum* complex, among which are *Th. bessarabicum*, *Th. distichum* and *Th. junceum*. These are all maritime grasses growing on the shores of the Baltic Sea, Mediterranean Sea and North Sea, except for *Th. distichum*, which grows on the shores of the Cape Province, South Africa. The species are usually rhizomatous, their spikes have a fragile rachis and they are predominantly self-fertilising.

The section *Lophopyrum* consists of the species of the *Th. elongatum* complex e.g., *Th. ponticum* and *Th. caespitosum*. These species occur along the coast of the Mediterranean Sea, as well as inland saline sites in the Middle East and European Russia. They are caespitose and self- or cross-pollinating.

The third section, *Thrichorphorae*, is composed of species of the *Thinopyrum intermedium* complex, e.g. *Th gentryi*, *Th intermedium* and *Th. podperae*. They are usually rhizomatous and cross-pollinating.

1.13.2 GENOME COMPOSITION

Controversy exists about the E- and J-genomes of the *Thinopyrum* sections. Jauhar (1988, 1990) was of the opinion that the genome of *Th. elongatum* is sufficiently different from the J genome to warrant its classification as the E genome. Observations by Cauderon & Saigne (1961), Pienaar *et al* (1988) Pienaar, (1990) and Dvorak (1981) showed that the J and E genomes are closely related, thus supporting the merging of the two into one genus. According to Dewey, the genus *Thinopyrum* includes three diploid species, ten segmental allotetraploids 2n = 28, nine segmental allohexaploids 2n = 42, two complex segmental octoploids 2n = 56 and a complex decaploid 2n = 70.

The genus *Thinopyrum* (derived from the Greek words meaning 'shore' and 'wheat') is based on the J genome (Löve, 1982). The J genome designation was first applied by Ostergren (1940) to designate the genomes of *Th. junceiforme* as J_1 and J_2 based on their autosyndetic pairing in a *Th. junceiformei* / *Elytrigia repens* hybrid. Karyotype analysis in various *Thinopyrum* species revealed that the chromosomes differed in length from approximately 7 – 10 μm.

1.13.3 *THINOPYRUM DISTICHUM*

Thinopyrum distichum, a tetraploid member of the *juncea* complex, is indigenous to South Africa. This species grows on the eastern, southern and western shores of the Cape Province. It is a hardy, slow-growing perennial that is well adapted to the highly saline and infertile coastal sand dunes (Pienaar, 1990). It proliferates predominantly through rhizomatous growth, but also reproduce sexually through self-fertilisation and produces few poorly developed seeds on a relatively large spike.

The two genomes of *Th. distichum* are designated J^d_1 and J^d_2. Pienaar (1981, 1983) found that hybrids from crosses of tetraploid and hexaploid wheat with *Th. distichum* had 4.81^{II} and 4.65^{II} per pollen mother cell (PMC), respectively. This suggested that the J_1^d and J_2^d genomes of *Th. distichum* are less related to each other than the J_1 and J_2 genomes of *Th. junceiforme*.

Two intrageneric crosses of *Th. distichum* with *Th. elongatum* (Host) D.R. Dewey and *Th. junceiforme* provided cytological evidence linking the genomes of *Th. distichum* with the J genome, proving that *Th. distichum* belongs to the same genus as *Th. junceiforme* and *Th. elongatum* (Pienaar, 1981, 1983; Pienaar *et al.*, 1988). The *Th. distichum* genome is closely related to the genomes of *Thinopyrum elongatum* (Host) Löve ($E^e = J^e$) and *Thinopyrum bessarabicum* (Savul & Rayss) Löve ($E^e = J^e$). Hybrids involving *Th. distichum*, *Th. elongatum* and *Triticum durum* had a new third genome which consisted of partial J^d and J^e genomes (Pienaar *et al.,* 1988), the J^d chromosomes thus easily substituting for the homoeologous J^e chromosomes and *vice versa*.

The J^e_1 genome of *Th. elongatum* appeared to be more closely related to the two genomes of *Th. distichum* than to the J_1 and J_2 genomes of *Th. junceiforme*. Kosina & Heslop-Harrison (1996) found that genomic DNA probes from *Th. distichum* and *Thinopyrum* (*Lophopyrum*) *elongatum* could not discriminate between the two wheatgrass genomes, J and E ($E = J^e$), in a trigeneric hybrid amphiploid involving *Triticum durum*, *Th. distichum* and *Th. elongatum*, thus confirming their close similarity.

Cytological information concerning the relationship between the J genome and the A, B & D genomes were obtained by studying meioses in hybrids between *Triticum* and *Thinopyrum*. A complex hybrid, *T. durum / Th. distichum // T. durum /3/ T. aestivum* (genome = $AABBDJ^d$) only revealed 11.52^I in stead of the expected 14^I. An average of 12.87^{II}, 1.52^{III} and 0.05^{IV} per PMC was

found (Pienaar *et al.*, 1988). The multivalents were presumably the result of pairing between the J^d genome and the A, B and D genomes and indicate a low level of homoeology. If this is the situation, it will be possible to transfer genes from *Th. distichum* to *Triticum* by chromosome recombination. Genes or genetic systems that enhance homoeologous pairing would improve the process.

Homoeology was shown between the J_1^e genome of *Th. elongatum* and the A and B genomes in *T. durum* (AABB) / *Th. elongatum* ($J_1^eJ_1^e$) allotriploid hybrids (ABJ_1^e) with an average of 2.6^{II} per PMC (Jenkins & Mochizuki, 1975). It was found that five of the seven *Th. elongatum* chromosomes show definitive homoeology with chromosomes of *Triticum*. In a *Th elongatum / Ae. squarrosa* hybrid of Dvorak (1971), up to 5^{II} were found per PMC, which indicate a better homoeology between the D genome and the J_1^e genome than between the J_1^e and A or B genomes. It is therefore possible to integrate genes of the J_1^e genome into *Triticum* genomes. The difference in pairing between homoeologous chromosomes of the J_1 and J_1^e genomes and the wheat genomes is the result of the presence or absence of genes that suppress or enhance homoeologous pairing (Dvorak, 1987).

Both C-banding patterns and meiotic configurations in the pollen mother cells in F_1 hybrids between *Th. distichum* (Thunb.) Löve and *T. turgidum* var. group *durum* (Morris & Sears, 1967) demonstrated that *Th. distichum* is a segmental allopolyploid. Karyotype analysis of *Th. distichum* showed that each chromosome pair could be identified on the basis of size and banding pattern (Liu & Wang, 1993; Littlejohn & Pienaar, 1994a). Littlejohn and Pienaar grouped the chromosomes into 14 pairs and two genomes based on the presence of many telomeric and interstitial bands. *Th. distichum* has five metacentric chromosome pairs, seven submetacentric and two heterobrachial chromosome pairs. Two of the chromosomes carry NOR loci. Restriction fragment length polymorphism analysis (RFLP), C-banding and *in situ* hybridisation could readily be used to identify *Th. distichum* chromosomes in addition lines (Fominaya *et al.*, 1997). Chromosome 5J, which corresponds to C-banded karyotype chromosome IV, has been shown to have a lower transmission rate than expected (Armstrong *et al.*, 1992) and it probably affects the functionality of the derived genome in the partial amphiploid. Further studies are required to assign the *Th. distichum* chromosomes to homoeologous groups. It is likely that mixed homoeology exists in these addition chromosomes (Littlejohn & Pienaar, 1994b), similar to the situation in *Th. elongatum* (Tuleen & Hart, 1988) and *Th. ponticum* (Podp.) Liu & Wang (Kim *et al.*, 1993).

1.14 TRITICALE

The fodder crop triticale (x *Triticosecale Wittmack*) is the first successful artificial species that was produced through intergeneric hybridisation (*Triticum* x *Secale*). The objective behind the development of triticale was to combine the hardiness of rye with the good breadmaking properties of wheat, thus increasing total grain production by extending the area of cereal cultivation. In a comparison of wheat and triticale cultivars, the yield of triticale was found to be comparable with those of wheat and rye controls under certain conditions (Ford *et al.*, 1984; Kempton *et al.*, 1986).

The first wheat-rye hybrid plants were reported by A.S. Wilson in 1876, but the plants were completely sterile. The first fertile sectors in spikes, which arose by spontaneous chromosome doubling, were only reported in 1891 by W. Rimpau. Initially wheat x rye crosses were restricted to combinations of hexaploid wheat, predominantly *T. aestivum* with diploid rye (RR). Later *T. durum* was also crossed successfully with rye. Hybridisation work has been greatly expanded following the use of colchicine for chromosome doubling and the introduction of techniques for culturing embryos on artificial media (O'Mara, 1948).

The first published name for the amphiploid between *Triticum* and *Secale* was *Triticosecale Wittmack* (Wittmack, 1899), while triticale is the unofficial name given by Muntzing (Muntzing, 1936). Three types of triticale can be distinguished;

(a) Primary triticales arising directly from the doubling of the chromosome complement of a wheat- rye cross.

(b) Secondary triticales derived from hybrids between triticales at the same or different ploidy level.

(c) Substitutional triticales in which chromosomes from genomes other than A, B or R (most frequently the D genome) have been substituted for chromosomes in the normal hexaploid triticale karyotype

Triticales at the tetraploid, hexaploid, octoploid and decaploid levels have been developed. Muntzing created variation by intercrossing triticales of different ploidy levels or by hybridising them with bread wheats. The hexaploid forms proved to be the most successful as an agricultural crop.

Tetraploid triticales have been synthesized directly from crosses between diploid rye and diploid wheat (Sodkiewics, 1984), but are extremely difficult to make and propagate because of low crossability and male sterility. The most successful primary tetraploid triticales are obtained from crosses of hexaploid triticale with diploid rye (Krolow, 1975). Such triticales have a complete rye genome and a mixed wheat genome of A and B chromosomes. Hexaploid triticales are synthesized by pollinating tetraploid wheat (usually *T. turgidum* var. durum) with rye and using embryo rescue to obtain F_1 plants. Colchicine treatment is used to double the chromosome number of the allohaploid F_1. Alternatively, tetraploid wheat and diploid rye may be treated with colchicine to double their chromosome numbers before hybridisation. Primary triticales tend to be tall and they show partial sterility and poor endosperm development. The problem may be avoided by making crosses among primary triticales, followed by a number of generations of selfing and selection (Rupert *et al.*, 1973; Gustafson *et al.*, 1985). The substitution of chromosomes in the rye genome with wheat chromosomes led to improved characteristics such as yield and grain quality (Gustafson & Zillingsky, 1973).

Wheat and its wild relatives evolved from a diploid primeval genome of seven chromosomes, which became modified during speciation and polyploidisation. A degree of genetic similarity, or homoeology has been retained between the chromosomes of the different genomes (A, B, D and others in alien species). Hexaploid bread wheat has two characteristics that facilitate the transfer of genes from related species. Firstly, bread wheat can tolerate the loss or addition of whole chromosomes without affecting the viability of the plant. Secondly, duplicate genes present in the other two homoeologous genomes often mask the effects of deleterious genes present on an alien chromosome substituted for a given wheat chromosome in the third genome (Sears, 1981; Riley & Law, 1984).

1.15 MOLECULAR MARKER ANALYSIS

1.15.1 AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS (AFLPs)

The use of amplified fragment length polymorphisms (AFLPs) was originally conceived to allow the construction of very high density DNA marker maps for application in genome research and positional cloning of genes (Zabeau & Vos, 1993). The AFLP technique is based on the selective amplification of restriction fragments from total, digested genomic DNA. The technique involves three steps: (1) restriction of the genomic DNA and ligation of oligonucleotide adapters, (2) selective amplification of a subset of all the fragments in the total digest, and (3) gel-based analysis of the amplified fragments.

The genomic DNA is digested with two restriction enzymes, one a frequent cutter (for example *MseI*), the other a rare cutter (for example *EcoRI*). Three different types of fragments are formed, *EcoRI-EcoRI* fragments, *EcoRI-MseI* fragments and *MseI-MseI* fragments. Two specific adapters, an *EcoRI*- and a *MseI* adaptor, are ligated to the restriction sites to prevent reannealing and to serve as "universal" primer binding sites for PCR (Vos *et al.*, 1995). A preselective PCR amplification is performed to increase the number of fragments for the selective PCR to follow. The sequence of the primers used is complementary to the adapter and the restriction site of the fragments. These preselective primers may also have an added selective nucleotide that will recognise a subset of restriction fragments having the complementary nucleotide downstream from the restriction site. The products of the preselective amplification are those fragments having one *MseI* and one *EcoRI* cut as well as the complementary internal nucleotide.

The product of the preselective PCR serves as template for a selective PCR reaction. Primers are radio-actively or fluorescently labelled during the PCR reaction and resolved by gel electrophoresis. Because only the one primer is labelled, only fragments containing that specific primer are detected on the gel. This ensures unambiguous detection of the single-strand amplified fragments in denaturing gels (Perkin-Elmer, 1995).

The AFLP technique is a random fingerprinting technique that may be applied to DNA of any origin or complexity. The method is easily standardised, readily automated, robust, reliable and reproducible. No prior sequence characterisation of the target genome is required. It is a cost-effective way to generate high-density genetic maps for marker assisted selection of desirable traits.

1.15.2 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

The technique was developed simultaneously in two laboratories (Welsh & McClelland, 1990; Williams *et al.*, 1990). The analysis is based on the amplification of genomic DNA using one short oligonucleotide primer under low stringency conditions and it results in the amplification of multiple products from loci distributed throughout the genome.

Denatured genomic DNA serves as template DNA. The primers (8-10 bases long) anneal to complementary sequences. Where primers bind to opposite strands close enough to another, the area between the primers is amplified in a polymerase chain reaction (PCR). These fragments are amplified in subsequent reactions. Amplified fragments are detected on agarose gels after ethidium bromide staining.

The advantages of the RAPD technique are that no prior sequence information are required and only a small amount of DNA are required for analysis (Kochert, 1994). The disadvantages are, however, that of reproducibility and the dominant nature of RAPD markers, which prevent distinction between homozygotes and heterozygotes (Reiter *et al.*, 1992).

1.15.3 DNA AMPLIFICATION FINGERPRINTING (DAF)

The technique was first demonstrated by Caetano-Anolles *et al.* in 1991. DAF analysis makes use of very short GC rich primers, as short as five-mer, but more typically seven-eight-mer long. A PCR amplification, similar as for RAPDs, is used to amplify genomic DNA fragments. The DAF products are separated on a polyacrylamide gel. Silver staining of the gels reveals DNA amplification profiles with high resolution.

DAF primers referred to as mini hairpin primers are oligonucleotides folded back on themselves to form hairpins. This makes it possible to have less than five selective nucleotides. Such primers have been reported to be superior to linear DAF primers (Caetano-Anolles & Gresshoff, 1996).

DAF analysis provides a more complex DNA profile than RAPD analysis and the polyacrylamide gel separation provides a higher resolution in band detection. It can be applied to a wide variety of organisms including humans, bacteria, plants and fungi (Caetano-Anolles *et al.*, 1995; Gresshoff *et al.*, 1997).

AIMS OF THE STUDY

The impact of salinity on crop production is becoming increasingly important, especially where existence farming is practised. It is thus necessary to develop salt tolerant cultivars adapted to local environments. Wide hybridisation was used in an attempt to transfer the salt tolerance from the indigenous salt tolerant wheatgrass, *Thinopyrum distichum*, to triticale, a fodder crop. Ears of *Thinopyrum distichum* were pollinated with pollen from various hexaploid triticale genotypes. Following colchicine treatment, an allopentaploid clone was recovered. Florets on ears of this plant were pollinated with pollen from various triticale genotypes to establish the B_1F_1. Ears of the triticale cultivar Rex were subsequently pollinated with pollen from the B_1F_1. The twenty-two B_2F_1 lines were allowed to self fertilise in order to establish B_2F_2 seeds with various *Thinopyrum* chromosome additions (Marais & Marais, 1998).

The transfer of *Thinopyrum* chromosomes or chromosome segments with genes for salt tolerance to triticale would benefit from the availability of molecular markers for the appropriate chromosomes. The main aim of the study was to use bulked segregant analysis (BSA) and monosomic or disomic addition lines, in combination with molecular marker techniques, AFLP, RAPD and DAF, to identify markers for *Thinopyrum* chromosomes contributing to salt tolerance.

Thinopyrum distichum can also be an important source of other adaptational genes for improving cultivated cereals. In order to facilitate the identification and transfer of such genes to cultivated cereals, it is essential to establish a full set of addition lines of *Thinopyrum* chromosomes to the triticale background. Molecular markers were used to group monosomic and disomic addition lines according to the specific *Thinopyrum* chromosome they retained.

CHAPTER 2

MATERIALS AND METHODS

2.1 PLANT MATERIAL

During the spring of 1995, ears of *Thinopyrum distichum* were pollinated with pollen from various hexaploid triticale genotypes and hybrid embryos could be rescued (Marais & Marais, 1998). The allopentaploid F_1 (n = 35, $J_1^dJ_2^d$ABR; *Thinopyrum* cytoplasm) grew vigorously and was highly salt tolerant. Following colchicine treatment, a clone (96M2-2) with 70 chromosomes was recovered. Florets on ears of this plant were cut open and it was placed among flowering plants of various triticale genotypes. B_1F_1 plants with 54-55 chromosomes were obtained which were near octoploids having the genomes AABBRR$J_1^dJ_2^d$. Ears of the triticale cultivar Rex were subsequently pollinated with pollen from the B_1F_1. The B_2F_1 (triticale cytoplasm) had chromosome numbers ranging from 45 to 49. Twenty-two B_2F_1 plants matured and set seed. At the onset of the study in 1998, B_2F_2 seeds of each family were available (Figure 2.1).

The B_2F_2 seeds of the 22 families and their B_2F_3 progeny were used as source material for the following (outlined in Figure 2.2):

i) Attempt to identify molecular markers for *Thinopyrum distichum* chromosomes associated with salt tolerance. Firstly, DNA was extracted from B_2F_2 plants, which were also evaluated for their salt tolerance. The salt tolerance data was used to compose salt sensitive and salt tolerant bulks. Secondly, monosomic and disomic addition B_2F_2 plants (43 or 44 chromosomes) were identified and their salt tolerance evaluated. The various bulks and salt tolerant addition plants were characterised for AFLP, RAPD and DAF polymorphisms in an attempt to find markers associated with a chromosome(s) conditioning salt tolerance.

ii) Attempt to identify diverse single chromosome addition plants having complete or near complete triticale genomes plus an additional random *Thinopyrum* chromosome. For this purpose, plants with 2n = 43/44 were identified and characterised for molecular markers (AFLP and RAPD). Cluster analysis was used to group the putative monosomic or disomic addition plants according to the specific *Thinopyrum* chromosomes they retained.

Figure 2.1 Derivation of triticale / *Thinopyrum distichum* amphiploids and backcross program.

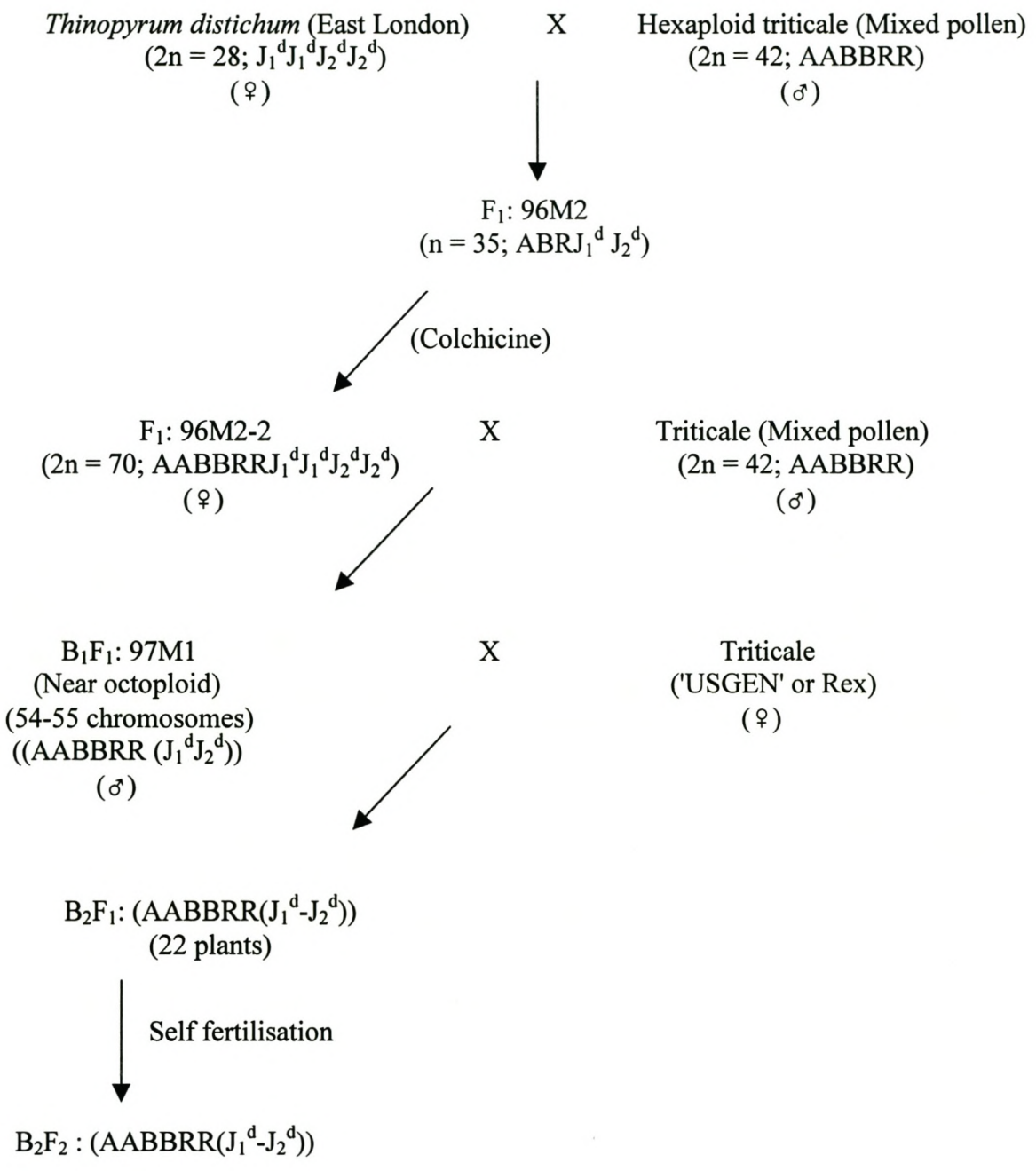
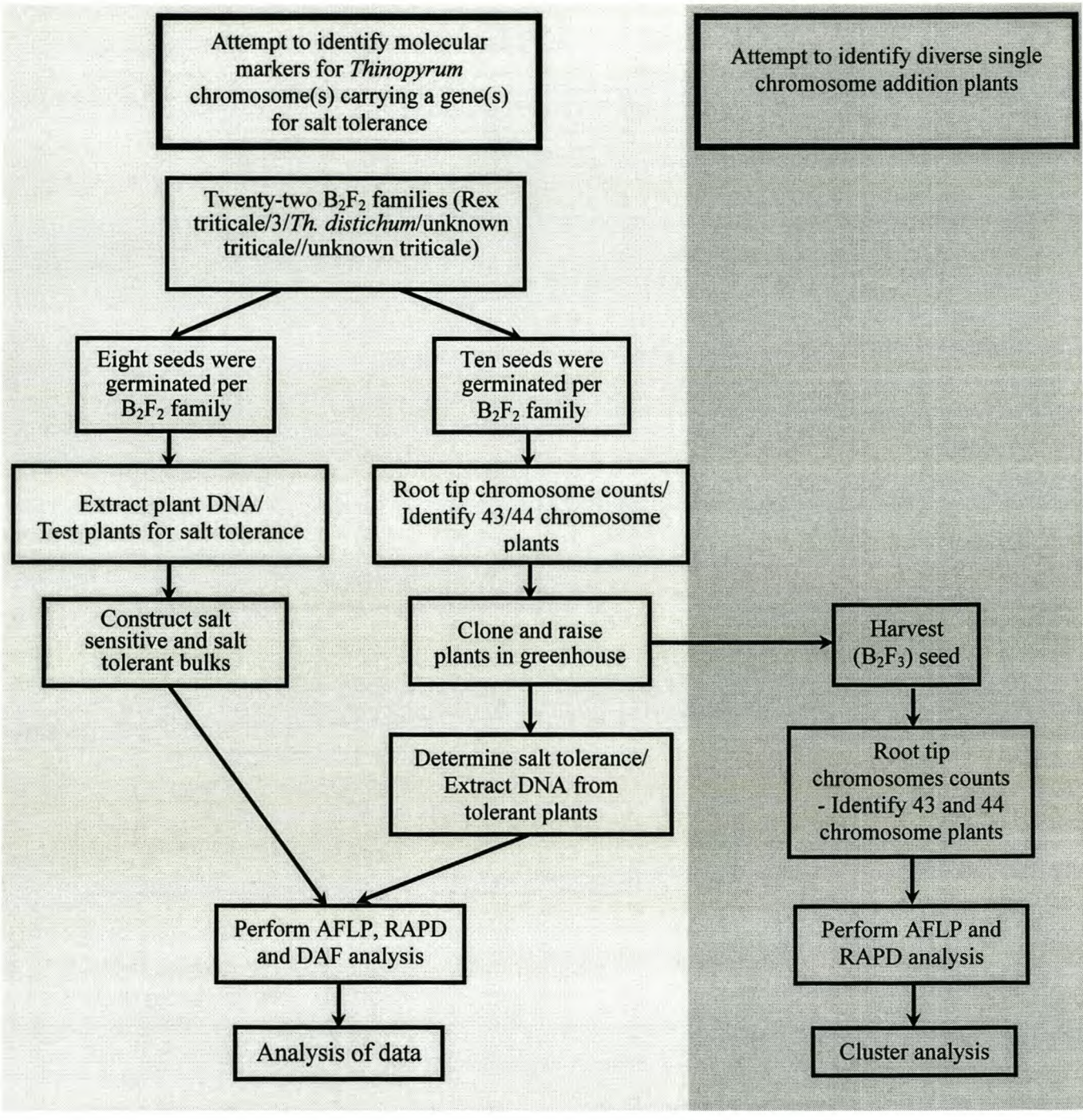


Figure 2.2 Outline of experimental approach.



2.2 IDENTIFICATION OF MOLECULAR MARKERS FOR *THINOPYRUM* CHROMOSOMES CONTRIBUTING TO SALT TOLERANCE.

2.2.1 EVALUATION OF THE B_2F_2 PROGENY FOR SALT TOLERANCE

A preliminary evaluation was made to determine whether the salt tolerance test would satisfactorily distinguish among tolerant and sensitive plants and to assess the variation for salt tolerance in the material. For this purpose, six seeds of each of the 22 B_2F_2 families (132 in total), with Rex and Henoch serving as salt tolerant and salt sensitive controls, respectively, were used.

2.2.1.1 Germination and establishment of plants for salt tolerance tests

Seeds were germinated on moistened Whatman filter paper circles in Petri dishes. The Petri dishes were placed in plastic bags to prevent drying out. Filter papers were periodically remoistened when dry. Petri dishes were incubated for 24 hours at 21°C and then transferred to a refrigerator at 2 - 4°C for 24 hours. Petri dishes were then returned to the incubator at 21°C to complete germination.

Seedlings were planted around the perimeter of a 2 λ (210 mm height) pot filled ¾ with coarse river sand. One seedling of a susceptible control (Henoch rye) and tolerant controls (Rex triticale and the *Thinopyrum*/ triticale B_1F_1) were planted in the middle of the pot. When the plants reached the tillering stage, they were tested for their salt tolerance.

2.2.1.2 Salt tolerance test

Seedlings were allowed to grow to the early tillering stage before the start of the experiment. Plants were kept in a growth cabinet with temperature maintained at 22-25°C and a 14h/10h daylight/night duration. The salt treatment involved the linear increase of the electrical conductivity (EC) of the nutrient solution (per 100 λ H_2O: Kynoch Sol-u-fert (164 g), Microplex (2 g), Calcium Nitrate (77 ml of a 180 $g\lambda^{-1}$ Ca and 125 $g\lambda^{-1}$ N solution)) by 4 dSm^{-1} on alternative days by dissolving equal amounts of NaCl and $CaCl_2$ (Table 2.1). Two litres of the appropriate salt solution were added to each pot to displace the previous solution. The EC was raised to 34 dSm^{-1} (seawater is known to have an EC of approximately 48 dSm^{-1}) and maintained at this level

till the salt sensitive plants started to die. Water levels were frequently readjusted to compensate for evapotranspiration. Rex and Henoch were also grown in normal nutrient solution as a control.

Plants were scored after the salt sensitive control plants died. The plant's appearance compared to the controls was noted and a score was given according to an empirical scale (Table 2.2). The salt sensitive Henoch rye was allotted a two on the salt tolerance scale and Rex a five.

Table 2.1 To determine the salt tolerance of plants, the electrical conductivity of the nutrient solution was increased on alternative days by the addition of equal amounts of NaCl and $CaCl_2$.

Day	EC (dS/m) (Approx.)	Weight (NaCl & $CaCl_2$ each) (g / 20 λ)
1	1.8	0
3	6	20
5	10	45
7	14	68
9	18	96
11	22	123
13	26	153
15	30	185
19	34	216
26	34	216
33	34	216
40	34	216
47	34	216

Table 2.2 Empirical scale of salt tolerance employed to evaluate a plant's tolerance

Number	Description
1 (Very Sensitive)	
2	Like Henoch
3 (Sensitive)	
4	
5 (Tolerant)	Like Rex
6	
7 (Very tolerant)	
8	Like 96M2 (primary *Th. distichum* / triticale hybrid)
9 (Extremely tolerant)	Like *Thinopyrum distichum*

2.2.1.3 DNA Extractions

Plant DNA extractions were done according to a protocol slightly adapted from Doyle & Doyle (1990) and Sambrook *et al.* (1989). The protocol was as follows: one gram of fresh leaf tissue was ground in 10 ml of 2% (w/v) CTAB isolation buffer (1.4M NaCl, 20 mM EDTA (pH 8), 100 mM Tris-HCl (pH 8) and 0.2% (v/v) BME) at 60°C in a mortar and pestle. The samples were incubated for 60 min at 60°C. After precipitation of the nucleic acids in isopropanol, the samples were incubated at -20°C for 1 hour. Samples were then centrifuged for 5 min at 5 000 rpm at a temperature of 10 - 25°C. The supernatant was carefully poured off and the pellet washed in wash

buffer (76% ethanol; 10 mM ammonium acetate). The Doyle & Doyle protocol was supplemented by a phenol: chloroform extraction protocol according to Sambrook *et al.* (1989). After the pellet was air-dried and suspended in 1ml of distilled water, the samples were transferred to 2.2 ml microfuge tubes. Five hundred µl of phenol and 500 µl chloroform:isoamyl alcohol (24:1; v:v) were added, mixed and centrifuged for 10 min at 12 000 rpm in a microfuge. Supernatant was transferred to a clean 2.2 ml microfuge tube. One millilitre of chloroform:isoamyl alcohol (24:1; v:v) was added, mixed and centrifuged as above. The supernatant of each sample was divided into two clean 2.2 ml microfuge tubes. Precipitation of DNA was done with 1/5 the volume 10 mM ammonium acetate and 2 volumes of 100% ethanol. Samples were mixed and centrifuged for 35 min at 12 000 rpm at a temperature of 4°C. The supernatant was drained off and 1 ml of 70% ethanol added. The samples were centrifuged for 30 min at 12 000 rpm at a temperature of 4°C. This step was repeated. Pellets were dried for 30 min in a 37°C oven and resuspended in 1 ml distilled water.

2.2.1.4 Quantification of genomic DNA

Concentrations of DNA extractions were determined on a 0.6% agarose gel in a 1 x TBE loading buffer. One µl of the sample DNA, 9 µl H_2O and 10 µl Ficoll Orange G were loaded, together with lambda DNA of standard concentrations (1.0, 3.0 and 5.0 µg/µl). Gels were run for 1 hour at 60V. Ethidium bromide (EtBr) was added to the agarose gel and bands detected under UV light. Concentrations were estimated by comparing band sizes and intensity to those of the lambda control. Genomic DNA sample concentrations were also determined with a Hoefer DynaQuant 200 Fluorometer as described in the manual.

2.2.2 CONSTRUCTION OF SALT TOLERANT AND SALT SENSITIVE BULKS FOR BULKED SEGREGANT ANALYSIS (BSA)

One-hundred-and-seventy-six B_2F_2 seeds were subsequently germinated to establish the plants used in the bulks (eight seeds of each of the 22 parental lines). Rex and Henoch seeds were simultaneously germinated to serve as salt tolerant and sensitive controls. Clones of the *Thinopyrum* / triticale alloploid (96M2-2-2) were included as a highly salt tolerant control. Before the onset of the salt tolerance test, DNA extractions were made of a random selection of the plants. This ensured that DNA from plants that would die due to the salt test would be available for making salt sensitive bulks. Following the completion of the salt test, DNA extractions were also

made of the plants that survived and of which DNA extracts have not been made previously. Four plants, allocated the same score, were grouped in a bulk. A total of 20 bulks were made of highly salt tolerant, moderately tolerant and salt sensitive plants (Table 2.3). DNA concentrations were determined on 0.6% agarose gels. Volumes containing 1 μg of DNA of each of the four plants were pooled and diluted to a concentration of 250 ng/μl. These were used to conduct RAPD, DAF and AFLP analyses.

Table 2.3 List of 20 bulks constructed and the salt tolerance of the plants represented in each bulk.

Bulk nr.	Salt tolerance	Bulk nr.	Salt tolerance
1	7--8	11	5
2	6--7	12	5
3	6--7	13	5
4	6--7	14	5
5	6--7	15	4
6	6	16	4
7	6	17	4
8	6	18	4
9	6	19	4
10	5	20	2

2.2.3 DERIVATION OF PUTATIVE MONOSOMIC OR DISOMIC ADDITION PLANTS AND THEIR CHARACTERISATION FOR SALT TOLERANCE

A further 220 B_2F_2 seeds were germinated in order to determine their chromosome number. Following germination (as described in section 2.2.1.1), two or more root tips of each seed were cut before the numbered seeds were transferred to pots.

2.2.3.1 Cutting root tips for chromosome counts

Roots from germinated seeds were cut when 1 - 2 cm in length between 08h00 and 10h30. Two or three roots were cut close to the kernel with clean scissors and placed in clean, numbered, 18 mm x 50 mm vials containing cold, double distilled water. Seedlings were placed in numbered compartments of a Sterilin dish containing moist filter paper. These were stored in a refrigerator at 4°C till planted. Vials with roots were placed on crushed ice in a foam box and kept for 29 hours in a refrigerator at 4°C.

2.2.3.2 Root fixation and staining

The water was drained from the vials and replaced with fixative (3 parts methanol : 1 part propionic acid). The roots were left for at least two days at room temperature. The fixative was drained off and replaced with distilled water for 30 min. Vials containing 1N HCl were heated in heating blocks to 60°C. The roots were transferred to these vials for 8 min. The roots were washed with distilled water for 2 min to stop hydrolysis. The water was drained and replaced with leuco-basic fuchsin (made according to Darlington and La Cour, 1960). This was placed in the refrigerator (4°C) for at least 2 hours. The leuco-basic fuchsin was drained and the roots rinsed twice with distilled water. Roots were rinsed with pH 4,5 sodium acetate buffer (3,16 g sodium acetate and 3,47 ml glacial acetic acid in 1 λ distilled water; pH adjusted to 4,5). The buffer was replaced with 2½ % pecticlear solution (0,5 g pecticlear from Serevac in 20 ml of above pH 4,5 buffer solution) and incubated for 35 min at 37°C. The pecticlear solution was drained and the roots stored in distilled water in a refrigerator (4°C) till chromosome counting.

2.2.3.3 Mounting

Root tips were cut off on a clean slide and placed in a drop of Rosner 1% aceto-carmine (Preparing 1% aceto-carmine :- 1 g carmine is added to 55 ml boiling distilled water. It is mixed well, cooled to 50°C and 45 ml of glacial acetic acid is added. The solution is slowly boiled in a reflux condensor (boiling stones) for 4 hours and filtered when cold. Root tips were cut and a cover slip placed on top. A filter or blotting paper was placed over the cover slip and firmly pressed with rolling action of the thumb to squash the material. Chromosomes were counted under an oil-immersion lens of the microscope using a green filter. Seedlings with 43 or 44 chromosomes were planted in a greenhouse.

2.2.3.4 Salt tolerance of the putative monosomic addition lines

Two clones of each plant were planted in one pot and their salt tolerance determined. A third clone was kept to retrieve seed from. As controls, two Henoch rye, two Rex and two clones of the primary *Thinopyrum* / triticale alloploids were planted in separate pots. Plants were evaluated for their salt tolerance (described in section 2.2.1.2).

2.3 MOLECULAR MARKER ANALYSES

2.3.1 AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) ANALYSIS

2.3.1.1 Primers

Primers were obtained from GIBCO / brl. The primers used and their sequences are listed in Table 2.4. A total of 25 primer combinations were used, (a) to screen for polymorphisms between salt tolerant and salt sensitive bulks, individual salt tolerant B_2F_2 plants with 43/44 chromosomes and, (b) to group the putative addition lines according to the specific *Thinopyrum* chromosome they retained.

2.3.1.2 Amplification

AFLPs were performed as described by Zabeau & Vos (1993) and adapted by Donini *et al.* (1997). Template DNA (500 ng) was restricted with 5 U *Sse*8387I (Pharmacia) and 5 U *Mse* (New England Biolabs) in a volume of 40 µl. The mix was incubated for 3 hours at 37°C before ligation of 5 pmol of *Sse* adapter and 5 pmol of *Mse* adapter. Samples were incubated overnight at 37°C. The bead purification step was omitted.

A two step amplification was done using a Hybaid Omnigene Cycler. A 1:9 dilution in 1 x $TE_{0.1}$ of the pre-amplification product was amplified using *Sse* + 0 and *Mse* + 0 primers. After a 10 x dilution of the PCR product, 5 µl of this solution was used for the second round of PCR using *Mse* + 2 or *Mse* + 3 and *Sse* + 2 or *Sse* + 3 oligomers. The *Sse* + 2 and *Sse* + 3 primers were end labelled with [γ - ^{33}P]ATP (Vos *et al.*, 1995). The primer combinations used are listed in Table 2.4. Denatured, labelled PCR product (5 µl) was loaded on a 6% polyacrylamide denaturing gel (6M urea in 1 x TBE buffer) and electrophoresed in 1 x TBE buffer at 80 W for approximately 2 hours. The gels were dried on Whatman 3MM paper for 1½ hours at 80°C and exposed to Biomax MR films (Kodak) for approximately 4 days (the duration of exposure were extended as the activity of the radio-active isotope decreased).

Table 2.4: *Sse* and *Mse* primer combinations applied in AFLP analysis in an attempt to group putative monosomic addition lines and to identify molecular markers associated with the salt tolerance gene(s) in *Th. distichum*.

	S12 - AC	S13 - AG	S27 – AGA
M11 - AA	✓	✓	✓
M12 - AC	✓	✓	✓
M13 - AG	✓	✓	✓
M14 - AT		✓	✓
M15 - CA		✓	✓
M16 - CC		✓	✓
M17 - CG		✓	✓
M32 - AAC		✓	✓
M53 - CCG		✓	✓
M64 - GAC		✓	✓
M74 - AAC		✓	✓

2.3.2 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

2.3.2.1 Primers

Oligonucleotides are available from Operon Technologies ™ (Alimede, California) and are distributed in primer kits containing 20 primers each. The 80 primers used and their sequences are listed in Table 2.5. Duplicate extracts of each of *Thinopyrum distichum* and Rex (made at separate occasions) were used to generate parental profiles with each of the primers in order to identify polymorphic bands. The duplication of each parental DNA profile provided a measure of repeatability, while a negative control determined whether contamination occurred. Primers generating polymorphic bands were used (a) to screen for polymorphisms between salt tolerant and salt sensitive bulks, individual salt tolerant B_2F_2 plants with 43/44 chromosomes and, (b) to group the putative addition lines according to the specific *Thinopyrum* chromosome they retained.

2.3.2.2 Amplification

RAPD reactions were performed in 0.5 ml thin-walled PCR reaction tubes. Ten nanograms of template genomic DNA and 10 pmol of primer were used in a 25 µl reaction mix.

Buffer (Advanced Biotechnologies, London) was added to a final concentration of 10% of the reaction volume (2,5 µl). The buffer contained 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C) and 0.1% Tween-20. In addition, 0.2 mM of each deoxynucleotidephosphate (dNTP), 2.5 mM $MgCl_2$ and 0.5 U of AB *Taq* DNA polymerase (Advanced Biotechnologies, London) were used in the reaction mix. The cycle program for amplification was as follows: 1 min at 94°C, 60 sec at 94°C, 20 sec at 35°C and 60 sec at 72°C for 45 cycles, ending in a 10 min cycle at 72°C to complete any incomplete fragments synthesised during the reaction.

2.3.2.3 Profiling

After completion of the cycle program, 15 µl of the RAPD amplification product and 6.0 µl "blue" loading Buffer type IV (0.25% bromophenol blue and 40% (W/V) sucrose on water - Sambrook *et al.*, 1989) were loaded on a 1.4% LE agarose gel. The running buffer consisted of 0.5 x TBE (1 x TBE: 0.089M Tris-borate, 0.089M boric acid, 0.002M EDTA) and ethidium bromide (10 µl/dm^3). The gels were run at 100 Volts for 3 to 3½ hours. Visualisation of the RAPD products were done using an ultraviolet transilluminator (UVP Inc.) and results documented by means of thermal printing. Visualisation was also done using a Biorad Gel Doc 1000. Polymorphisms were identified and scored either as the presence or absence of a specific amplification product.

Table 2.5 Sequences of the 80 Operon primers used.

Primer Code		Primer Code	
Primer Kit OPA	**Sequence 5' to 3'**	**Primer Kit OPK**	**Sequence 5' to 3'**
OPA 1	CAGGCCCTTC	OPK 1	CATTCGAGCC
OPA 2	TGCCGAGCTG	OPK 2	GTCTCCGCAA
OPA 3	AGTCAGCCAC	OPK 3	CCAGCTTAGG
OPA 4	AATCGGGCTG	OPK 4	CCGCCCAAAC
OPA 5	AGGGGTCTTG	OPK 5	TCTGTCGAGG
OPA 6	GGTCCCTGAC	OPK 6	CACCTTTCCC
OPA 7	GAAACGGGTG	OPK 7	AGCGAGCAAG
OPA 8	GTGACGTAGG	OPK 8	GAACACTGGG
OPA 9	GGGTAACOCC	OPK 9	CCCTACCGAC
OPA 10	GTGATCGCAG	OPK 10	GTGCAACGTO
OPA 11	CAATCGCCGT	OPK 11	AATGCCCCAG
OPA 12	TCGGCGATAG	OPK 12	TGGCCCTCAC
OPA 13	CAGCACCCAC	OPK 13	GGTTGTACCC
OPA 14	TCTGTGCTGG	OPK 14	CCCGCTACAC
OPA 15	TTCCGAACCC	OPK 15	CTCCTGCCAA
OPA 16	AGCCAGCGAA	OPK 16	GAGCGTCGAA
OPA 17	GACCGCTTGT	OPK 17	CCCAGCTGTG
OPA 18	AGGTGACCGT	OPK 18	CCTAGTCGAG
OPA 19	CAAACOTCGG	OPK 19	CACAGGCGGA
OPA 20	GTTGCGATCC	OPK 20	CTCTCGCGAG

Table 2.5 (continued)

Primer Code		Primer Code	
Primer Kit OPL	**Sequence 5' to 3'**	**Primer Kit OPY**	**Sequence 5' to 3'**
OPL 1	GGCATGACCT	OPY 1	GTGGCATCTC
OPL 2	TGGGCGTCAA	OPY 2	CATCGCCGCA
OPL 3	CCAGCAGCTT	OPY 3	ACAGCCTGCT
OPL 4	GACTGCACAC	OPY 4	GGCTGCAATG
OPL 5	ACGCAGGCAC	OPY 5	GGCTGCGACA
OPL 6	GAGGGAAGAG	OPY 6	AAGGCTCACC
OPL 7	AGGCGGGAAC	OPY 7	AGAGCCGTCA
OPL 8	AGCAGGTGGA	OPY 8	AGGCAGAGCA
OPL 9	TGCGAGAGTC	OPY 9	AGCAGCGCAC
OPL 10	TGGGAGATGG	OPY10	CAAACGTGGG
OPL 11	ACGATGAGCC	OPY 11	AGACGATGGG
OPL 12	GGGCGGTACT	OPY12	AAGCCTGCGA
OPL 13	ACCGCCTGCT	OPY 13	GGGTCTCGGT
OPL 14	GTGACAGGCT	OPY 14	GGTCGATCTG
OPL 15	AAGAGAGGGG	OPY 15	AGTCGCCCTT
OPL 16	AGGTTGCAGG	OPY16	GGGCCAATGT
OPL 17	AGCCTGAGCC	OPY17	GACGTGGTGA
OPL 18	ACCACCCACC	OPY 18	GTGGAGTCAG
OPL 19	GAGTGGTGAC	OPY 19	TGAGGGTCCC
OPL 20	TGGTGGACCA	OPY 20	AGCCGTGGAA

2.3.3 DNA AMPLIFICATION FINGERPRINTING (DAF) ANALYSIS

2.3.3.1 Primers

Primers were obtained from Integrated DNA Technologies (IDT), Inc. The hexamer primers used and their sequences are listed in Table 2.6. DAF analysis was only used to screen for polymorphisms between salt tolerant bulks, individual salt tolerant addition plants and salt sensitive bulks. The following were run on each gel: *Thinopyrum distichum*, Rex, Bulks 1 and 2 (most salt tolerant bulks), Bulks 19 and 20 (most salt sensitive bulks), 97M901-6.2, 97M901-21.6, 97M901-169, a negative control and a 100 bp ladder.

2.3.3.2 Amplification

DAF reactions were performed in 0.2 ml thin-wall PCR reaction tubes. Genomic DNA was diluted to 0.25 ng/µl of which 1 µl was used. A final concentration of 0.3 µM of primer was used in a 10 µl reaction mix. Stoffel fragment (AmpliTaq® DNA Polymerase) was added to a final concentration of 0.3 U/µl. In addition, 1 µl of a 10 x Stoffel buffer (100 mM Tris-HCl,

10 mM KCl, pH 8.3 at 25°C), 0.2 mM of each deoxyribonucleotidephosphate (dNTP) and 2.5 mM $MgCl_2$ were added to the reaction mix. The cycle program for amplification was as follows: 95°C for 30 s, 55°C for 2 min and 72°C for 30 s, repeated for 35 cycles.

2.3.3.3 Polyacrylamide gels

A 40% acrylamide stock solution was made containing 39.2 g acrylamide and 0.8 g N'N-bisacrylamide dissolved in 100 ml de-ionised distilled water. The 10% acrylamide working solution was made using 10 g urea, 25 ml of the 40% acrylamide stock solution, 20 ml of a 5 x TBE (1.78M Tris-HCl, 1.78M boric acid and 40 mM EDTA) and 5 ml glycerol, diluted to a final volume of 100 ml by adding de-ionised distilled water. To prepare a gel, 100 µl of ammonium persulphate solution (10%, w/v) and 10 µl N,N,N',N'-Tetramethylethylendiamine (TEMED) were added to 7.5 µl of the 10% acrylamide working stock. The gel mix was injected into the gel cassette (0.75 mm spacers and comb) with a syringe. Thirty minutes were allowed for the gel to set before it was placed in the electrophoresis apparatus. A 1 x TBE solution was used as buffer. Excess urea was flushed from the wells using a syringe. Gels were pre-electrophoresed at 200 V for 10 min. Wells were again flushed with buffer before samples were loaded (1 µl of sample DNA and 3 µl of loading buffer). Electrophoresis was done at 200 V for 1½ hours, until the slowest dye reached ± 1 cm from the bottom of the gel.

2.3.3.4 Silver staining

Gels were transferred to a small container and fixed for 30 min in 7.5% glacial acetic acid (Gelbond was not used). This was followed by three washing steps of 3 min each in distilled water at room temperature. Staining was done in 0.1% $AgNO_3$/0.056% formaldehyde for 45 min at 37°C, with silver solution pre-heated to 37°C. The gel was washed in distilled water to remove silver solution, but not for more than 10 seconds. Developing was in 3% Na_2CO_3 / 0.056% formaldehyde / 8 µM sodium thiosulphate (formaldehyde and sodium thiosulphate were added just before being used) at 8°C till bands in 100 basepair ladder were well developed. Developing was stopped in cold (4°C) 7.5% glacial acetic acid for 7 min and the gels rinsed in distilled water.

Table 2.6 DAF primer sequences used in molecular marker analysis.

Primer	Sequence
B13	5'-GCA GGC AC-3'
B14	5'-GCA GGC AT-3'
B15	5'-GCA GGC AG-3'
B16	5'-GCA GGC AA-3'
B17	5'-GCA GGT CC-3'
B25	5'-GCA GGT GC-3'
B27	5'-GCA GGT GG-3'
B28	5'-GCA GGT GA-3'
B30	5'-GCA GGT AT-3'
B46	5'-GCA GGG AT-3'

2.4 GROUPING OF PUTATIVE MONOSOMIC AND DISOMIC ADDITION PLANTS ACCORDING TO THE SPECIFIC *THINOPYRUM* CHROMOSOME THEY RETAINED

2.4.1 IDENTIFICATION OF MONOSOMIC AND DISOMIC ADDITION PLANTS

B_2F_2 plants described in section 2.2.2 were allowed to mature. The ears of plants with 43 or 44 chromosomes were covered with glassine bags to ensure self-fertilisation. Five B_2F_3 seeds of each B_2F_2 plant were germinated and chromosome counts made in an attempt to identify those with monosomic or disomic *Thinopyrum* addition chromosomes (43 or 44 chromosomes). DNA extractions were made of plants with 43 or 44 chromosomes (see Figure 2.1) and used in RAPD and AFLP marker analyses (described in section 2.3) in an attempt to group them according to the specific *Thinopyrum* chromosome they retained.

2.4.2 COLCHICINE TREATED PLANT

A B_2F_2 plant was identified with a chromosome number of 22 (probably a haploid having a single additional J^d chromosome). The plant was cloned and the clones treated with 0.1% colchicine solution containing 2% DMSO and 0.001% GA_3 (Valkoun *et al.*, 1990). The roots and shoots were trimmed and the plants were placed in the solution (covering the roots and plant base) for 8 hours at a temperature of 18°C. Roots were then rinsed and placed in water at 4°C overnight whereafter clones were planted in pots in a greenhouse at 18°C. The doubled haploid was included in the attempt to use molecular marker analysis to group addition lines.

2.4.3 CLUSTER ANALYSIS

Gels were analysed and polymorphic bands were scored as 1 (present) or 0 (absent). The data were used to calculate a genetic similarity matrix with the simple matching coefficient. Addition lines were grouped by cluster analysis with the similarity matrix and the unweighted pair-group method (UPGMA) (Statistica edition 99). From each distance matrix, a tree was constructed using the neighbour-joining method.

CHAPTER 3

RESULTS

3.1 IDENTIFICATION OF MOLECULAR MARKERS FOR *THINOPYRUM* CHROMOSOMES CONTRIBUTING TO SALT TOLERANCE

3.1.1 PRELIMINARY EVALUATION OF B_2F_2 FAMILIES FOR SALT TOLERANCE

Plants of the 22 B_2F_2 families were tested for salt tolerance. Rex, B_1F_1 progeny of the *Thinopyrum* / triticale cross (both relatively salt tolerant) and Henoch (a salt sensitive cultivar) were included as controls.

Henoch was the first to respond to the salt treatment, starting to wither after approximately 10 days (at an EC of 22). Henoch watered with normal nutrient solution maintained normal development and growth. After approximately 30 days, the salt stressed Henoch controls died and the experiment was terminated. Although the salt stressed Rex appeared normal, it was evident that growth was impaired when compared to the unstressed Rex. The B_1F_1 controls generally showed good tolerance and were mostly allocated scores of around six, however, a few sensitive plants were observed.

The B_2F_2 plants varied widely in their tolerance levels (Table 3.1) with scores ranging from two to eight. The variation might be attributed to the contribution of different *Thinopyrum* chromosomes retained in addition to the triticale chromosomes. The variation might also be attributed to heterogeneity among the plants with regard to the triticale chromosomes they possess as the original wide cross and the first backcross to triticale were done with pollen from various hexaploid triticale genotype.

The majority of plants (62 of the 132) did not survive the salt test and were given a score of two. One plant, 97M901-22-169, showed a very high level of salt tolerance. It appeared to be in a better condition than the salt stressed Rex controls and was given a score of eight (comparable to the amphiploid, see Table 2.2). Chromosome counts showed that 97M901-22-169 had 48 chromosomes, probably the full 42 complement of the triticale parent and six additional chromosomes from the *Thinopyrum* parent. It may have contained more than one chromosome carrying genes for increased salt tolerance and could be useful in attempts to identify molecular markers associated with the salt tolerance trait. A DNA extract was made for further use in molecular marker analysis.

The salt tolerance test distinguished sufficiently between salt tolerant and salt sensitive plants to allow the construction of bulks of salt tolerant and salt sensitive plants for bulked segregant analysis.

Table 3.1 Salt tolerance data recorded in a preliminary evaluation of the variation in 22 B_2F_2 families.

B_2F_2 Family 97M901-	Number of plants with tolerance score:						
	8	7	6	5	4	2	1
1				1	2	3	
2		2		1		3	
3		1		1	1	3	
4				1	3	2	
5				3	2	1	
6					2	4	
7				1	2	3	
8			1	1	3	1	
9				1	2	3	
10					4	2	
11				1	2	3	
12			3			3	
13				1	1	4	
14			1	2	3		
15			1	2		3	
16				1	1	4	
17				1	3	2	
18				2	1	3	
19				2	3	1	
20					1	5	
21						6	
22	1				2	3	
Total	1	3	6	22	38	62	0

3.1.2 CONSTRUCTION OF SALT TOLERANT AND SALT SENSITIVE BULKS FOR BULKED SEGREGANT ANALYSIS (BSA)

The salt tolerance of one-hundred-and-seventy-six B_2F_2 plants was determined and bulks made, each bulk consisting of four plants that were given the same score. Bulk 1 consisted of the most salt tolerant plants, bulk 2 the second most tolerant and so forth, to bulk 20, composed of the most salt sensitive of the plants (Table 2.3). The bulks were subsequently used in AFLP and RAPD analyses.

As in the preliminary evaluation, the plants varied widely in their salt tolerance levels. In the second evaluation, plants that performed well under the salt stress came predominantly from three families (15, 17 and 18), while plants in two of the families (2 and 20) did very poorly under saline stress. In the preliminary screening, family 2 appeared to contain more salt tolerant plants.

3.1.3 DERIVATION OF PUTATIVE MONOSOMIC AND DISOMIC ADDITION LINES AND THEIR CHARACTERISATION FOR SALT TOLERANCE

The chromosome numbers of two-hundred-and-twenty-two B_2F_2 seedlings were determined in order to identify addition lines (42 chromosomes from the triticale parent plus one or two additional chromosomes from the *Thinopyrum* parent). Chromosome numbers ranged from 22 (possibly 21 triticale chromosomes plus one additional *Thinopyrum* chromosome) to 50 (possibly 42 triticale chromosomes plus 8 additional *Thinopyrum* chromosomes). The chromosome number of several of the plants could not be determined because cells in mitosis could not be found after several attempts. A total of 25 plants with 43 chromosomes and 21 with 44 chromosomes were identified (Table 3.2). The salt tolerance of these putative addition plants was determined. The plants varied in their salt tolerance level, but only two plants, 97M901-6.2 and 97M901-21.6, appeared to have exceptionally high levels of salt tolerance. It was thus assumed that the additional chromosomes were from *Thinopyrum distichum* and contained a gene(s) contributing to the salt tolerance of the plant. DNA extracts were made of the two plants and used in AFLP, RAPD and DAF analyses to identify markers for these *Thinopyrum distichum* chromosomes.

Table 3.2. Variation in chromosome numbers observed in 22 B_2F_2 families.

Chr. nr.	B_2F_2 family: 97M901-																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Total
Less than 42	1	2	3	2	3	0	2	6	0	1	0	1	2	0	0	1	3	0	7	1	1	1	37
42	5	1	2	4	1	3	3	1	0	2	1	2	4	1	4	0	4	4	3	1	3	1	50
43	2	2	3	2	1	0	1	0	1	1	1	0	1	0	3	1	1	0	0	1	2	2	25
44	0	1	0	0	0	2	0	0	1	0	1	2	1	4	1	0	0	2	0	1	3	2	21
More than 44	1	3	0	1	3	4	0	1	6	3	4	0	0	4	2	3	2	4	0	6	1	4	52
Not counted	1	1	2	1	2	1	4	2	2	3	3	5	2	1	0	5	0	0	0	0	0	0	35
																							220

3.1.4 MOLECULAR MARKER ANALYSIS

3.1.4.1 Amplified fragment length polymorphism (AFLP) analysis

Twenty-five AFLP primer combinations were used to search for polymorphic bands between the triticale and *Thinopyrum distichum* parental plants (Table 2.4). Amplification products were separated on polyacrylamide gels and visualised on Biomax MR films. Bands identified in the profiles of *Thinopyrum distichum* and absent in the Rex profiles were then scored in the primary amphiploid (96M2-2-2), salt tolerant and salt sensitive bulks, as well as salt tolerant monosomic and disomic addition plants. *Mse* and *Sse* primers with two selective nucleotides were applied first. These primer combinations resulted in banding patterns with a large number of similar sized bands in each profile. The hybridisation temperature was increased to increase binding stringency of the primers, but this did not reduce the number of bands. *Mse* and *Sse* primers with 3 selective nucleotides each were then applied, which alleviated the problem to a degree. Triticale has a large genome, resulting in a large number of fragments when cut with restriction enzymes. This might explain the large number of bands in each lane.

Of the *Thinopyrum* specific bands identified, five were also associated with salt tolerant bulks, but absent in the salt sensitive bulks. A summary of these markers is given in Table 3.3. Two polymorphic bands were identified with the AFLP primer combination S27 and M64 (Figure 3.1). The first band of approximately 300 bp was identified only in the *Thinopyrum* and bulk 2 profiles. The second band was approximately 290 bp and visible in *Thinopyrum*, 96M2-2-2 and bulk 1 profiles. None of the moderately salt tolerant or salt sensitive bulks displayed the band.

A polymorphic band was identified with the AFLP primer combination S27 and M11 (Figure 3.2). Two bands, approximately 310 bp, present in the *Thinopyrum* profile, were also present in the 96M2-2-2 and bulk 1 profiles. A band of similar size as the smaller of the two fragments could be identified in the bulk 20 profile, but the larger fragment could only be identified in bulk 1. Therefore, only the larger of the two fragments was considered as a potentially useful polymorphism.

A band of approximately 230 bp was identified with the AFLP primer combination S27 and M12 (Figure 3.3). The band was present in the profile of *Thinopyrum* and bulk 2. Although the band was not very clear, it was clearly polymorphic between the salt tolerant and sensitive bulks.

The fifth marker was identified with the AFLP primer combination S27 and M16 (Figure 3.4). The band, present in the *Thinopyrum*, 96M2-2-2 and bulk 2 profiles was approximately 190 bp in size.

Three of the markers were identified only in the bulk 2 and two only in the bulk 1 profiles. This suggests that the plants represented in the two bulks contained different *Thinopyrum* chromosomes or chromosome segments contributing to salt tolerance. Salt tolerance is polygenic, therefore genes on different chromosomes may contribute to a different extent to the total salt tolerance of the plant.

Three additional AFLP markers were identified for the *Thinopyrum* chromosome(s) present in 97M901-21.6 (Figure 3.8 and Figure 3.10(a) and (b)) and one marker for 97M901-6.2 (Figure3.10(b)). A summary of the markers identified is given in Table 3.5. None of the markers identified with the monosomic or disomic addition plants was also identified employing BSA and the same AFLP primers.

Table 3.3 Summary of AFLP markers identified in salt tolerant bulks.

Primer Combination	Size (bp)	Identified in:
S27 / M64	300	*Th. dist.*, bulk 2
S27 / M64	290	*Th. dist.*, 96M2-2-2, bulk 1
S27 / M11	310	*Th. dist.*, 96M2-2-2, bulk 1
S27 / M12	230	*Th. dist.*, bulk 2
S27 / M16	190	*Th. dist.*, 96M2-2-2, bulk 2

Figure 3.1 Biomax MR film developed of [γ - ^{33}P]ATP labelled DNA fragments amplified with AFLP primers S27 and M64. The GIBCO 30-330 bp AFLP™ DNA Ladder was used to size the AFLP products. Lanes 1, 2 and 3 are, respectively, *Th. distichum*, Rex (triticale) and the primary amphiploid 96M2-2-2. Lanes 4 to 13 represent the bulks of tolerant to sensitive plants, bulk 1 being composed of the most tolerant plants to bulk 20, which was composed of the most sensitive plants. The arrows indicate polymorphic fragments between the salt tolerant and sensitive bulks.

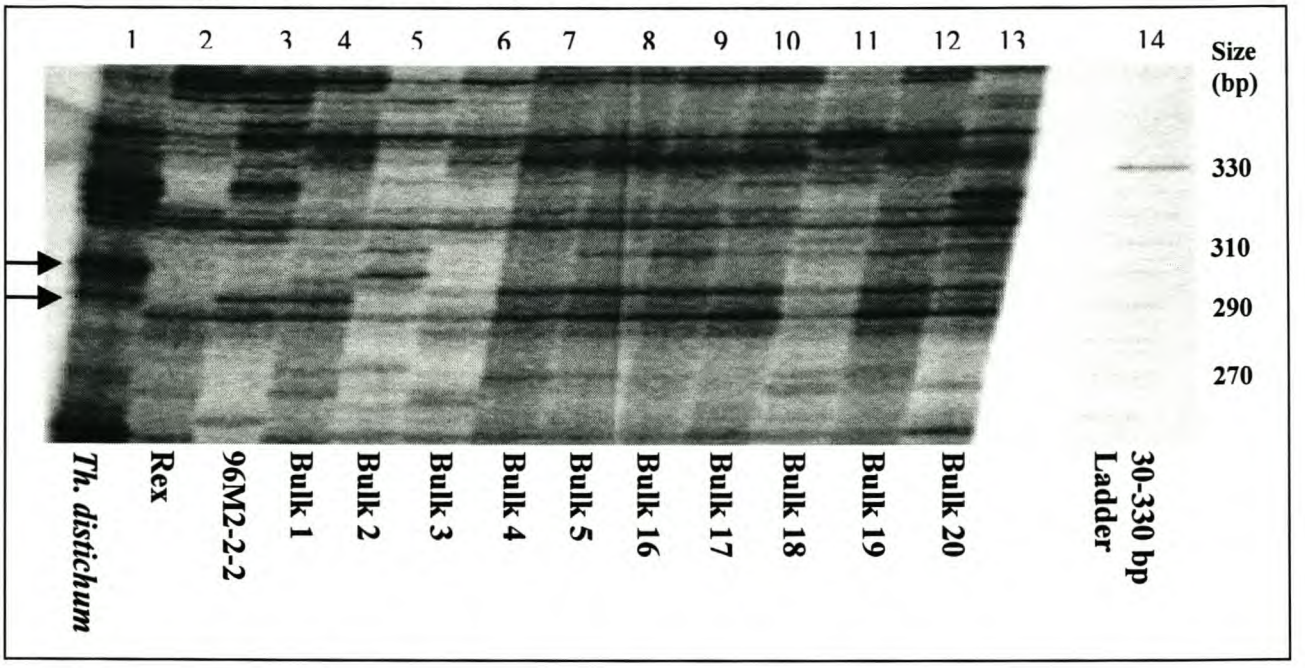


Figure 3.2 Biomax MR film developed of [γ - ^{33}P]ATP labelled DNA fragments amplified with AFLP primers S27 and M11. The GIBCO 30-330 bp AFLP™ DNA Ladder was used to size the AFLP products. Lane 2 and 3 are *Th. distichum* and Rex, respectively, the two parental lines, while lane 1 is the primary amphiploid, 96M2-2-2. Lanes 4 to 23 represent the bulks of tolerant to sensitive plants, bulk 1 being composed of the most tolerant plants to bulk 20, which was composed of the most sensitive plants. The arrow indicates the polymorphic fragment between the salt tolerant bulk and sensitive bulks.

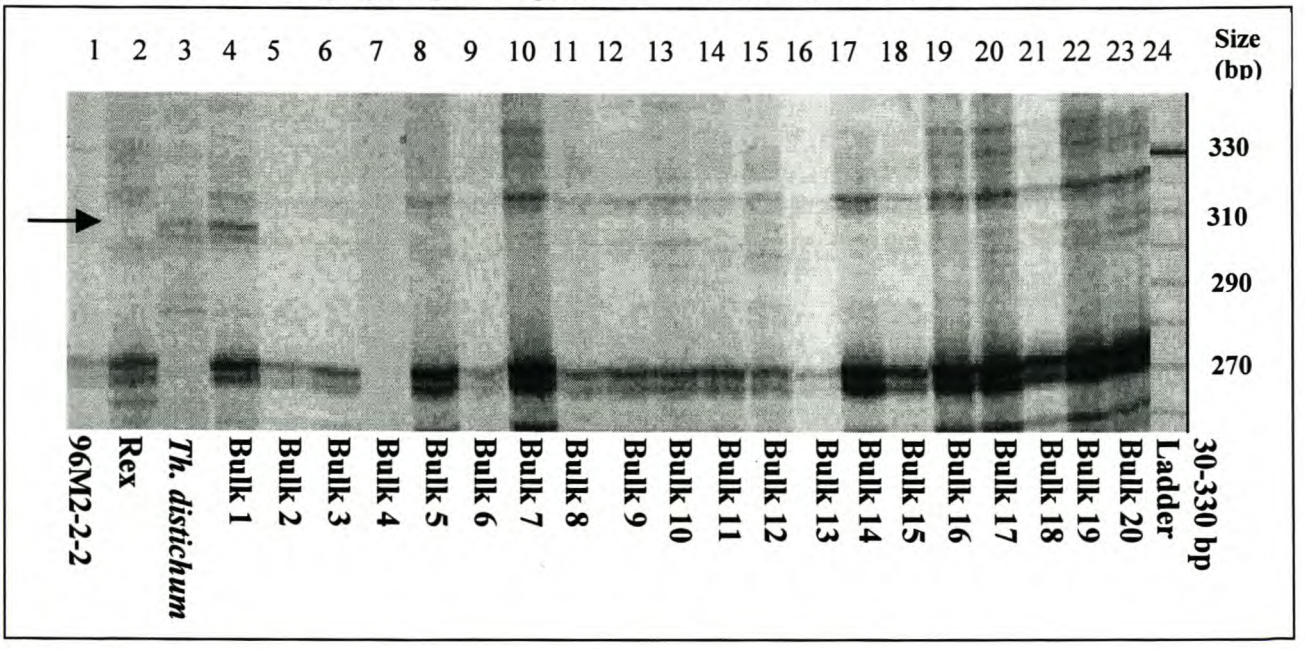


Figure 3.3 Biomax MR film developed of [γ - ^{33}P]ATP labelled DNA fragments amplified with AFLP primers S27 and M12. The GIBCO 30-330 bp AFLP™ DNA Ladder was used to size the AFLP products. Lanes 1, 2 and 3 are, respectively, *Th. distichum*, Rex (triticale) and the primary amphiploid 96M2-2-2. Lanes 4 to 13 represent the bulks of tolerant to sensitive plants, bulk 1 being composed of the most tolerant plants to bulk 20, which was composed of the most sensitive plants. The arrow indicates polymorphic fragment between tolerant and sensitive bulks.

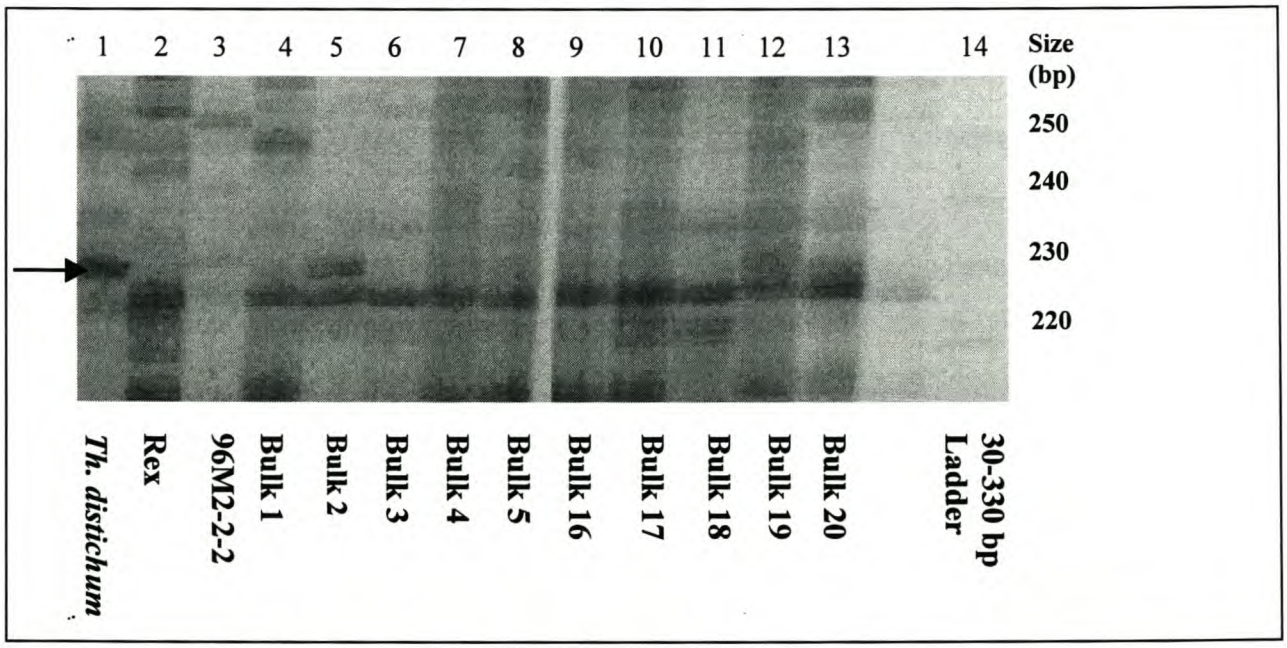
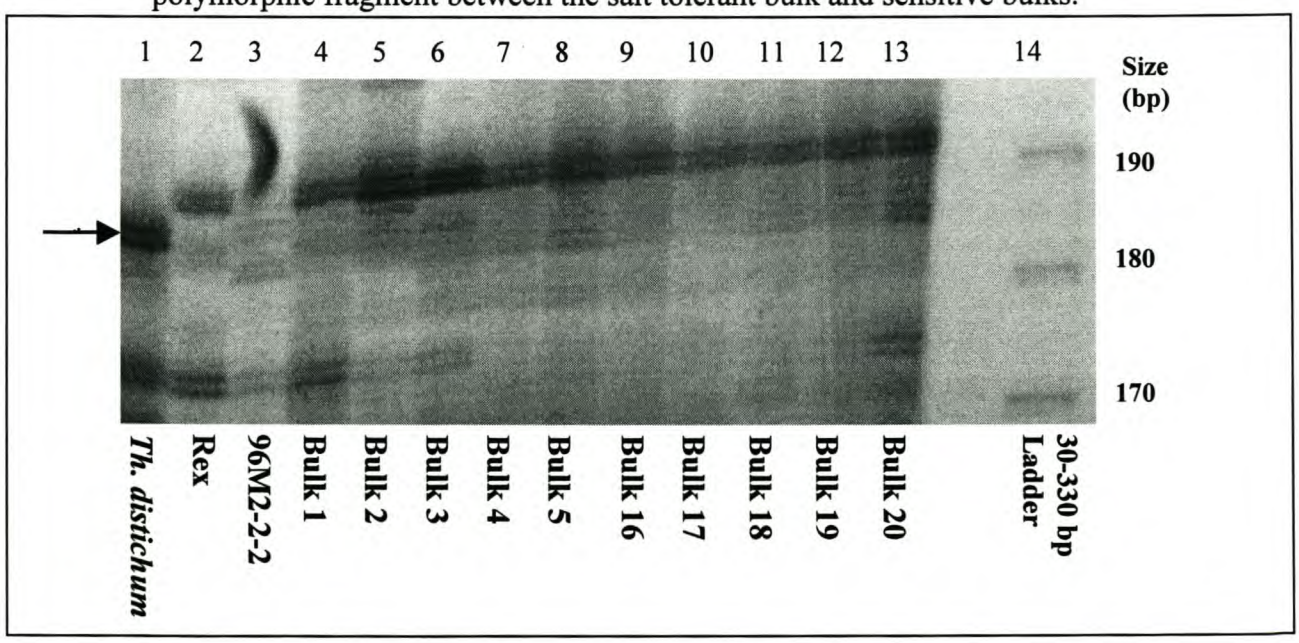


Figure 3.4 Biomax MR film developed of [γ - ^{33}P]ATP labelled DNA fragments amplified with AFLP primers S27 M16. The GIBCO 30-330 bp AFLP™ DNA Ladder was used to size the AFLP products. Lanes 1, 2 and 3 are, respectively, *Th. distichum*, Rex (triticale) and the primary amphiploid 96M2-2-2. Lanes 4 to 13 represent the bulks of tolerant to sensitive plants, bulk 1 being composed of the most tolerant plants to bulk 20, which was composed of the most sensitive plants. The arrow indicates the polymorphic fragment between the salt tolerant bulk and sensitive bulks.



3.1.4.2 Random amplified polymorphic DNA (RAPD) analysis

A total of 80 primers were used in amplification reactions resulting in numerous fragments. Results varied for some primers and were not always repeatable, but the conditions as described under the section 2.3.2 of Materials and Methods could be used for the majority of primers. RAPD fragments were separated on agarose gel and visualised by ethidium bromide staining. Bands identified in both the *Thinopyrum distichum* profiles and absent in Rex profiles were scored in the salt tolerant and salt sensitive bulks, as well as the individual salt tolerant plants. Two RAPD markers were identified that were associated with salt tolerant bulks, but absent in the salt sensitive bulks.
A summary of the markers is given in Table 3.4.

The first polymorphism was identified with RAPD primer OPK14 (5'-CCCGCTACAC-3') and produced a fragment of approximately 580 bp (Figure 3.5). The band was also visible in the profile of bulk 1, but in none of the other profiles. Although the band was not very clear, it was repeatable. A repeatable, polymorphic band was also identified with the RAPD primer OPK7 and was approximately 330 bp in size (Figure 3.6). The band, present in the *Thinopyrum* profile, was also present in the bulk 2 and bulk 3 profiles, as well as in 97M901-6.2, a salt tolerant plant with a disomic *Thinopyrum* chromosome addition.

Two additional RAPD markers were identified appearing to be associated with the *Thinopyrum* chromosome(s) in 97M901-6.2 (Figure 3.11(a) and (c)) and three markers with a 97M901-21.6 (Figures 3.11(a) and 3.12(a) and (b)) addition chromosome. A summary of the markers is given in Table 3.5.

As with the AFLP markers, the RAPD markers were not present in all bulks of salt tolerant plants. The OPK7 marker was present in bulk 2, bulk 3 and 97M901-6.2 opposed to the AFLP markers that were only identified in bulk 2 profiles. This may indicate the presence of more than one chromosome or chromosome segment in bulk 2 contributing to salt tolerance, the one identified with the AFLP primers, the other with the RAPD primer. It is possible that one or more plant in bulk 2 may have this chromosome(s). Unfortunately the individual plants were not tested, so this could not be confirmed.

Table 3.4 Summary of RAPD markers identified in salt tolerant bulks.

Primer	Size (bp)	Identified in:
OPK 14	580	*Th. dist.*, bulk 1
OPK 7	330	*Th. dist.*, bulk 2, bulk 3, 97M901.6.2

Figure 3.5 Ethidium bromide stained agarose gel of amplification products of RAPD primer OPK14. Lanes 1 and 2 are Rex and *Th. distichum*, respectively, the two parental lines. Lanes 3 to 6 represent the bulks of tolerant to sensitive plants, bulk 1 including the most tolerant plants and bulk 20, the most sensitive plants. Lanes 7 to 10 represent individual salt tolerant plants. The arrow indicates the polymorphic band.

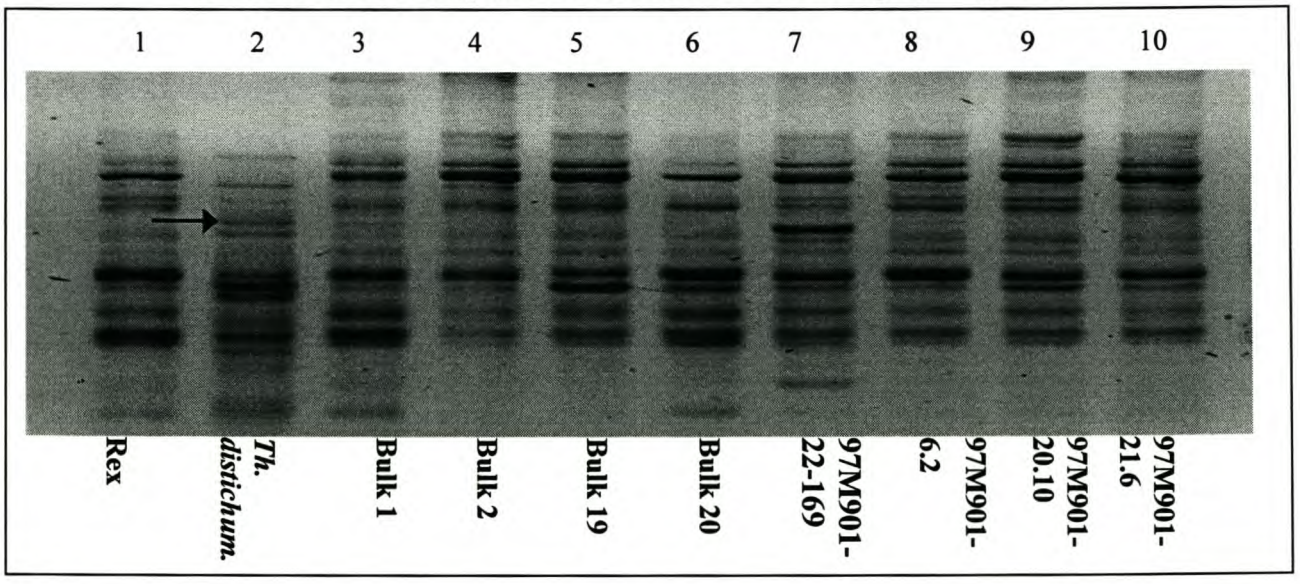
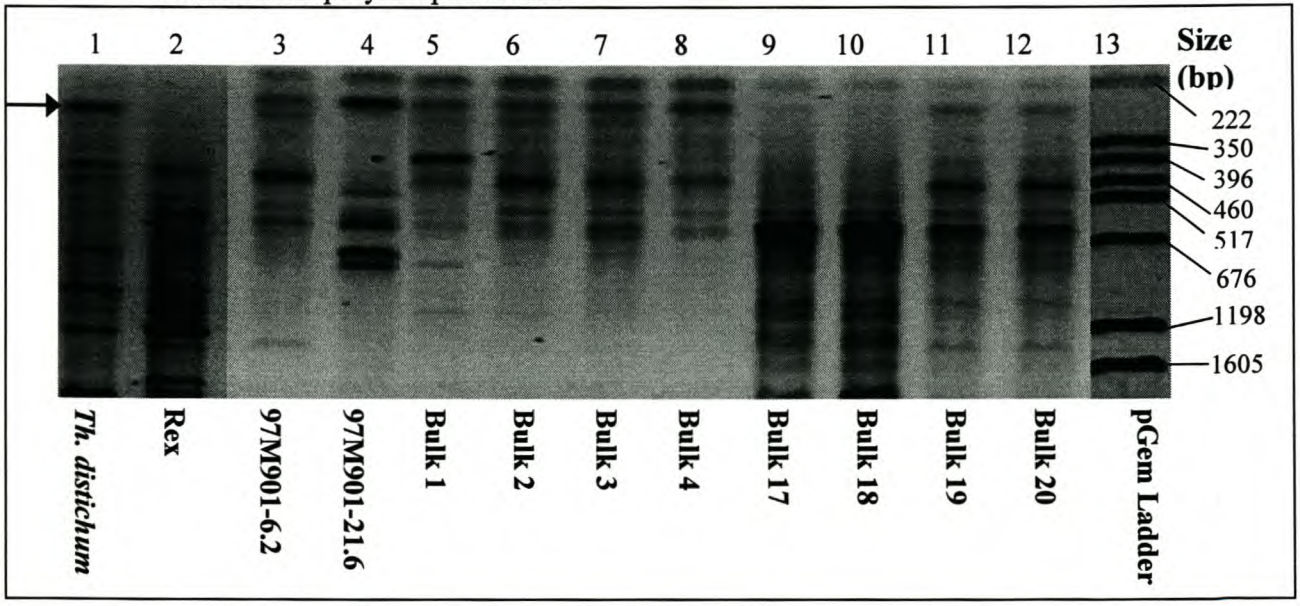


Figure 3.6 Ethidium bromide stained agarose gel of amplification products of RAPD primer OPK7. Lanes 1 and 2 are *Th. distichum* and Rex, respectively, the two parental lines. Lane 3 and 4 are two of the *Thinopyrum* chromosome addition plants identified that were salt tolerant. Lanes 5 to 12 represent the bulks of tolerant to sensitive plants, bulk 1 including the most tolerant plants and bulk 20, the most sensitive plants. The arrow indicates the polymorphic band.

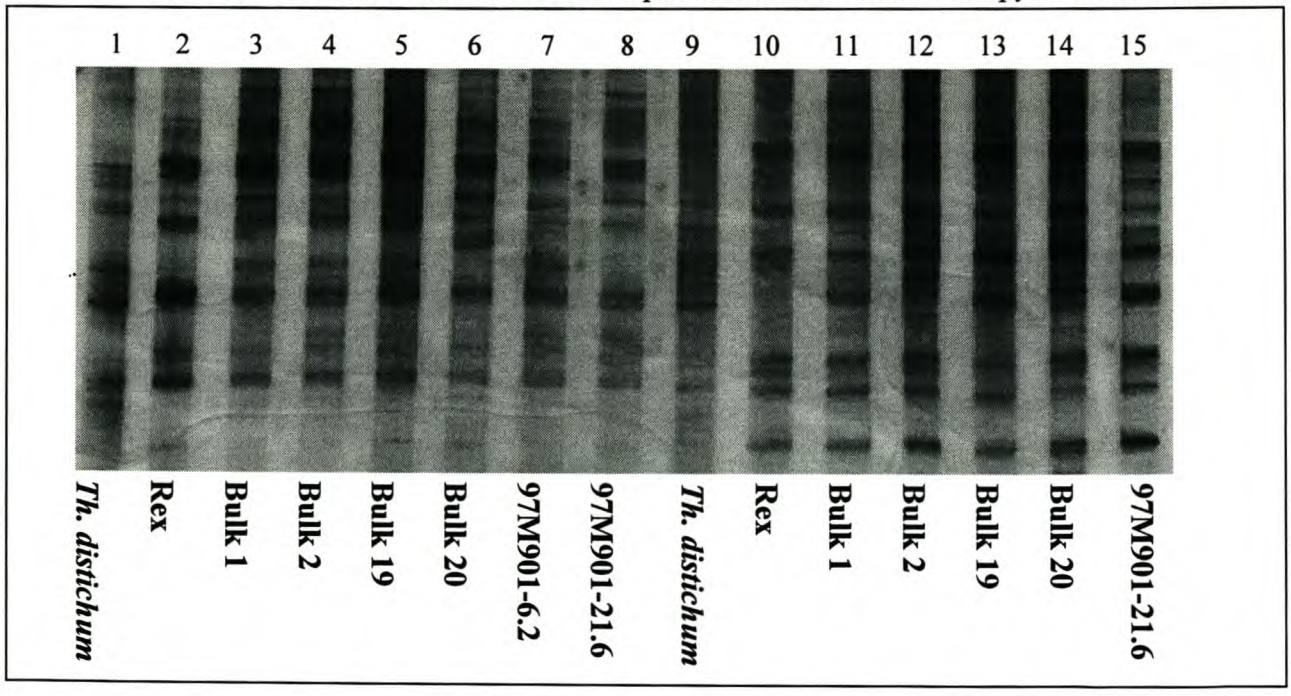


3.1.4.3 DNA amplification fingerprinting (DAF) analysis

Ten DAF primers were available for analysis. The experimental conditions as described in section 2.3.3 were used, but no results were obtained. Several of the reaction conditions were altered in an attempt to optimise the technique, but ultimately only four of the primers gave results (two are shown in Figure 3.7). Although the profiles were of high resolution and many polymorphic bands could be identified between the *Thinopyrum* and Rex profiles, none of the *Thinopyrum* specific bands could be identified in the salt tolerant bulks or individual tolerant plants.

The reason for the difficulty with the DAF technique is unclear. Personal communication with Prof. Gresshoff (co-inventor of the technique) could not resolve the problems. It is, however, a very efficient technique, combining the sensitivity of RAPD and the high resolution of polyacrylamide gel elëctrophoresis.

Figure 3.7 Silver stained DAF gel of the primers B14 and B15, the first 8 lanes were produced using B14 (5'-GCA GGC AT-3') and the last 7 using B15 (5'-GCA GGC AG-3'). Lanes 1 and 9 are the *Thinopyrum distichum* profiles and lanes 2 and 10 are the profiles of Rex. They are followed by the profiles of bulks 1 and 2 (bulks of the most salt tolerant plants) and then bulks 19 and 20 (bulks of the most salt sensitive plants). Lanes 7 and 8 and lane 15 are individual salt tolerant plants with additional *Thinopyrum* chromosomes.



3.2 GROUPING OF PUTATIVE MONOSOMIC ADDITION PLANTS ACCORDING TO THE SPECIFIC *THINOPYRUM* CHROMOSOME THEY RETAINED

Seventeen monosomic and five disomic B_2F_3 addition plants were identified. The plant treated with colchicine, 97M901-20.10, was also included as a disomic addition plant. Molecular marker analyses (RAPD and AFLP) and cluster analysis were used to group the addition plants according to the specific *Thinopyrum* chromosome they retained. The B_2F_2 monosomic addition plants (97M901-6.2 and 97M901-21.6), from which the two B_2F_3 disomic addition plants (23 and 17) derive, were included.

Twenty-five AFLP primer combinations were used to identify polymorphic bands between the triticale and *Thinopyrum* parental plants. Similarly, the Operon primer kits, OPA, OPK, OPL and OPY (a total of 80 primers) were used to produce RAPD profiles of the Rex and *Thinopyrum* parents. Polymorphic bands present in the *Thinopyrum* profiles and absent in the Rex profiles were scored for in the monosomic or disomic addition plant profiles. If the band was present in the progeny profile, a 1 was used to denote presence and while a 0 indicated the absence of a band. Nine AFLP bands (Figures 3.8, 3.9 and 3.10) and 8 RAPD bands (Figures 3.11 and 3.12), polymorphic between the parental profiles, could be scored in the progeny. A summary of the markers is given in Table 3.5 and Table 3.6.

Table 3.5 Summary of AFLP markers used for grouping addition lines.

Primer Combination	Size (bp)	Identified in:
S13 / M32	>330	*Th. dist.*, 17, 84, 114, 127, 146, 222, 20.10, 21.6
S13 /M14	190	*Th. dist.*, 17, 36, 114, 121, 127, 132, 183, 210, 21.6
S13 / M16	165	*Th. dist.*, 23, 222, 229, 6.2, 21.6
S27 / M74	320	*Th. dist.*, 36, 229
S13 / M74	320	*Th. dist.*, 71, 178, 229
S27 / M16	254	*Th. dist.*, 84, 127, 146,
S13 / M15	228	*Th. dist.*, 146, 197, 210
S27 / M32	305	*Th. dist.*, 178, 189, 229
S27 / M53	270	*Th. dist.*, 183, 197, 229, 20.10

Figure 3.8 Biomax MR film developed of [γ - ^{33}P]ATP labelled DNA fragments amplified with AFLP primers S13 and M32. The fragments on the gel were larger than the GIBCO 30-330 bp AFLP™ DNA Ladder used to size the AFLP products. Lanes 1, 2 and 3 are, respectively, the primary amphiploid 96M2-2-2, *Th. distichum* and Rex (triticale). Lanes 5 and 6 represent the salt tolerant, monosomic and disomic addition lines whereas lane 4 is the colchicine treated haploid plant. Lanes 7 to 29 are putative monosomic or disomic addition lines. The arrow indicates the *Th. distichum* specific fragment also identified in the progeny.

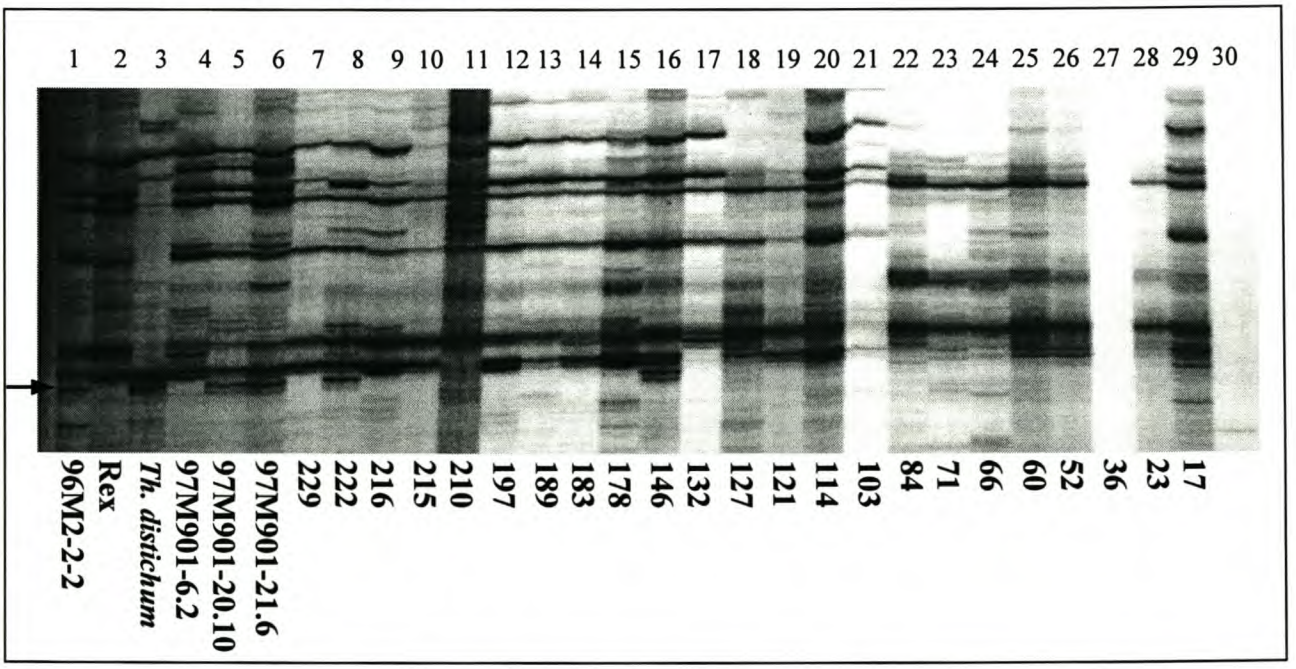


Figure 3.9 Biomax MR film developed of [γ - ^{33}P]ATP labelled DNA fragments amplified with AFLP primers S27 and M74. GIBCO 30-330 AFLP™ DNA Ladder were used to size the AFLP products. Lanes 1, 2 and 3 are, respectively, the primary amphiploid 96M2-2-2, *Th. distichum* and Rex (triticale). Lanes 5 and 6 represent the salt tolerant, monosomic and disomic addition lines whereas lane 4 is the colchicine treated haploid plant. Lanes 7 to 29 are putative monosomic or disomic addition lines. The arrow indicates the *Th. distichum* specific fragment also identified in the progeny.

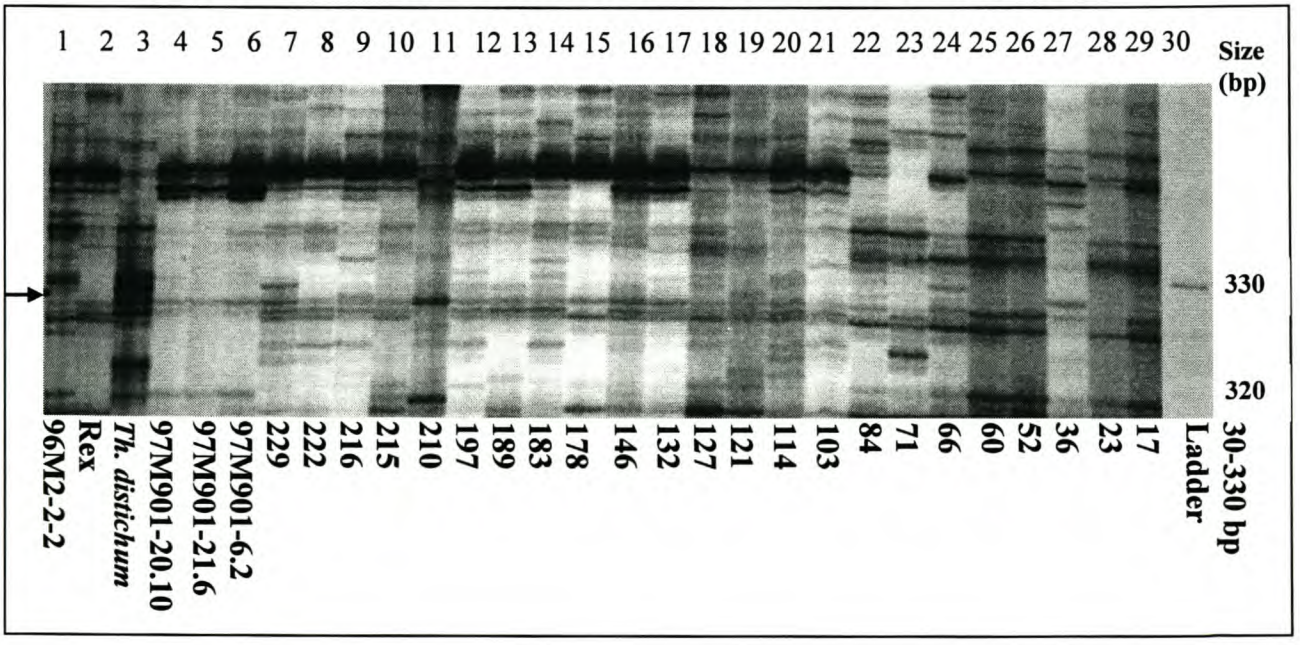


Figure 3.10 Biomax MR films developed of [γ - ^{33}P]ATP labelled DNA fragments amplified with AFLP primers. Lanes 1, 2 and 3 are, respectively, the primary amphiploid 96M2-2-2, *Th. distichum* and Rex (triticale). Lanes 4 and 5 represent the salt tolerant, monosomic and disomic addition lines whereas lane 5 is the colchicine treated haploid plant. Lanes 7 to 29 are putative monosomic or disomic addition lines. The arrow indicates the *Th. distichum* specific fragment also identified in the progeny.

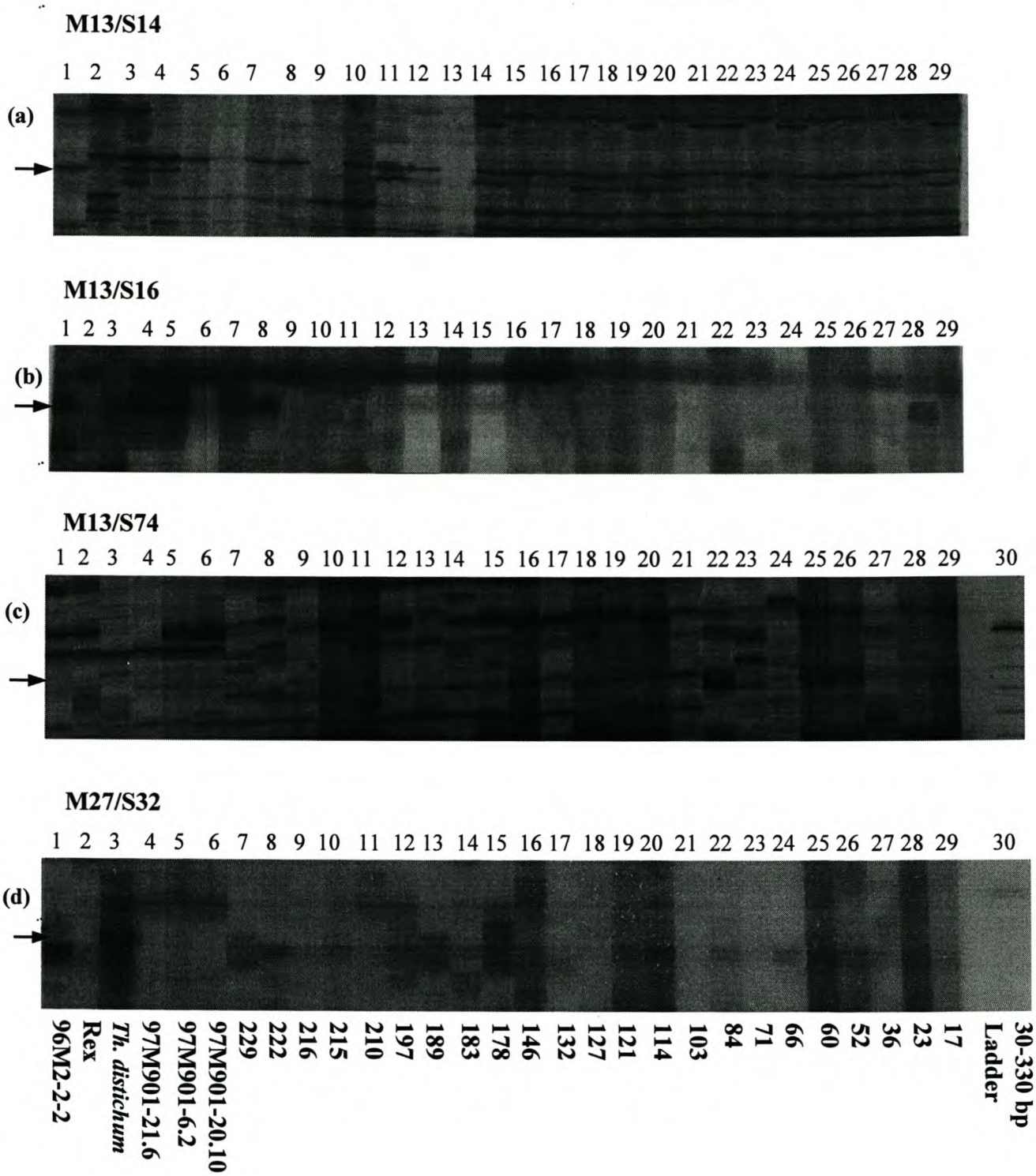


Table 3.6 Summary of RAPD markers used for grouping addition lines.

Primer	Size (bp)	Identified in:
OPA 16	705	*Th. dist.*, 17, 52, 103, 114, 146, 197, 216, 229, 21.6
OPY 10	380	*Th. dist.*, 17, 52, 71, 103, 114, 121, 178, 21.6
OPY 3	650	*Th. dist.*, 17, 52, 121, 146, 21.6
OPL 17	1060	*Th. dist.*, 36, 60, 127, 132, 189, 197, 215,
OPY 3	1060	*Th. dist.*, 23, 103, 114, 121, 6.2
OPY 6	625	*Th. dist.*, 52, 71, 178, 215, 216
OPK 6	390	*Th. dist.*, 23, 52, 60, 66, 103, 146, 189, 197, 222, 6.2
OPK 20	560	*Th. dist.*, 84, 121, 197, 210

Figure 3.11 Ethidium bromide stained agarose gels of amplification products of RAPD primers. Lane 1 and 2 are *Th. distichum* and Rex, respectively, the two parental lines. Lanes 3 to 25 are putative monosomic or disomic addition lines. Lane 26 is the colchicine treated plant, while lanes 27 and 28 is the salt tolerant monosomic and disomic addition plants. The arrows indicate the *Thinopyrum* specific fragment also identified in the progeny.

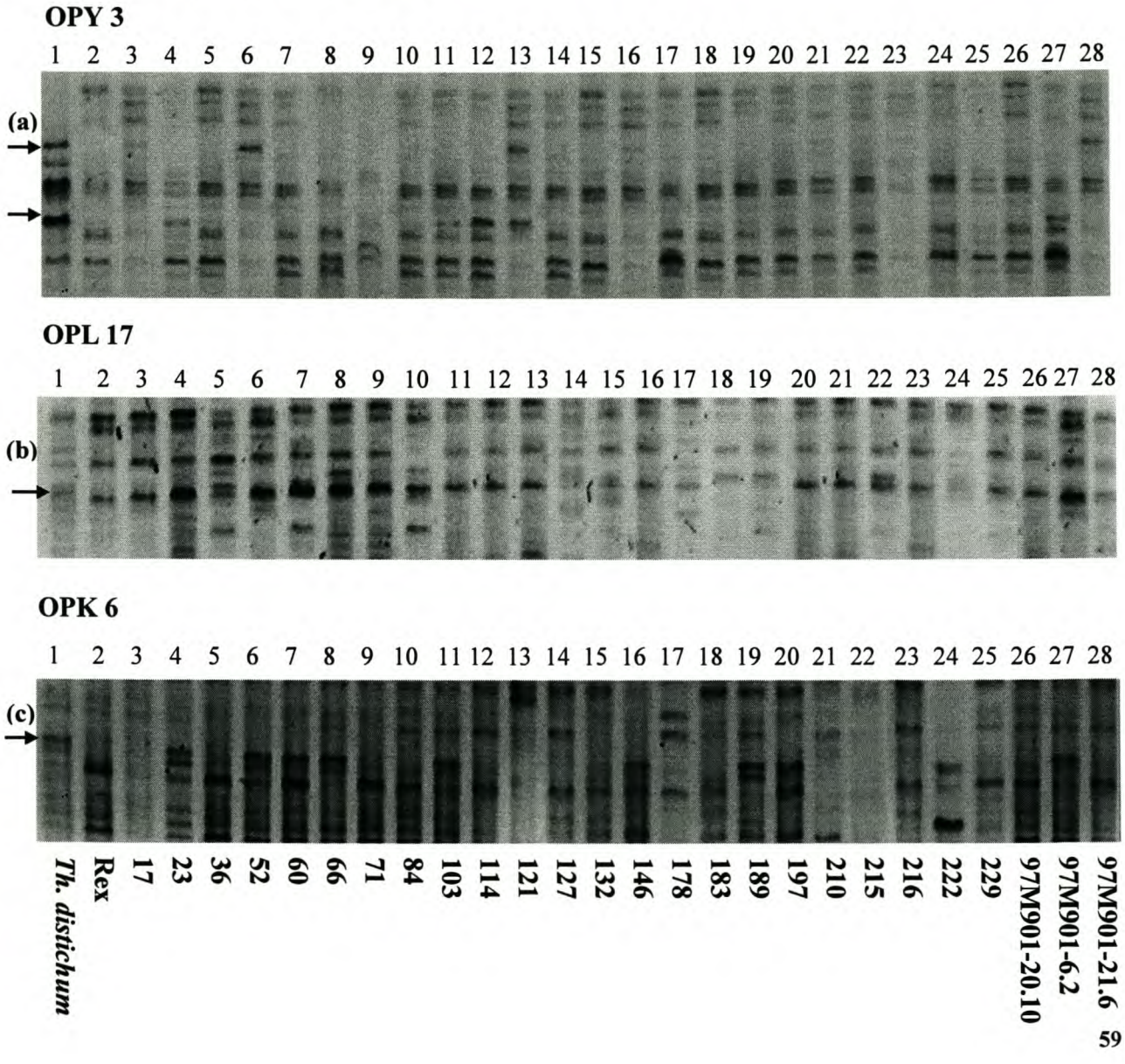
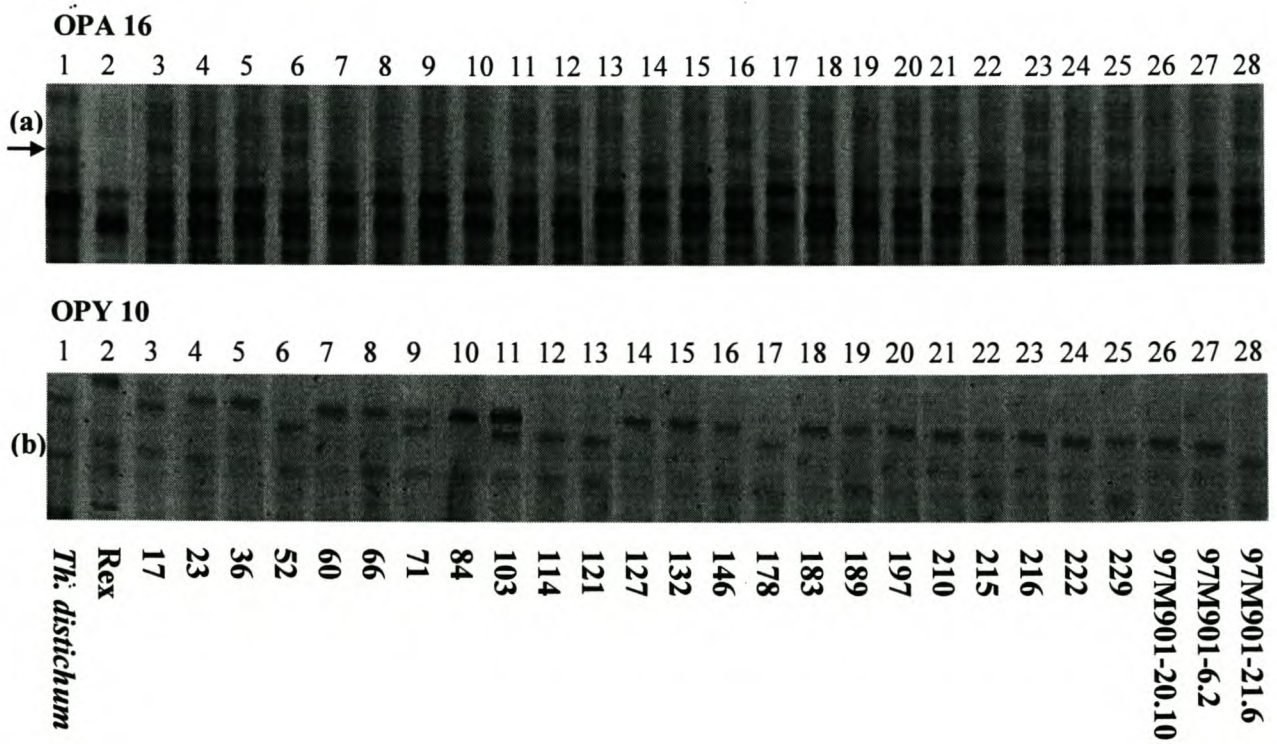


Figure 3.12 Ethidium bromide stained agarose gels of amplification products of RAPD primers. Lanes 1 and 2 are *Th. distichum* and Rex, respectively, the two parental lines. Lanes 3 to 25 are putative monosomic or disomic addition lines. Lane 26 is the colchicine treated plant, while lanes 27 and 28 are the salt tolerant monosomic and disomic addition plants. The arrows indicate the *Thinopyrum* specific fragment also identified in the progeny.



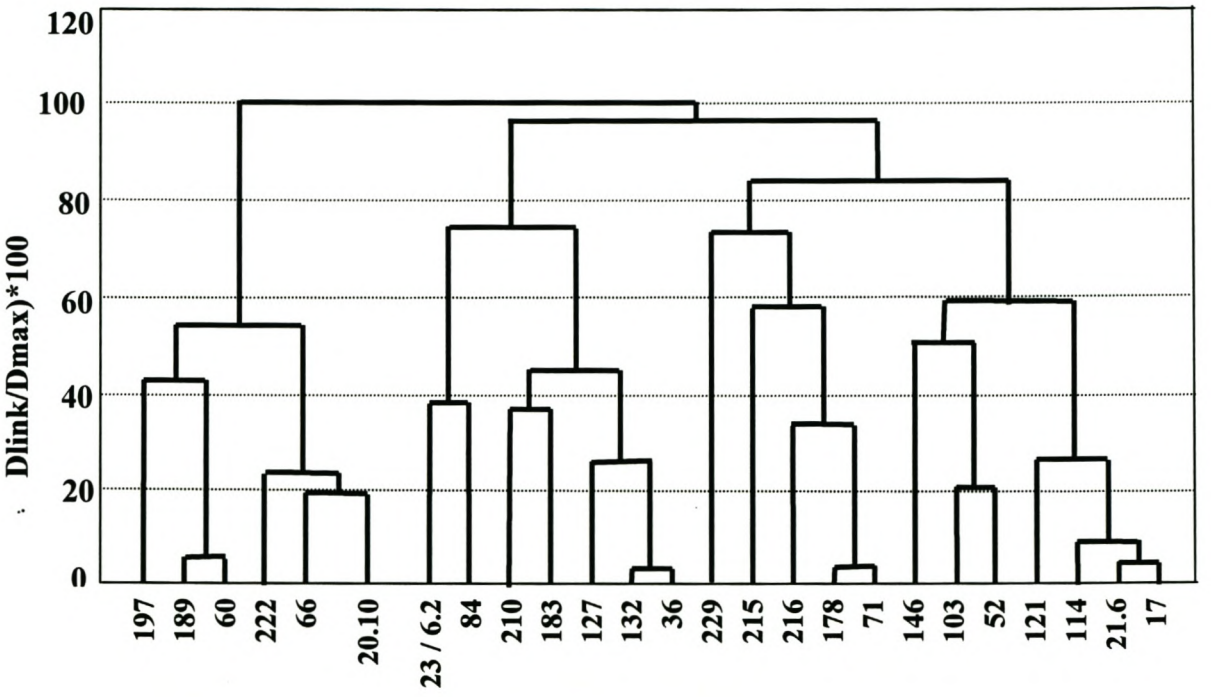
3.2.1 CLUSTER ANALYSIS

The distance matrix was created using the profile data obtained through marker analysis. Unweighted pair-group averages were used to create a tree diagram for the 26 variables (Figure 3.13). Six groups could be distinguished with a 60% probability or higher. If every *Thinopyrum* chromosome was represented in the monosomic or disomic addition plants, 14 groups would be expected ($n = 14 = J_d^1 J_d^2$). The two parental lines, 97M901-6.2 and 97M901-21.6, were grouped with their respective B_2F_3 progeny, 23 and 17.

Table 3.7 Similarity matrix used in grouping addition plants according to specific *Thinopyrum* chromosomes retained.

	17	23	36	52	60	66	71	84	103	114	121	127	132	146	178	183	189	197	210	215	216	222	229	20.10	6.2	21.6
17	1.000	0.000	0.258	0.600	0.000	0.000	0.258	0.258	0.447	0.800	0.600	0.447	0.316	0.548	0.224	0.316	0.000	0.183	0.258	0.000	0.316	0.258	0.183	0.316	0.000	0.913
23		1.000	0.000	0.258	0.408	0.577	0.000	0.000	0.577	0.258	0.258	0.000	0.000	0.236	0.000	0.000	0.333	0.236	0.000	0.000	0.000	0.667	0.236	0.000	1.000	0.236
36			1.000	0.000	0.408	0.000	0.000	0.000	0.000	0.258	0.258	0.577	0.816	0.000	0.000	0.408	0.333	0.236	0.333	0.408	0.000	0.000	0.236	0.000	0.000	0.236
52				1.000	0.316	0.447	0.516	0.000	0.671	0.400	0.400	0.000	0.000	0.548	0.447	0.000	0.258	0.365	0.000	0.316	0.632	0.258	0.183	0.000	0.258	0.548
60					1.000	0.707	0.000	0.000	0.354	0.000	0.000	0.354	0.500	0.289	0.000	0.000	0.816	0.577	0.000	0.500	0.000	0.408	0.000	0.000	0.408	0.000
66						1.000	0.000	0.000	0.500	0.000	0.000	0.000	0.000	0.408	0.000	0.000	0.577	0.408	0.000	0.000	0.000	0.577	0.000	0.000	0.577	0.000
71							1.000	0.000	0.289	0.258	0.258	0.000	0.000	0.000	0.866	0.000	0.000	0.000	0.000	0.408	0.408	0.000	0.236	0.000	0.000	0.236
84								1.000	0.000	0.258	0.258	0.577	0.000	0.471	0.000	0.000	0.000	0.236	0.333	0.000	0.000	0.333	0.000	0.408	0.000	0.236
103									1.000	0.671	0.447	0.000	0.000	0.408	0.250	0.000	0.289	0.408	0.000	0.000	0.354	0.289	0.204	0.000	0.577	0.408
114										1.000	0.600	0.447	0.316	0.365	0.224	0.316	0.000	0.183	0.258	0.000	0.316	0.258	0.183	0.316	0.258	0.730
121											1.000	0.224	0.316	0.183	0.224	0.316	0.000	0.183	0.516	0.000	0.000	0.000	0.000	0.000	0.258	0.548
127												1.000	0.707	0.408	0.000	0.354	0.289	0.204	0.289	0.354	0.000	0.289	0.000	0.354	0.000	0.408
132													1.000	0.000	0.000	0.500	0.408	0.289	0.408	0.500	0.000	0.000	0.000	0.000	0.000	0.289
146														1.000	0.000	0.000	0.236	0.500	0.236	0.000	0.289	0.471	0.167	0.289	0.236	0.500
178															1.000	0.000	0.289	0.000	0.000	0.354	0.354	0.000	0.408	0.000	0.000	0.204
183																1.000	0.000	0.289	0.408	0.000	0.000	0.000	0.289	0.500	0.000	0.289
189																	1.000	0.471	0.000	0.408	0.000	0.333	0.236	0.000	0.333	0.000
197																		1.000	0.471	0.289	0.289	0.236	0.333	0.289	0.236	0.167
210																			1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.236
215																				1.000	0.500	0.000	0.000	0.000	0.000	0.000
216																					1.000	0.000	0.289	0.000	0.000	0.289
222																						1.000	0.236	0.408	0.667	0.471
229																							1.000	0.289	0.236	0.333
20.1																								1.000	0.000	0.289
6.2																									1.000	0.236
21.6																										1.000

Figure 3.13 Dendogram of 24 putative monosomic and disomic addition plants and 2 parental lines, grouped according to the *Thinopyrum* specific chromosomes they retained.



CHAPTER 4

DISCUSSION

4.1 IDENTIFICATION OF MOLECULAR MARKERS FOR *THINOPYRUM* CHROMOSOMES CONTRIBUTING TO SALT TOLERANCE

4.1.1 EVALUATION OF B_2F_2 FAMILIES FOR SALT TOLERANCE

The B_2F_2 plants varied widely in their salt tolerance (Table 3.1), with scores ranging from two to eight. Plants within the 22 B_2F_2 families did not show consistent scores except for line 14. This indicates that some plants had more favourable chromosome combinations. From the chromosome counts done on B_2F_2 plants, it was found that the B_2F_2 possessed 0 to eight *Thinopyrum distichum* chromosomes (the plants had chromosome counts of 42 to 50), thus contributing to the variation in salt tolerance. One of the plants tested, 97M901-22-169, which exhibited a high level of salt tolerance, had 48 chromosomes, probably consisting of 42 Rex chromosomes and six additional *Thinopyrum* chromosomes. The B_1F_1 with 54-55 chromosomes (possibly 12 or 13 additional *Thinopyrum* chromosomes; almost complete J_1^d and J_2^d genomes) also showed high salt tolerance levels. Forster *et al.* (1987), Gorham *et al.* (1986) and Dvorak & Ross (1986) found that some of the full or partial hybrids between *Th. bessarabicum* and hexaploid wheat show enhanced salt tolerance. Forster *et al.* (1986) showed that the full expression of salt tolerance resulted from a full complement of *Th. bessarabicum* chromosomes and that the salt tolerance decreased with a decrease in number of *Th. bessarabicum* chromosomes. Several *Th. elongatum* chromosomes were found to contribute to the salt tolerance of addition lines (Dvorak *et al.*, 1988). It thus appears that, as in *Th. elongatum*, several of the *Th. distichum* chromosomes contribute to salt tolerance.

4.1.2 MOLECULAR MARKERS IDENTIFIED THROUGH BULKED SEGREGANT ANALYSIS (BSA)

Bulked segregant analysis (BSA) was used, in combination with AFLP and RAPD analysis, to identify seven possible markers for *Thinopyrum distichum* chromosomes carrying genes for salt tolerance. Polygenes determine salt tolerance and these may be located on different chromosomes. Two of the AFLP markers were associated with bulk 1 and three with bulk 2. The RAPD marker OPK7 was present in bulk 2, bulk 3 and 97M901-6.2, while the OPK14 marker was

identified in bulk 1. The markers identified in the different bulks may be linked to different chromosomes or chromosome segments of the *Th. distichum* genomes. The plants represented in the different bulks (and within the bulks) very likely contained different combinations of *Thinopyrum* chromosomes or chromosome segments encoding for salt tolerance. Some of the associations may obviously be incidental and the markers need to be validated in follow-up experiments. Salt tolerant B_2F_3 plants, as well as backcross derivatives from the cross: *Th. distichum* / 4 x rye (*Secale cereale*) // 2 x rye (Marais & Marais, 1998), are being identified for the purpose of confirming the markers.

The success of BSA for tagging quantitative trait loci (QTL) based on phenotypic information is dependent on the magnitude of the phenotypic effect of the individual QTL, the population size sampled and the influence of non-genetic factors on the phenotype. Several factors, e.g. environmental factors, soil composition, aeration, etc., influence the salt tolerance of plants. This may result in plants performing better or worse under saline conditions than predicted by their genome composition. Plants with high salt tolerance may not survive the salt tolerance test due to, for example high humidity, while a salt sensitive plant will survive due to, for example good soil composition. The evaluation of plants in this study was, however, done under controlled conditions, neutralising this effect to a large extent. The high levels of aneuploidy, heterogeneity among plants with regard to the triticale chromosomes they possess and chromosome rearrangements existing in the material does, however, influence the evaluation. The heterogeneity can, for instance, be seen in the polymorphisms between the lines with regard to the Rex specific bands they contain.

The use of AFLP and RAPD analyses and their respective advantages and disadvantages have been discussed extensively in the literature (Devos & Gale, 1992; Williams *et al.*, 1990; King *et al.*, 1993; Welsh & McClelland, 1990). These techniques are frequently used in combination with bulked segregant analysis (BSA) to identify markers associated with traits of interest (Williams *et al.*, 1997; Grattaplaglia *et al.*, 1996). Wang & Paterson (1994) concluded that DNA pooling strategies for mapping QTL may only be successful in tagging QTL with a high phenotypic effect (0.75 standard deviations (SD) and higher). Grattaplaglia *et al.* (1996) found that a BSA approach missed important QTL due to sampling effects in the bulk composition. Several bulks were used in this study consisting of highly salt tolerant to highly salt sensitive plants, which would allow the identification of different molecular markers in the different bulks. The markers identified were only identified in bulks of highly salt tolerant plants and may therefore be associated with QTL with high phenotypic effect.

4.1.3 MOLECULAR MARKERS FOR *THINOPYRUM* CHROMOSOMES IN MONOSOMIC AND DISOMIC ADDITION PLANTS

Molecular marker analysis (AFLP and RAPD) identified three potential markers for the *Th. distichum* chromosome(s) present in 97M901-6.2 and 6 markers for chromosome(s) in 97M901-21.6. Only one of the markers identified with BSA was also identified in 97M901-6.2. It is possible that the *Thinopyrum* chromosomes present in the 97M901-6.2 and 97M901-21.6 were not well represented in the bulks.

Selection for a trait of interest located on a specific chromosome will benefit from the availability of genetic markers spaced along the chromosomes of interest. AFLP and RAPD analysis are frequently used for this purpose. From a total of 120 primers tested, King *et al.* (1993) identified 10 RAPD markers specific to chromosome $5E^b$ of *Th. bessarabicum*. Chromosome $5E^b$ is thought to play a major role in the high salinity tolerance of *Th. bessarabicum*.

The markers identified in this study are not necessarily closely linked to the gene(s) that improve the salt tolerance. Polymorphic markers that are closely linked to the gene(s) contributing to salt tolerance would enable the selection of plants with small chromosome segments carrying the genes of interest. If some of the markers identified in this study are in fact linked to chromosomes carrying genes for salt tolerance, they will be valuable in future attempts to transfer the relevant *Thinopyrum* chromosome segments to cultivated cereals.

4.2 GROUPING OF PUTATIVE MONOSOMIC AND DISOMIC ADDITION PLANTS ACCORDING TO THE SPECIFIC *THINOPYRUM* CHROMOSOME THEY RETAINED

Alien addition lines can be identified by gross morphology, karyotype of the added chromosomes, isozyme analysis and genetic tests. *Thinopyrum distichum* chromosomes were characterised according to morphology and C-band distribution (Littlejohn & Pienaar, 1994). These were subsequently used to identify *Th. distichum* chromosomes in crosses with Inia 66 (Littlejohn & Pienaar, 1995) and *Triticum turgidum* (Fominaya *et al.*, 1997). Proteins and isozymes, together with *in situ* hybridisation have been used for the detection of complete and translocated arms of *Th. bessarabicum* chromosomes in a *T. aestivum* background. These diagnostic markers were used to characterise a distinct set of *Th. bessarabicum* disomic additions to wheat (William & Mujeeb-Kazi, 1995).

This study made use of molecular markers (AFLP and RAPD) to group the 24 putative monosomic and disomic addition lines according to the specific *Thinopyrum* chromosomes they retained. Of the possible 14 addition lines (n = 14 =$J_d^1J_d^2$), six broadly similar groups with one to six plants per group were identified. Littlejohn and Pienaar (1994) identified 11 of the possible 14 addition lines of *Th. distichum* chromosomes by C-banding. Of the 39 meiotically stable lines with 44 chromosomes C-banded, between one and five plants of each addition were identified, except for chromosome J^{dx}, which was identified in 15 of the 39 lines. The addition lines and substitution lines differed morphologically from one another and from Inia 66. The advantage of the molecular marker techniques is its sensitivity, which enables the identification of small chromosome segments that might have been missed by identification through gross morphology, karyotyping of additional chromosomes or isozyme analysis.

While members of each group are likely to carry the same additional *Thinopyrum* chromosomes, this may not necessarily be the case as the interpretation of the marker results is complicated by heterogeneity among plants with regard to the triticale chromosomes they possess. It is also likely that chromosome rearrangements occurred during backcrossing which may further complicate data. Zhang *et al.* (1996) used RAPDs to characterise seven partial amphiploids of the hybrid *Triticum aestivum* x *Thinopyrum ponticum*. RAPD results obtained from the partial amphiploids showed that the *Th. ponticum* genomes in four of the partial amphiploids were not intact genomes of the perennial wheatgrass, but modified synthetic genomes composed of chromosomes or chromosome segments of the wheatgrass. This rearrangement of genomes was also found by Banks *et al.* (1993) and Dvorak (1976). Pienaar (1981) showed that pairing of homoeologous J^d chromosomes occur in partial amphiploids and that it is therefore likely to have rearranged genomes of mixed homoeology in disomic addition lines. This may also explain why specific chromosomes are more frequently found in addition lines than others are. If J_1J_2 bivalents were formed during meiosis, their inheritance during backcrossing and self-fertilisation would be stabilised, while chromosomes not pairing would be lost.

Nonetheless, it is now possible to select addition plants from each group that are likely to represent different *Thinopyrum* chromosomes. The data will also be useful in future attempts to find further addition plants carrying the remaining *Thinopyrum* chromosomes. Several *Thinopyrum* specific bands were identified in marker analysis that was not identified in any of the addition plants. These probably originate from *Thinopyrum* chromosomes not represented in the addition lines.

CHAPTER 5

CONCLUSION

In this study, bulked segregant analysis (BSA), in combination with AFLP and RAPD analyses, identified seven possible markers for *Thinopyrum distichum* chromosomes carrying genes for salt tolerance. The distribution of the markers in these bulks suggests that more than one *Thinopyrum* chromosome is carrying genes for salt tolerance.

Three molecular markers were identified for the *Thinopyrum distichum* chromosome(s) present in a salt tolerant monosomic addition plant, while six molecular markers were identified for the addition chromosomes in a disomic addition plant. The validity of the markers should, however, be confirmed in follow-up experiments.

Twenty-four plants with monosomic or disomic additions of *Thinopyrum* chromosomes were identified and characterised for molecular markers. Cluster analysis was used to group the putative plants into six broadly similar groups according to the specific *Thinopyrum* chromosomes they retained. It is now possible to select addition plants from each group that are likely to represent different *Thinopyrum* chromosomes. The data will also be useful in future attempts to find further addition plants carrying the remaining *Thinopyrum* chromosomes.

CHAPTER 6

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