STRATEGIES FOR CLONING ION TRANSPORTERS IN SALT-RESISTANT PLANTS

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Contents

A List of Abbreviations

Amp	Ampicillin
AD	absorbance density
akt	Gene encoding *Arabidopsis* high-affinity K^+ Transporter
aha	Gene encoding *Arabidopsis* H^+-ATPase
at-clc	Gene encoding *Arabidopsis* voltage-gated chloride channel
bp	Base pair
BSA	Bovine serum albumin
clc	Gene family encoding voltage-gated chloride channel
clc-Nt1	Gene encoding *N. tabacco* voltage-gated chloride channel
CFTR	Cystic fibrosis transmembarne conductance regulation
cpm	Counts per minute
CsCl	Caesium chloride
2,4 D	2,4-Dichlorophenoxyacetic acid
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
ds DNA	Double-stranded DNA
ds m^{-1}	Deci Siemens per metre
EDTA.Na_2	Diaminoethane-tetraacetic acid, disodium salt
ena1	Gene encoding a putative *S. cerevisiae* Na^+-ATPase

GEF (sclcc)	Gene encoding *S. cerevisiae* voltage gated chloride channel
GUS	β-galucoronidase repoter gene
hkt1	Gene encoding *Arabidopsis* high-affinity K^+ Transport
IPTG	Isopropyl-β-thiogalactoside
kb	Kilobase
kDa	Kilodalton
kst1	Gene encoding *Solanum tuberosum* K^+ transporter
lha	Gene encoding tomato (*lycopercion esculentum*)
min	Minute(s)
MMLV-RT	Moloney Murine Leukemia Virus - Reverse Transcriptase
MOPS	3 [N-morpholino] propanesulfonic acid
MSMO	Murashige and Skoog basal salts with minimal organics
nhaA, nhaB, nhaC	Gene encoding *E. cloi* Na^+ /H^+ antiporter
nhaBv	Gene encoding *Vibrio alginolyticus* Na^+ /H^+ antiporter
nhpA	Gene encoding *E. hirae* Na^+ /H^+ antiporter
nhe	Gene encoding human Na^+ /H^+ antiporter
OD	Optical density
Oligo	Oligonucleotide
ORF	Open reading frame
PCR	Polymerase Chain Reaction

PEG 8,000	Polyethylene glycol 8,000
Pfu	Plaque forming unit
pH	Hydrogen ion concentration, $-\log_{10}$
Pma	Gene encoding *N. plumbaginifolia* H^+ATPase
PMF	Proton motive force
PPFD	Photosynthetic photon flux density
psi	Pounds per square inch
p-type	Plasma membrane-type
Pvp	Polyvinyl pyrrolidone
rpm	Revolutions per minute
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
sod2	Gene eencoding *S. pombe* Na^+ /H^+ antiporter
ss DNA	Single-stranded DNA
Tm	Melting temperature
v-type	Vacuolar-type
v-PPase	Vacuolar pyro-phosphatase
(v/v)	Volume/volume
(w/v)	Weight/volume
X-Gal	5-bromo-4-chloro-3-indoyl β-D galactoside

z-sod2	Gene encoding Z. *rouxii* Na^+ /H^+ antiporter

ABSTRACT.

Most crop species upon which we depend are glycophytes, which lack the mechanisms required to tolerate ion excesses present in high concentration in saline soils. Therefore, it is important to understand how salt-resistant plants respond to external salinity. What is known is that a number of physiological processes are involved in salt-tolerance and each of these processes requires the co-ordinated expression of a number of genes.

One way to identify putative genes encoding Na^+ and Cl^- transporters from salt-resistant plants is to use an approach involving heterologous probes. Genomic Southern blots of barley and sugar beet were performed using a range of H^+-ATPase, Na^+ / H^+ antiporter and Cl^- channel sequences as probes. Some sequence homology to both p-type and v-type H^+-ATPases, and to *clc-0* (a voltage gated Cl^- channel) was found. However, Northern analysis showed that only in barley was the *clc-0* homologue expressed; the transcript was ~1.4 kb and probably not poly-adenylated.

Attempts to isolate a voltage gated Cl^- channel from barley genomic library constructed in λ Dash II by colony hybridization using *clc-0* as probe were unsuccessful.

In 1996 the first plant voltage-gated Cl^- channel, *clc-nt1* was cloned from tobacco using an RT-PCR approach. A similar strategy was then adopted in this study. Seventeen putative *CLC* sequences were found in the databases and these were aligned. Three regions of consensus were identified that were suitable for designing degenerate PCR primers.

Two PCR approaches were used, conventional PCR and RT-PCR, to identify *clc* homologues in sugar beet, barley and red beet. The conventional PCR approach used cDNA libraries and genomic DNA as template, but failed to identify any *clc* homologues.

RT-PCR was performed on oligo dT- and random primed cDNA from root and leaf tissues of high-salt-grown plants. A 600 bp fragment, *bv600,* from sugar beet leaves, and two shorter fragments, *bv160* and *bv163*, from red beet cell suspension cultures, were amplified and demonstrated to be authentic plant sequences. These fragments were then sequenced and this information used to interrogate the databases using BLAST searches. The fragment *bv600* was identified by BLAST as homologous to an ATP-dependent protease. Similar comparisons using the *bv160* and *bv163* sequences suggested that they are almost identical, but BLAST failed to identify any homologues

in the databases.

The red beet fragment *bv160* was used to identify a 7 kb sugar beet genomic fragment, *bvFAS1.7*. This fragment was sequenced and analyzed by a range of computer programs. The *bvFAS1.7* fragment, containing the *bv160* sequence, probably encodes a reverse transcriptase although this is by no means certain. More recent alignments of all of the *clc* sequences, and of the six plant sequences deposited in the databases, suggests that better (*i.e.* less degenerate) primers can now be designed which should improve the success of an RT-PCR approach in cloning *clc* homologues in salt-resistant plants.

CHAPTER ONE INTRODUCTION

1.1. Introduction.

Within the next 50 years the population of the Earth will increase dramatically, but with our existing agricultural practices, we will not able to provide adequate nourishment to all of these citizens. At present, there are now more malnourished people alive than at any other time in human history (United Nations FAO Commission, 1989) unless suitable action is taken now, this situation will worsen. The introduction this century of Industrial Agricultural Systems (IAS) to parts of the developing world has to some extent delayed the impending crisis, but it is well established that these systems are neither sustainable in the long-term, nor affordable in developing nations. Currently, land is being lost from agriculture at an alarming rate (61,000 square km *p.a.*, World Resources, 1991); the traditional solution has been to abandon land that becomes low yielding and develop new regions, but of the 32 % of suitable uncultivated land left, 15 % is tropical or temperate forest, and 10 % is grazed. Ploughing these habitats is not desirable; such a policy will lead to strong political opposition and inevitable conflict. Perhaps more importantly, such strategy will not provide a long-term solution, but merely further delay the inevitable crisis. The only practical solution to this problem is biotechnological. The IASs have inadvertently established an unhealthy dependence on 5 species, which accounts for >80 % of the calories consumed by humans. The ingenuity of plant breeds over the last 100 years has resulted in the extension of the range of these crops and food supplies have increased enormously, but these crops are now geographically constrained by temperature, rainfall, soil composition, *etc.* The inherent genetic diversity of these crops, it seems, has been almost exhausted. What is now required are crops with a different genetic composition that will produce high yield in a regions of low productivity, but this must be achieved using agricultural practises that are sustainable.

To conclude, we need new high yielding and nutritious crops that can survive in regions hither-to unexploited by intensive agriculture; they should require minimal levels of input (fertiliser, pesticide, *etc.*) and of soil disturbance for cultivation. These plants do not exist

and there is no prospect that they can be developed by conventional means by the end of the next century. In short, plant biotechnology provides the only acceptable solution to what is already the greatest challenge of the 21st Century.

1.2. Importance of Salinity to Agriculture

One of the major causes for the loss of existing agricultural land, and a major constraint preventing the development of new areas, is soil salinity. Soil salinization is one of man's oldest environmental problems. The historical records show that human civilisations never remain established in one locality for more than 800 to 2000 years. The major reason for the decline in many of these civilisations seems to have been the destruction of the land resource (Carter and Dale, 1974). For example, historical records show a shift in agriculture in the Tigris-Euphrates basin of ancient Mesopotamia from the cultivation of wheat to the more salt-tolerant barley as the fertile but poorly drained soils became increasingly saline (Jacobson and Adams, 1958). This was subsequently followed by a serious decline of approximately 65 % in the yield of barley and is considered to have played an important role in the decline of the Sumerian civilisation in Mesopotamia (Ashraf, 1994).

The processes of soil salinization have continued relentlessly throughout history and there are few countries practising irrigation agriculture today that are not affected. In fact, the long term viability of irrigation agriculture has been seriously questioned (Jacobson and Adams, 1958). The global extent of saline soils is believed to range between 400 and 950 x 10^6 ha., and it has been estimated that one third of the 230 x 10^6 ha. currently under irrigation is affected by salinity (Flowers, 1977).

Moreover, the distribution of the 343.5 million ha. of saline soils throughout the world was evaluated by Massound (1974), of this, 53.5 million ha was located in Africa, 17.4 million ha. in Australia, 1.96 million ha. in Mexico and central America, 6.2 million ha. in North America, 69.4 million ha. in South America, 91.7 million ha. in North and Central Asia, 83.3 million ha. in South Asia, and 20.0 million ha. in South-East Asia.

Vast areas of arid and semi-arid regions of the world where crop production is based on irrigation, are suffering from increased soil salinity (Boyko 1966, 1968). In these areas

salinity occurs mainly as a result of either, accelerated redistribution of salts in the soil profile due to high water tables, or the use of insufficient irrigating water to leach salts out of soil, such as from canals tube wells, or sea water. Therefore, water from these sources which contains variable amounts of salts, and their uncontrolled use, has caused considerable salinization worldwide (Ashraf, 1994).

For example, in Pakistan, soil salinity is one of the most common irrigated crop stress problems, because the climate varies from arid to semi-arid. World-wide, 40,000 ha are being lost annually from agriculture due to salinity (Al-khatib, 1993). It is estimated that about a quarter of a million hectares of good agricultural land in South Western Australia have become unproductive due to soil salinity (Malcolm, 1982).

Salinity problems have caused a continuing degradation of arable land since ancient times, in some cases land has been reduced by salinity within a short time. This is certainly true in California where irrigated agriculture in most areas is less than 100 years old. Nonetheless, of the 8.6 million ha. of arable land in California, 4.5 million acres are now affected by salinity, and this damage by salinity is expected to increase from 4.5 to 5.2 million ha. by the turn of the century. In the San Joaquin valley, which has 4.5 million ha. of land under irrigation, in 1984 more than 0.4 million acres were assessed as severely affected by salinity (Lewis, 1984). Therefore, it is expected that by the year 2000, a further one million ha. could be lost due to salinity.

1.3. Plant Responses and Adaptations to High Salinity.

Soil salinization is a more complex agricultural phenomenon than a simple increase in sodium and chloride concentrations (Tanji, 1990). Calcium sulphate and carbonates may be present in excessive amounts, together with toxic concentrations of boron or selenium. At the same time, some nutrients (particularly phosphorus and nitrogen) may be available in such low amounts that they limit growth (Gorham, 1992).

In sodic soils (which have a high cation exchange capacity dominated by Na^+, with carbonate as the major anion) plants face multiple stress factors caused by a shortage of potassium, high pH, impenetrable soil structure and water logging (Gorham, 1992). In many saline habitats and agricultural soils there are seasonal variations in salinity caused

by periodic inundation with sea water and variations in rainfall, evapo-transpiration and irrigation (Gorham, 1992). Furthermore, salinity levels are rarely uniform, often being highest in the surface layers where evaporation occurs. It may also be extremely localised with highly saline areas bordering relatively non-saline soils, such soils support a limited number of plants. Therefore, plants have to adapt to soils which vary spatially, temporally and chemically with respect to salinity and associated stresses (Gorham, 1992).

Salt tolerant plants (halophytes) have adapted to highly saline conditions which at times exceed that of sea water (500 mM NaCl), although maximum growth often occurs at lower salt concentrations (Ungar, 1991). Oceans and the shallower seas of the world comprise a major environment colonized by various forms of plant life. In addition, the seashores, salt marshes and salt deserts of the world's arid regions represent habitats having green plants with similar adaptations to high salinity (halophytes). Coastal salt-marsh plant communities range from an assemblage of highly salt tolerant species occurring in lower salt marsh zones subjected to almost daily inundation by saline water, to those on upper marsh areas that are submerged in saline water during high spring and autumn tides. Upper salt marsh communities are more diverse and usually contain salt-tolerant ecotypes of a small number of glycophytes, in addition to highly adapted halophytic species, which must have a very long evolutionary history of adaptation in such habitats (Ashraf, 1994). At the other extreme, the growth of salt sensitive glycophytes (plant which grow in non-saline soils where the water is sweet rather than saline) is severely limited at concentrations as low as 50 mM NaCl (Gorham, 1992).

Most crop species upon which we rely for food are glycophytes which have no pre-history in saline habitats and hence they have no requirement of adaptation to salt. In addition, most glycophytes have developed in soils with a low Na^{+} content, and lack the mechanisms required to tolerate the water deficit and the ion excesses prevailing in saline soil (Greenway & Munns, 1980). Moreover, many glycophytes are particularly intolerant of salts being inhibited by NaCl concentrations too low (25-50 mM) to produce a significant water deficit (Lessani and Marschner, 1978).

Crop species have been categorized by Maas and Hoffman (1977) into four groups on the basis of their tolerance: tolerant, moderately tolerant, moderately sensitive and sensitive. Barley, cotton, sugar beet are tolerant because their threshold salinity level (maximum

salinity without yield loss) ranges from 6.9 to 8.0 dS m^{-1}, whereas wheat, soyabean are moderately tolerant, their threshold salinity level ranging from 4.9 to 6.8 dS m^{-1}. Moderately sensitive crops include potato, tomato, sugar cane and rice, whereas most fruit trees, carrot and onion are in the sensitive group where the threshold salinity level is below 2.0 dS m^{-1}.

1.4 The Deleterious Effect of Salts on Plant Cells

The deleterious effect of salt on plant cells has three components: osmotic stress, ion toxicity (Na^+ and Cl^-) and disturbance of mineral ion balance (Ashraf, 1994).

Under saline conditions, the low osmotic potential of salt solutions makes it necessary for plants exposed to these media to adjust their osmotic potential, otherwise they would experience osmotic desiccation, because water would move osmotically from the cells into the soil (Greenway and Munns, 1980; Maas and Nieman, 1978; and Flowers, *et al.*, 1977). Because the growth of cells is primarily correlated with turgor potential, decreased turgor is the major cause of inhibition of plant growth under saline conditions (Ashraf, 1994). Therefore, transfer of a salt-sensitive plant from a non saline to a highly saline medium will result in rapid and irrecoverable wilting (Gorham, 1992).

The major response of halophytes and other salt-tolerant plants to high salinity is osmotic adjustment that can occur due to ion uptake from the soil solution or by internal synthesis of organic solutes. However, to regain turgor , a desiccated plant cell must reverse the water potential gradient (outside low) so that water flows back into the cell. This is achieved by the accumulation of osmotically active solutes. An energetically cheap way of achieving this is to take up Na^+ and Cl^- ions from the external medium and sequester them in the vacuole. If the solute potential of the vacuole (ψ_s^{vac}) can be made more negative than that of the surrounding soil, water will flow in and turgor will rise. However, for the cytoplasm to rehydrate, it is necessary for ψ_s^{cyt} to decrease in parallel to ψ_s^{vac}, and this is achieved by the accumulation of non-toxic compatible solutes (e.g. glycine betaine, proline, sugars; Flowers *et al.*, 1977). By contrast, some glycophytes are unable to adapt osmotically under salt stress because of reduced accumulation of ion from

the external medium. However, these organic compounds are considered as major organic osmotica in many higher glycophytes (Ashraf, 1994).

The second problem is that unless the influx of salt is regulated it will rapidly reach toxic concentrations within the plant. Although transpiration is reduced by salinity, in some cases it is sufficient to saturate the aeral parts of the plant with toxic levels of NaCl within a few days unless there is a barrier to the entry of salt (Gorham, 1992). Salt tolerance is thus largely a question of maintaining a balance between allowing sufficient salt to enter the shoot for osmotic adjustment, and preventing the accumulation of toxic levels within the plant (Gorham, 1992). However, the adaptation of plants to NaCl involves metabolic reactions (synthesis of organic solutes) and transport phenomena such as ion extrusion at the plasma membrane and vacuolar compartmentation (Serrano and Gaxiola, 1994).

Therefore, total ion activity and the relative proportion of ions in the external environment have considerable adverse effects on plant growth. Ions that are often found in excess in saline soils include Cl^-, SO_4^{2-}, HCO^{2+} Na^+, Ca^{2+}, and Mg^{2+}, whereas K^+ and NO_3 are found less often (Ashraf, 1994). High internal salt concentrations are reported to be responsible for the reduction in growth and death of salt-sensitive plants (Gorham, 1992). Salt may also interfere with the ability of the plants to acquire or assimilate essential nutrients (Grattan and Grieve, 1992). In other words, excess Na^+, and Cl^- may disturb mineral nutrition by inhibiting the uptake of essential nutrient ions such as potassium, nitrates, calcium, magnesium, and phosphate (Greenway and Munns, 1980; Lynch and Lauchli, 1985; Gorham, 1992). Moreover, uptake and translocation of K^+ and Ca^{2+} are greatly reduced by salt stress (Kuiper, 1984).

Halophytes are distinguished from glycophytes by their ability to accumulate ions to high concentrations, particularly in the leaf cells (Flowers, *et al.*, 1977). Therefore, the ability of most salt-tolerant species to accumulate high tissue salt concentrations is at least as important as the ability to restrict uptake (Gorham, 1992).

Several different physiological mechanisms of tolerance enable higher plants to tolerate high level of salts. Salt inclusion and salt exclusion have been recognized in different plants in relation to salinity tolerance (Maas and Nieman, 1978). Salt excluders have the ability to restrict the uptake of salts into the shoot. This may be due to low rates of salt

uptake, active efflux to the soil, or to both (Winter, 1982; Winter and Preston, 1982). By contrast, salt includers take up large quantities of salt and store it in the shoot. In this case, the high amounts of salts in the cytosol of the plant cells present problems for many physiological/biochemical events taking place there. Therefore, all salt includers carry out compartmentation of salts into the vacuole and become succulent. Other salt includer species possess special glands on their leaf or stem surface to excrete high concentrations of salts, for example, the salt bladders of the *Atriplex* species.

1.5. Physiological Strategies for Resistance to Salinity.

Three key physiological strategies are found in salt-resistant plants that confer some measure of tolerance. These are:-

1. Maintenance of a high cytoplasmic K^+/Na^+ ratio.

2. Maintenance of a low cytoplasmic Cl^- concentration.

3. Tolerance and/or avoidance of desiccation (osmoregulation).

1.5.1. Maintenance of High Cytoplasmic K^+/Na^+ Ratios.

Several separate mechanisms may be involved here. These are as follows.

1.5.1.1. Better Discrimination for K^+ Uptake

Cellular K^+ uptake plays a major role in plant growth and development (Mengel and Kirkby, 1982). Essential physiological functions of K^+ fluxes in plant cells include enzyme activation, osmoregulation, control of membrane potential, turgor-controlled whole-leaf movements such as solar trashing, and opening and closing of stomatal pores. Potassium uptake transporters in plant cells interact with several other metals (cation), such as Na^+ and Al^{3+}, that are detrimental to plant growth (Greenway and Munns, 1980).

Uptake of K^+ is mediated by both high-affinity and low-affinity mechanisms. High-affinity pathways are utilized when the extracellular K^+ concentration is in the micromolar range; low-affinity pathways operate when the extracelluar K^+ concentration is in the millimolar range (Assmann and Haubrick, 1996).

(a). High-affinity K^+-uptake Mechanism.

High-affinity uptake of K^+ is especially important in the transport of K^+ from the roots (Schroeder, *et al,* 1994) . The first high-affinity K^+ transporter gene was recently isolated by complementation of a K^+ transport deficient yeast with a cDNA library made from wheat roots growing in low K^+ (Schroeder *et al,* 1994). *In situ* hybridisation showed expression of *hkt1* to be localized in cortical cells of roots and mesophyll cells along the vascular tissue in leaves (Fig. 1.1). *hkt1* mRNA was expressed in *Xenopus* oocytes and the resultant currents were initially interpreted as arising from a H^+/K^+ symport (Schachtman and Schroeder, 1994). However, a subsequent study by the same group indicated that this was a misinterpretation of the data, and provides evidence that *HKT1* actually functions as an Na^+/K^+ co-transporter (Rubio, *et al.,* 1995). This result is unexpected, given the known toxicity of Na^+ to plants. In support of this controversial conclusion is the observation that two point mutations in *hkt1* were identified that increase Na^+ tolerance in *hkt1*-complemented yeast cells relative to yeast cells complemented wild-type *hkt1* (Rubio, *et al.,* 1995). Atomic absorption spectrophotometry showed that Na^+-resistant mutants have higher K^+:Na^+ ratios than yeast cells expressing wild-type *hkt1* (Rubio, *et al.*, 1995). It will be of interest to ascertain whether such mutations can be exploited to improve the salinity tolerance of crop species.

(b). Low-affinity K^+-uptake Mechanism.

The pathway for low-affinity K^+ uptake is believed to be provided by inward-rectifying K^+ channels, allowing potassium to enter along an electrochemical gradient when potassium in the soil is relatively abundant (Smart, *et al.*, 1996). Rectification is a property of some ion channels whereby they are open at only some potentials and are closed at others, resulting in the passing of current in one direction only. The first two

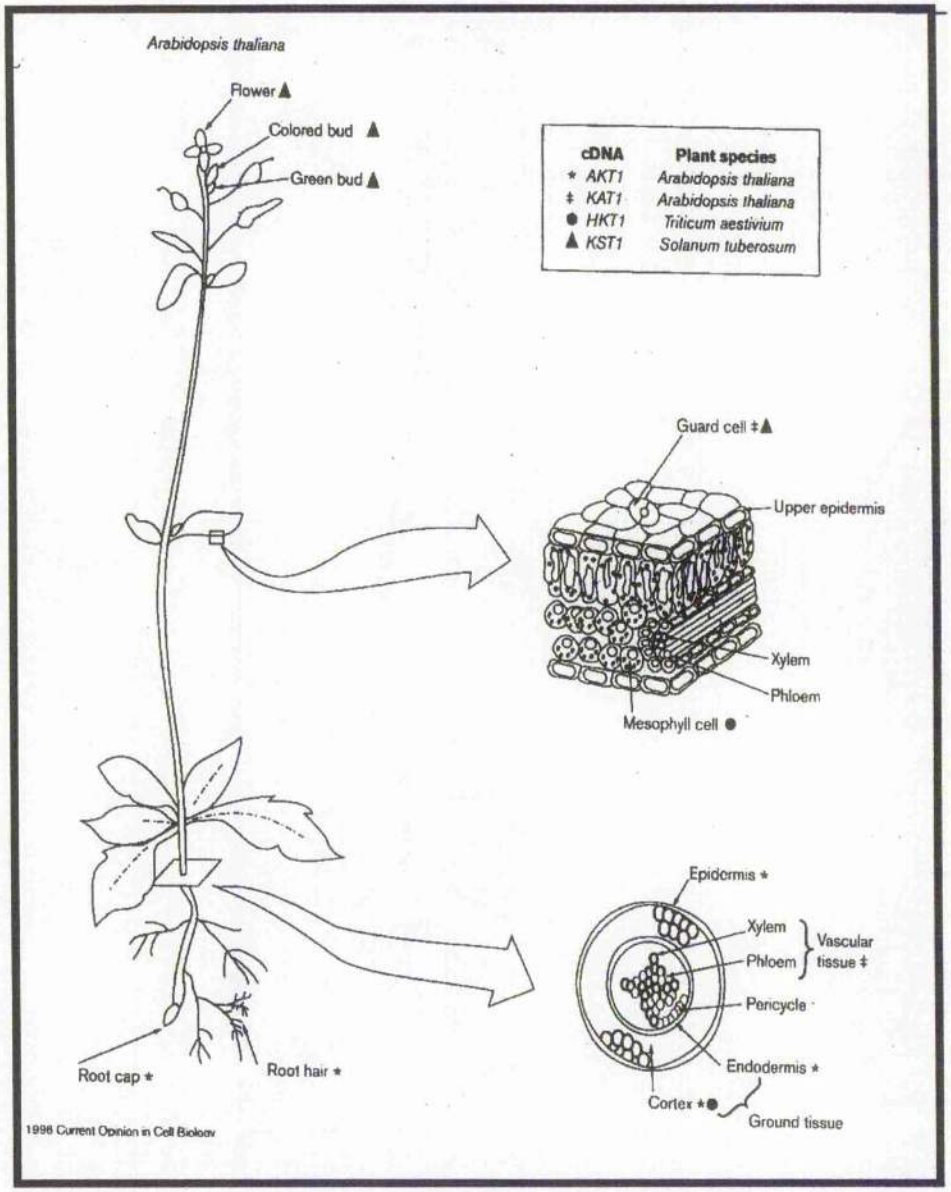


Figure. 1.1. The Localization of K^+ Transporters in *Arabidopsis thaliana.*

General anatomy of a dicotyledonous plant, as exemplified by the model plant *Arabidopsis thaliana.* The localization of known plant K^+ transporters in leaves, stems and roots are shown.
(From Assman and Haubrick, 1996).

plant genes encoding K^+ channels were reported in 1992. The genes *akt1* (Sentenac *et al.*, 1992) and *kat1* (Anderson, *et al.*, 1992) were both cloned from *Arabidopsis thaliana* cDNA libraries by complementing yeast mutants lacking endogenous potassium transporters and screening for growth on a low K^+ media.

Histochemical analysis of transgenic *Arabidopsis* plants containing the *kat1* promoter fused to the β-glucoronidase reporter gene has shown that *kat1* is primarily expressed in guard cells (Nakamura, *et al.*, 1995; see Figure 1). As the influx of K^+ into guard cells is involved in stomatal opening, the expression pattern of *kat1* suggests that it may represent the inward K^+ channel involved in the control of stomatal apertures (Nakamura, *et al.*, 1995; Assmann and Haubrick, 1996). Constructs of the *atk1* promoter and the *β*-glucoronidase reporter genes show that this channel is localized to non-vascular tissue of mature root (Lagarde, *et al.*, 1996).

In addition to *kat1* and *akt1*, two other plant K^+ channel genes have been cloned. The genes *akt2* and *akt3* were independently identified by screening of *Arabidopsis* cDNA and genomic libraries with *kat1* fragments and degenerate oligonucleotides from the conserved pore region (Ketchum and Slayman, 1996).

The protein KST1 is a major inward rectifying K^+ channel in *Solanum tuberosum* and was identified using *kat1* to screen a cDNA library made from epidermal fragments (Muller-Rober, *et al.*, 1995). The predicted *kst1* sequence has an amino acid similarity of 81% with *kat1* and 67% with *akt1*. Muller-Rober, *et al.*, 1995 demonstrated that *kst1* was found to be expressed in green, coloured, and open flower buds, epidermal fragments, sink leaves, guard cells and all floral organs (Figure 1). Additionally, Southern analysis indicated that *kst1* has two copies in the potato genome (Muller-Rober, *et al.*, 1995). Patch clamp measurements have identified inward rectifying K^+ channels capable of mediating K^+ uptake. Further, *kst1* is sensitive to low pH, and when coupled with a H^+-ATPase provide an ideal mechanism for the uptake of K^+ into cells (Muller-Rober, *et al.*, 1995). In line with the properties of a K^+-selective channel, KST1 cation selectivity was determined as $K^+ > Rb^+$ and NH_4^+ ; Na^+ and Li^+ are not transported.

Locating the pathway for sodium influx in higher plants has so far proved somewhat elusive. Modification of sodium influx (essentially 'blocking a leak') may prove more

energy efficient that letting sodium in and then pumping it out again, and this may prove to be an important strategy for engineering sodium resistance in plants.

Gorham, *et al.*, 1986 demonstrated that under moderately saline conditions hexaploid wheats (AABBDD genome) accumulate less sodium and more potassium in expanding and young leaves than tetraploid (AABB) wheat. This K^+ : Na^+ discrimination factor has been recognized in the D-genome *Triticum* and a series of investigations has localized the trait to the 4D chromosome through the use of chromosome substitution lines in which B-genome chromosomes are replaced singly by their D-genome homologues (Gorham *et al.*, 1987).

Examination of whether differences in the permeability of Na^+ through K^+ uptake channels can account for differences K^+/Na^+ discrimination have been made. Patch-clamp studies in wheat and other glycophytes have found little sodium permeation through voltage-gated potassium channels (Schachtman *et al.*, 1991; Gassmann and Schroeder, 1994; Tyerman *et al.*, 1997). Experiments using whole plants have indicated that transport of $^{22}Na^+$ to the shoot tissue is reduced in plants containing this discrimination trait (Gorham, *et al.*, 1990). Ion transport across the plasma membrane of root cells is responsible for solute distribution at the whole plant level.

Membrane-potential ($\Delta\psi$) –dependent uptake of Na^+ and Na^+/H^+ antiport activity by plasma membrane vesicles were measured in a tetraploid (AABB) and a hexaploid (AABBDD) wheat. Inhibition of Na^+ influx by Ca^{2+} was greater in the hexaploid, but overall it was concluded that neither of the measured transport processes was responsible for the discrimination trait on 4DL (Allen, *et al.*, 1995).

The potassium homeostasis of plant cells seems to be controlled by feedback inhibition of the uptake system by intracellular potassium (Glass, 1976.& 1983). This could be caused by the highly co-operative binding of K^+ to an intracellular allosteric site of the channel which when occupied blocks transport (Glass, 1976). It was reported that the roots of a moderate-tolerant tomato species (*Lycopersicon cheesmanii*) and moderately-tolerant corn (*Zea mexican*) exhibited a higher rate of potassium uptake than the roots of a salt-sensitive domestic tomato (*L. esculentum*) and a salt-sensitive corn variety (Rush and Epstein,

1981; Hajibagheri, *et al.*, 1989). Also, salt-adapted tobacco cells have enhanced K^+ uptake capacity (Watad, *et al.*, 1991).

1.5.1.2. Active Na^+ Efflux from Cells.

Sodium is relatively abundant, and K^+ relatively scarce, in most natural environments, but within the protoplasm of living cells the converse is true. As living cells are not completely impermeable to Na^+, the low concentration of Na^+ in the protoplasm requires its continuous extrusion, usually against an electrochemical gradient (Rodriguez-Navarro, *et al.*, 1994). Therefore, active extrusion of sodium occurs by either a primary active Na^+-pumping (ATPase?) or a secondary active (Na^+/H^+ antiport?) mechanism coupled to an electrochemical proton gradient (Serrano and Gaxiola, 1994).

1.5.1.2.1. Primary Active Pumps

Most living cells exclude Na^+, and create a Na^+-concentration gradient across their cell membrane. In animal cells, this gradient is generated by the (Na^+, K^+)-ATPases (Skou, 1988) which plays a role in most active transport mechanisms that are Na^+ coupled. In addition, Na^+/H^+ antiporters often function to maintain cell pH homeostasis (Gristein, 1988). In contrast, in eukaryotic cells with walls, the membrane potential is generated by a H^+-pumping ATPase (Serrano, 1985). Consequently, sodium-pumping ATPase are not widespread in non-animal cells, where sodium extrusion is probably mediated by an Na^+/H^+ antiport mechanism (Serrano, 1985 & 1991; Padan, 1992). Therefore, in plant cells, both an Na^+/H^+ antiport mechanism (Colombo, *et al.*, 1979; Hassidim, *et al.*, 1990) and a primary sodium pump coupled to ATP hydrolysis (Cheeseman, 1982) have been proposed. Evidence for these systems, however, is very indirect and no information exists on either the proteins or the genes involved.

In 1954, Edward J. Conway and co-workers demonestrated that yeast cells are capable of actively and specifically excluding Na^+, by a system independent of that mediating K^+

uptake. Since then, many reports on the biochemical characteristics of this transport system failed to establish unambiguously the mechanisms involved (Eddy, 1982).

Although, precise details of the mechanisms for K^+ and Na^+ transport in Na^+ resistant plant cells is not available, a model has been established in yeast. Genes encoding the K^+ and Na^+ transport systems have now been cloned (Gaber, *et al.*, 1988, Haro, *et al.*, 1991, Ko and Gaber, 1991; Garciadeblas, *et al.*, 1993).

Haro, *et al.*, (1991) demonstrated that Na^+ tolerance in yeast showed a large dependence on *ena1*, a gene encoding a putative Na^+-ATPase, and on *trk1*, a gene required for the expression of high-affinity K^+ uptake (Gaber, *et al.*, 1988). Transformation of the acutely Na^+-sensitive *S. cerevisiae Δtrk1Δena1* double mutant with the *trk1* and *ena1* genes, restored both the discrimination between K^+ and Na^+, and Na^+ efflux capacity indicating that these two genes play a crucial role in Na^+ resistance (Haro, *et al.*, 1993).

The question now is whether homologous genes result in a similar response in salt-resistant plants. It seems evident that although many mechanisms (including some not considered here) can protect plants from Na^+ stress, the exclusion of Na^+ from the root, either by decreasing influx or increasing efflux, would be a very effective mechanism of protection (Gorham, 1992). The increased Na^+-tolerance of a *Nicotiana tabaccum* cell culture line was attributed to an enhanced capacity to take up K^+ (Watad, *et al.*, 1991). Clearly, identification of the genes and their corresponding protein that code for K^+ and Na^+ transport could be used in protein engineering programs to reduce the salt sensitivity of some plant species.

The *ena1* (*pmr2*) gene which plays a central role in Na^+ and Li^+ tolerance in *S. cerevisiae* (Garciadeblas, *et al.*, 1993) was cloned by its ability to complement a low Li^+ efflux yeast strain for growth on high Li^+ (Haro *et al.*, 1991). In addition, a second Na^+-ATPase gene, *ena2*, has now been isolated from *S. cerevisiae* (Garciadeblas, *et al.*, 1993). The putative protein encoded by *ena2* differs only in thirteen amino acids from the protein encoded by *ena1* (Garciadeblas *et al.*, 1993). However, *ena1* and *ena2* are the first two genes of a tandem array of four highly homologous genes (*ena1-ena4*) which probably

have similar function (Garciadeblas, *et al.*, 1993). However, the major pathway for Na^+ efflux in *S. cerevisiae* appears to be mediated by ENA1, and it has been estimated that the combination of ENA2, ENA3 and ENA4 accounts for about half of the activity (Garciadeblas, *et al.*, 1993). Homologues of *ena1* may occur in plants, as suggested by Cheeseman (1982). Over-expression of *ena1* in *S. cerevisiae* was achieved with a construct of the *ena1* promoter driving the *ena1* structural gene (Benito, 1997). The phenotype of this transformant was as follows: 5-fold higher content of the ENA1-protein in plasma membrane; lower Na^+ and Li^+ effluxes; slightly higher Na^+ tolerance and much higher Li^+ tolerance (Benito, 1997).

Banuelos *et al.*, (1995) demonstrated the functional expression of *ena1* of *S. cerevisiae* in *Schizosaccharomyces pombe* by transforming the *ena1* gene into a *sod2::ura4* deletion mutant of *S. pombe*. The ENA1 protein was expressed and surviving colonies recovered on high Na^+ media, demonstrating that Na^+-ATPases (ENA1) and Na^+ / H^+ antiporters (SOD2) provide an equally efficient mechanism for Na^+ efflux.

1.5.1.2.1.1. Plasma Membrane ATPase

In cells of higher plant and fungi, the major ion pump in the plasma membrane is a p-type H^+-ATPase (Serrano, 1985, 1990, 1991; Sussman, 1994). In animal cells the major ion pump is a Na^+/K^+-ATPase. The plasma membrane has a primary role in the coupling of metabolic energy to solute transport across the plant cell membrane (Leonard, 1984) and the p-type H^+ATPase couples the energy released by ATP hydrolysis to the translocation of H^+ to the cell exterior. Through this activity, the p-type H^+-ATPase functions to establish an inwardly-directed proton electrochemical gradient across the plasma membrane which consists of an acid-exterior (pH gradient) and negative-interior membrane electrical potential gradient (Sze, 1985). Coupling of the potential energy conserved in this proton electrochemical gradient ($\Delta\mu_H$) established by the H^+-ATPase to the energetically 'uphill' movement of other solutes is believed to be mediated by other secondary transport systems associated with the plasma membrane, which can act as H^+ /solute symports, antiports or electrically driven uniports (Leonard, 1984).

The basic function of these H^+ pumps is to energize the plasma membrane to regulate intracellular and extracellular pH, drive active nutrient uptake (chemical balance), and modulate cell turgor (Serrano, 1989 and DeWitt, 1994). Proton pumps also influence cellular elongation. When the H^+-ATPases in the plasma membrane are stimulated, the outward transport of hydrogen ions decreases the pH in the surrounding cell wall; this causes certain enzymes in the cell wall, which are activated at a lower pH, to begin to break cross-linking between microfibrils. This degradation loosens the cell wall, thereby allowing the cell to expand because of turgor pressure (Moore, *et al.*, 1998).

The *in vivo* activity of the p-type H^+-ATPase, as assessed by proton efflux measurement, seems to increase during osmotic adaptation in carrot (Reuveni *et al.*, 1987) and tobacco (Watad *et al.*, 1986) cell cultures. However, it has been demonstrated that the halophytic plant *Atriplex nummularia*, when exposed to high salinity during growth, the *in vitro* activity of the plasma membrane p-type H^+-ATPase increases. Apparently, this phenomenon does not occur in non halophytic plant (Braun, *et al.*, 1986).

Several plant genes have now been cloned which encode proteins with structural characteristics of p-type cation-translocating ATPase. It has been recognized that there was not just one gene and corresponding polypeptide, but rather a multigene family (Assmann and Haubrick, 1996). Harper *et al.*, (1994) reported that the *Arabidopsis* H^+-ATPase genes family has at least ten members. In addition, a recent study indicates there are a minimum of seven genomic *Lycopersicon* H^+-ATPase (*lha*) sequences encoding plasma membrane in tomato (Ewing and Bennett, 1994), and at least four plasma membrane H^+-ATPase (*pma*) gene have been identified in tobacco (Boutry, *et al.* 1989).

Using information from protein sequences, as well as knowledge of a highly conserved acid sequence found in all p-type H^+-ATPases, an oligonucleotide was synthesised and used to isolate clones encoding H^+-ATPase from an oat cDNA library (Harper and Sussman, 1989). The oat clone was then used to screen *A. thaliana* cDNA and genomic libraries from which different gene clones were isolated, *aha1*, *aha2* and *aha3* for *Arabidopsis* H^+-ATPase (Sussman, *et al.*, 1991). It is presumed that different family members will have different promoter sequences, allowing expression in a cell-specific, tissue-specific or developmental-stage-specific manner (Sussman, 1994). The location of

the *aha3* gene product was studied in *Arabidopsis* by expression of a construct of the *aha3* promoter sequence 5' of the β -glucuoronidase (GUS) reporter gene; in this case expression of GUS was found to be located on the plasma membrane of companion cells of vegetative tissue (DeWitt *et al.*, 1991; DeWitt and Sussman, 1995). Histochemical localization of GUS activity under the light microscope demonstrated that *aha-2* is expressed in root hairs and surface tissues of the root (Sussman, 1994). Furthermore, *aha9,* appears to be restricted to stamens (Michelet, *et al.*, 1994), and *aha10* to integument layers of developing seed (Harper, *et al.*, 1994). In conclusion, it is believed that several H^+-ATPase generate the proton-motive force used by sucrose carriers in the phloem, *aha-2* encodes the one that drives mineral absorption from the soil into roots, *aha-10* is involved in the flow of nutrients that nourish the embryo, and *aha-9* provides the proton-motive force involved in anther function (Sussman, 1994).

There are, in addition, two other clones which are much more distantly related to the *aha* gene family (~22% identity to *aha1*; Huang, *et al.*, 1994), and are, therefore, assigned the abbreviations *axa1* and *axa2* to signify the possibility that they represent a p-type ATPase which transports cations other than protons (Sussman, 1991).

Ewing *et al.*, 1990 identified and characterized the first two cDNA clones (*lha1* and *lha2*) from tomato (*Lycopercsion esculentum*) which probably encode isoforms of plasma membrane H^+-ATPase. Recently, Ewing and Bennett, (1994) identified seven genomic sequences (designated *lha1-lha7*) that encode isoforms of tomato (*Lycopercion esculentum*) plasma membrane H^+-ATPase by screening a genomic library with the highly conserved ATP-binding domain of the cDNA *lha1*. Three of these genes are expressed differentially in plant. However, *lha1* is expressed at similar levels in all tissues examined, *lha2* is most highly expressed in immature and mature leaves and *lha4* is most highly expressed in roots and hypocotyls. In addition, the isoforms *lha3, lha5, lha6, lha7* are expressed at very low or undetectable levels in all organs examined (Ewing and Bennett, 1994).

Boutry, *et al.*, (1989) reported the isolation and preliminary characterization of three different clones from a root cDNA library of *Nicotiana plumbaginifolia* encoding distinct isoforms of H^+-ATPases. However, these isoforms were isolated by hybridisation to *S. cerevisiae* H^+-ATPase genes (*pma*; Boutry, *et al.*, 1989). The largest one (*pma2*) exhibits

a homology of 73 % at the amino acid level with a limited protein sequence obtained from purified oat plasma membrane H^+-ATPase (Schaller and Sussman, 1988), and an 82 % similarity with the *Arabidopsis thaliana aha* genes (Harper, *et al.*, 1989). It is, therefore, concluded that the *N. plumbaginifolia pma2* gene encodes a plasma membrane H^+-ATPase (Boutry, *et al.*, 1989). Three members of the *pma* gene family of p-type H^+-ATPases have now been isolated from genomic and cDNA libraries of *N. plumbaginifolia* (Perez *et al.*, 1992). The three genes are between 65 % and 96 % identical at the deduced amino acid sequence level, and because of their high similarity, it is most likely that these three genes encode H^+-translocating ATPases with similar function. The three genes are expressed in leaf, stem, flower and root tissues, albeit at different levels, according to the organ and gene (Perez, *et al.*, 1992). A fourth gene *pma4* has now been identified with an even higher amino sequence identity (95 %-97 %) with the *Arabidopsis aha1-3* genes (Moriau, *et al.*, 1993) suggesting there may be two *pma* sub-families. Measured *pma4* transcript levels indicated that this gene is expressed at similar levels in root, stem, leaf, and flower tissues (Moriau, *et al.*, 1993), contrary to the *pma1-3* subfamily which display organ-specific differential expression (Perez, *et al.*, 1992).

1.5.1.2.1.2. Vacuolar ATPase

In plant cells electrogenic H^+ pumps play a central role in energizing the plasma membrane as well as the vacuolar membrane (Sze, 1985). The plasma membrane p-type H^+-ATPase extrudes H^+ from the cell, forming a membrane potential (negative inside) and a pH (acid outside) gradient. Two distinct electrogenic H^+ pumps acidify the vacuolar compartment: a H^+-ATPase (Rudnick, 1986) and a H^+-PPase (O'Neill and Spanswick, 1984). Ion transport across the tonoplast of plant cells play an important role in the control of cell homeostasis, cytoplasmic pH regulation, sequestration of toxic ions and xenobiotics, regulation of cell turgor, and the storage of amino acids, sugars and CO_2 in the form of malate, and finally possibly as a source for elevating cytoplasmic calcium (Raschke, *et al.*, 1988). All of these activities are driven by the two primary active H^+-transport mechanisms present in the vacuolar membrane. These two mechanisms employ high energy metabolites to pump protons into the vacuole, establishing a proton

electrochemical potential that provides the driving force for secondary transport of numerous ions and metabolites (Barkla and Pantoja, 1996). Plant vacuolar, or v-type H^+-ATPases represent a ubiquitous class of proton pumps that are found on a variety of cellular organelles including lysosomes, endosomes, secretory and storage vesicles and protein storage organelles as well as the tonoplast of higher plants, fungi and yeast (Sze, *et al.*, 1992). On the other hand, the H^+-PPase is abundant only in the vacuolar membranes of plant cells, where it establishes a H^+ gradient of similar, and often greater, magnitude than the H^+-ATPase on the same membrane (Rea, *et al.*, 1992). However, unlike the v-type H^+-ATPase, it derives free energy from the hydrolysis of cytoplasmic pyrophosphate (PPi) and appears to be present only in plants and phototrophic bacteria (Barkla and Pantoja, 1996).

In plant cells accumulating NaCl, the driving force for secondary active Na^+ transport into the vacuole is believed to be provided by the PMF (proton motive force) generated across the tonoplast by the v-type H^+-ATPase and the v-PPase, and both H^+ pumps may therefore, play a fundamental role in energizing Na^+/H^+ antiport activity in cells accumulating significant quantities of NaCl (Barkla and Pantoja, 1996). Several groups have suggested mechanisms for the regulation of v-type H^+-ATPase and v-PPase. Matsumoto and Chung, (1988), suggested that exposure of barley roots to NaCl (200 mM) induce the synthesis of v-type H^+-ATPase subunits, thus accounting for the increased transport activity. Barkla, *et al.*, 1995 demonstrated that in the halophytic plant *M. crystallinum*, growth in 400 mM NaCl increases the activity and the level of expression of the v-type H^+-ATPase but he found that the activity and the level of expression of the v-PPase enzyme decreased.

Plants have been shown to have several isoforms for the different subunit of the v-type H^+-ATPase. In carrot, three different genes for 70-KDa catalytic nucleotide-binding subunit A have been cloned and sequenced (Zimniak, *et al.*, 1988; Gogarten, *et al.*, 1992). Four genes encoding the 70-KDa subunit in tobacco have been cloned (Nanda, *et al.*, 1992). In Barley, two different clones for the B subunit were identified (Berkelman, *et al.*, 1994). Moreover, Manolson, *et al.*, (1988) reported the cloning of cDNA encoding the plant sequence for the 57-KDa subunit B from *Arabidopsis thaliana*. However, in

Arabidopsis thaliana a single copy encoding the v-PPase has been cloned (Sarafian, *et al.*, 1992).

1.5.1.2.2. Secondary Active Transport (Na^+/H^+ antiporters).

Antiporters are secondary transporters, membrane proteins that couple electrochemical gradients of ions or organic solutes to drive energetically 'uphill' transport of other solutes (Padan and Schuldiner, 1994). Furthermore, antiporters play a vital role in all living cells. In most bacteria, they are involved in the pH homeostasis of the cytoplasm, as well as in the extrusion of Na^+ which, at high concentrations, is toxic to the cytoplasm (Padan and schuldiner, 1993a).

Moreover, Na^+/ H^+ antiporters are ubiquitous membrane transport proteins. They have been found in the cytoplasmic membrane of microorganisms and animal cells (Padan and Schuldiner, 1993b, 1994). They have also been found in membranes of various intracellular organelles, in mitochondria, in plant vacuoles and in the storage granules of animal cells (Padan and Schuldiner, 1994).

The driving force for the Na^+/H^+ antiporter in bacterial cells is an electrochemical potential of H^+ across the membranes which is established mainly by the function of the respiratory chain. The driving force for the antiporter in mammalian cells (usually called exchanger) is an electrochemical potential of Na^+ which is established by the function of Na^+, K^+-ATPase. The antiporters in *Escherichia coli* have been studied extensively and to date the genes for three cell membrane antiporters have been identified *nhaA* (Karpel, *et al.*, 1988), *nhaB* (Pinner, *et al.*, 1992) and *chaA* (Ivey, *et al.*, 1993).

1.5.1.2.2.1. Sodium Efflux in Bacteria

The molecular biology of the Na^+/H^+ antiporters in prokaryotes was initiated by the study of an *E. coli* mutant which led to the development of a general strategy for the cloning of antiporter genes from bacteria by functional complementation (Pinner, *et al.*, 1992). When the *E. coli* gene *nhaA* is present in high copy number it confers resistance to host

cells and increases their Na^+/H^+ antiport activity in membranes. A *ΔnhaA* knock-out strain of *E. coli* was constructed by transposon-mediated inactivation of the endogenous *nhaA* gene. The *ΔnhaA* mutant was hypersensitive to Na^+ (and Li^+) in the growth media and provides an excellent system for cloning bacterial antiporter genes by functional complementation (Pinner, *et al.*, 1992).

A second (*ΔnhaB*) *E. coli* gene coding for a Na^+ / H^+ antiporter has now been identified; hydropathy analysis indicates the *nhaB* gene product has 12 putative transmembrane helices (Pinner, *et al.*, 1992). The *nhaA* gene is regulated by a lysR-type regulatory protein called NhaR (Rahav-Manor, *et al.*, 1992), and its transcription is stimulated by high Na^+ concentrations, and by elevated cytoplasmic pH (Karpel, *et al.*, 1991). When either of the *nhaA* or *nhaB* is present at high copy number in the cells, the Na^+/H^+ antiporter activites increase (Goldberg, *et al.*, 1987; Karpel, *et al.*, 1988 Pinner, *et al.*, 1992). It has been postulated that *nhaB* provides a backup Na^+ extrusion system in *E. coli* which is effective when *nhaA* which is inactive (Pinner, *et al.*, 1993). The stoichiometry of NHAA is estimated to be $2H^+$ for every Na^+ (Taglichit, *et al* 1993), and that of NHAB exchange to be $3H^+$ for 2 Na^+ (Padan and Schuldiner, 1994). However, NHAB differs from NHAA by having a higher affinity for the Na^+ ion, and by demonstrating pH independence over a broad range of pH (Pinner, *et al.*, 1992).

The *E. coli* deletion mutant *ΔnhaA, ΔnhaB,* and the double mutant *ΔnhaAΔnhaB,* have been constructed and are freely available (Pinner, *et al.*, 1992; Padan and Schuldiner, 1994). These mutants provide an excellent cloning system for Na^+/H^+ antiporters from other organisms by functional complementation (Padan and Schuldiner, 1994).

A novel gene (*nhaC*) has been cloned from a DNA library from the alkalinophile *Bacillus firmus* OF4, which functionally complements the *ΔnhaAΔnhaB* mutant strain of *E. coli* (Ivey, *et al*,. 1991). The *nhaC* gene confers enhanced Na^+ (Li^+) resistance upon the mutant and is accompanied by increased Na^+/H^+ antiport activity of the transformant (Ivey, *et al*,. 1991). Little homology between *nhaC* and *nhaA* exists suggesting this represents a new class of bacterial Na^+ /H^+ antiporter. Although, there was no significant similarity between the deduced protein products of *nhaC* and *nhaA*, there was a small

region of significant similarity between *nhaC* gene product and the protein encoded by human Na^+/H^+ antiporter gene *cd28* (Sardet, *et al*,. 1989).

The gene *nhaA* encoding Na^+/H^+ antiporter has been cloned from a DNA library of the marine bacterium *Vibrio alginolyticus* which complements the *ΔnhaA* mutant strain of *Escherichia coli* (Nakamura, *et al.*, 1994). Since the Na^+/H^+ antiporter activity of V. *alginolyticus* observed at alkaline external pH is also electrogenic (Nakamura *et al.*, 1992), the antiporter of *V. alginolyticus* is functionally similar to NHAA. *Vibrio alginolyticus* is halophilic and requires 0.5 M NaCl for optimal growth, therefore, the Na^+ /H^+ antiporter is likely to be constitutively expressed in this marine bacteria (Nakamura, *et al.*, 1994). The deduced amino acid sequence of Na^+/H^+ antiporter has a partial similarity with NHAB from *E. coli*, and NHAC from *Bacillus firmus* (Nakamura, *et al.*, 1994). A second Na^+ /H^+ antiporter has been found in the marine *V. alginolyticus* and cloned by functional complementation of the double *ΔnhaAΔnhaB* mutant strain of *E. coli* and named NHABv (Nakamura, *et al.*, 1994).

A gene encoding a Na^+/H^+ antiporter, *nhaB*, was cloned from *V. parahaemolyticus* by functional complementation of *E. coli* cells (Nozaki,*et al.*, 1996). The hydropathy profile of the resulting predicted protein suggested the presence of 12 membrane-spanning domains. The amino acid sequence of NHAB showed high homology with the sequence of the NHAB Na^+/H^+ antiporter of *E. coli* (Pinner, *et al.*, 1992) and of *Haemophilus influenzae* (Fleischmann, 1995). Furthermore, the NHAB Na^+/H^+ antiprort system of *V. parahaemolyticus* is similar to that of NHAA of *E. coli* and V. *parahaemolyticus* with respect to its pH dependence (active at pH 8.5 but not at pH 7.0), but differs from that of the *E. coli* NHAB system (active at both pH 7.0 and 8.5; Nozaki *et al.*, 1996). Moreover, no significant sequence similarity was found between NHAB and the NHAA of *V. parahaemolyticus*. Thus, these two systems appear to be unrelated, although their function is similar (Nozaki, *et al.*, 1996).

During the course of studies on these antiporters the gene coding for a third Na^+/H^+ antiporter (NHAD) has been isolated from *V. parahaemolyticus* (Nozaki, *et al.*, 1998). It has been demonstrated that the activity of this antiporter is pH-dependent with highest activity in the range pH 8.5 to 9.0, and no activity at pH below pH 7.5. Thus the NHAA and NHAB Na^+/H^+ antiporters found in *Vibrio* species have similar pH profiles (Nozaki,

et al., 1998), but in contrast *V. alginolyticus* does not appear to posses a homologue of *nhaD* (Nozaki, *et al.*, 1998).

In gram-positive (fermenting anaerobic) bacteria such as *Enterococcus hirae*, sodium can be extruded by two transport mechanisms: a primary Na^+-ATPase and secondary Na^+/H^+ antiport (Kakinuma and Igarashi, 1989). Both systems are viewed to be important in Na^+ excretion, the Na^+/H^+antiporter at neutral and acidic pH, and the Na^+-ATPase at alkaline pH where $\Delta\mu_H$ is limiting (Kakinuma, 1987). However, using the double mutant of *E. hirae* that is defective in both mechanisms, the *napA* gene was cloned by complementation which codes for a hydrophobic protein of 383 amino acids containing 12 probable transmembraneous helices (Waser, *et al.*, 1992). Disruption of the *napA* gene leads to the loss of Na^+/H^+ exchange activity as measured in whole cells or membrane vesicles (Waser, *et al.*, 1992). Moreover, the NAPA antiporter shows significant homology to the K^+/H^+ antiporter in *E. coli* (Reizer, *et al.*, 1992), and only weak homology to NHAA and NAHB. The NAPA protein exhibits properties different from those of the two *E. coli* Na^+/H^+ antiporters encoded by *nhaA* and *nhaB*.

1.5.1.2.2.2. Sodium Efflux in Yeast

A new locus (*sod2*) was identified in *S. pombe* by expression of an *S. pombe* library in wild type cells, a screening for improved sensitivity to Li^+ (Jia, *et al.* 1992). Over-expression of *sod2* increased Na^+ export capacity and conferred Na^+ tolerance to wild type cells (Jia, *et al.*, 1992). The predicted *sod2* gene product can be placed in the broader class of transporters which possess 12 hydrophobic transmembrane domains (Jia, *et al.*, 1992) and show limited similarity to *nhaA* (Jia, *et al.*, 1992) and *nhaC* (Ivey, *et al.*, 1991). Disruption of *sod2* yields cells incapable of exporting Na^+, that are hypersensitive to Na^+ and Li^+ and which grow slowly between pH 3.5 and 7.5 (even in low Na^+ media). These results suggest the role of *sod2* in pH homeostasis and in Na^+ extrusion (Jia, *et al.*, 1992). The increasing sensitivity of the mutants with pH is reminiscent of the *ΔnhaA E. coli* strain (Padan, *et al.*, 1989; Dibrov, *et al.*, 1991). The functional expression of the *S. cerevisiae ena1* gene in *S. pombe Δsod2*$^-$ mutants restores the Na^+ and Li^+ tolerances of

this strain, suggesting that under certain conditions the two Na^+ efflux systems (Na^+-ATPase and Na^+ /H^+ antiporter) are interchangable (Banuelos, *et al.*, 1995).

Genes more closely related to *sod2* have been isolated from the marine yeast *Zygosaccharomyces rouxii* (Watanabe, *et al.*, 1995) and *Saccharomyces cerevisiae* (Prior, *et al.*, 1996). Cells of the salt-tolerant yeast *Z. rouxii* maintain a low intracellular level of sodium ions (Na^+) even when grown in medium containing high concentrations of NaCl (Onishi, 1959). Like plants, the H^+-ATPase in the plasma membrane of *Z. rouxii* participates in the extrusion of H^+ from cells (Watanabe, *et al.*, 1991). The H^+ gradient formed by its function might be utilized for the uptake of some solutes (*e.g.* sugar, amino acids, *etc.*,) *via* secondary active transport mechanisms, as described in *S. cerevisiae* (Eddy, 1982). Therefore, it is possible that the extrusion of Na^+ from the cell is also coupled to H^+ gradients across the plasma membrane. However, in order to clarify the relationship between the salt-tolerance of *Z. rouxii* and the function of Na^+/H^+ antiporters, a gene was isolated from *Z. rouxii* (Watanabe, *et al.*, 1995) which exhibited homology to the Na^+ /H^+-antiporter gene (*sod2*) from *S. pombe* (Jia, *et al.*, 1992). This newly isolated gene (*z-sod2*) encodes a product of 791 amino acids, which is larger than the product encoded by its *S. pombe* homologue. The expression of *z-sod2* was constitutive and independent of NaCl-shock. Unlike wild type cells, disruption of *z-sod2* in *Z. rouxii* severely reduced growth in 3 M NaCl with no effect on the osmotolerance (growth in 50% sorbitol), strongly implicating the gene product in salt-tolerance of *Z. rouxii* (Watanabe, *et al.*, 1995) . Knock-out mutants, *Δz-sod2*, did not decrease the salt-tolerance of *Z. rouxii* to the level which might be expected in other yeasts (*i.e. Δena1, Δsod2*), suggesting that genes other than *z-sod2* might participitate in the salt-tolerance of *Z. rouxii* (Watanabe, *et al.*, 1995).

In *S. cerevisiae* the ENA1 Na^+-ATPase is active mainly at alkaline pH values, and the existence of another system, possibly a H^+/cation antiporter, operating at acidic pH values has been predicted (Ortega and Rodriguez-Navarro, 1986). This hypothesis was partially confirmed by the observation that the disruption of all four *ena* genes did not completely eliminate Na^+ and Li^+ effluxes. However, the *nha1* gene encoding a putative Na^+ /H^+ antiporter in *S. cerevisiae* was cloned by selection based on increased NaCl tolerance

(Prior, *et al.*, 1996). The putative protein is similar to the Na^+ / H^+ antiporters from *S. pombe* (SOD2) and *Z. rouxii* (ZSOD2). Over-expression of *nha1* increased sodium and lithium tolerance mainly at acidic and neutral pH values (partially pH-dependent), whilst disruption leads to an increased sensitivity (Prior, *et al.*, 1996).

Therefore, in yeast ZSOD2 of *Z. rouxii* and NHA1 of *S. cerevisiae* are closely related to *sod2* of *S. pombe*, and in the former two, both are curical for lithium and sodium tolerance (Watanabe, *et al.*, 1995; Prior, *et al.*, 1996). Thus, *SOD2* may represent a new family of eukaryotic Na^+/H^+ exchangers that use ΔpH (acidic outside) to export Na^+ ions out of the cytosol (Dibrov, *et al.*, 1997).

1.5.1.2.2.3. Sodium Efflux in Mammals.

The plasma membrane Na^+/H^+ antiporter of higher animal eukaryotes is reported to perform the following functions: intracellular pH regulation (Aronson and Boron, 1986), regulation of intracellular volume, net transepithelial H^+ and Na^+ secretion and signal transduction (Sardet, *et al.*, 1990). In addition, the exchanger is rapidly activated in response to a variety of external signals (including sperm, growth and factors, hormones; Sardet, *et al.*, 1990), and is also involved in an array of processes in the cell cycle (Padan and Schuldiner, 1994). The Na^+/H^+ antiporter, first analyzed in kidney (Murer *et al.*, 1976), operates in all cells as a major H^+-exchanging system driven by the inward directed Na^+ chemical gradient (Aronson and Boron, 1986; Grinstein, 1988) and is inhibited by amiloride (L'Allemain, *et al.*, 1984). Moreover, it is fully active at acidic pH (pH 6 and below), and virtually turned off at neutral pH (Sardet, *et al.* 1989). A human gene *nhe1* was cloned by the functional complementing of a mouse fibroblast mutant lacking Na^+ /H^+ exchanger activity with human genomic DNA (Pouyssegur, *et al.*, 1984; Franchi, *et al.*, 1986a, 1986b; and Sardet, *et al.* 1989).

However, the *nhe-1* c-DNA clone facilitated the isolation of several isoforms referred to as *nhe-2, nhe-3, nhe-4* and *β-nhe* (Tse, *et al.*, 1991), all of which exhibit 45-70 % identity to *nhe-1* at the amino acid level and possess a similar hydropathy profile. However, the putative fourth and sixth transmembrane helices of these forms showed the

highest degree of conservation. In addition, tissue distribution studies reveal that *nhe-1* is expressed at varying levels in all tissues examined, whereas *nhe-3* is expressed mostly in kidney and intestine (Tse, *et al.*, 1992). Conversely, *nhe-4* is most abundant in stomach, with intermediate levels found in colon and small intestine, and trace amounts in kidney, brain, uterus and skeletal muscle cells (Orlowski, *et al.*, 1992).

1.5.1.2.2.4. Sodium Efflux in Plants.

Sodium / proton antiporters are postulated to function at the plasma membrane and the tonoplast of plant cells where they are envisaged to pump Na^+ out of the cytoplasm and into either the apoplast or into the vacuole (Mennen, *et al.*, 1990). In contrast to micro-organism and animal cells, the presence of a Na^+/H^+ antiporter is not an ubiquitous in plant cells (Mennen, *et al.*, 1990).

Sodium transport is better characterised at the vacuolar membrane than at the plasma membrane. Secondary active transport of Na^+ into the vacuole via the tonoplast Na^+/H^+ antiporter is believed to be energized by the PMF generated by the activity of the v-type H^+-ATPase and/or the v-PPase. In salt resistant plants, it is viewed that an active antiporter functions to sequester Na^+ into the vacuole, thereby avoiding toxic levels of Na^+ from accumulating in the cytoplasm (Barkla and Pantoja, 1996). Sodium / proton antiport activity is reported to increase with increasing salt stress (Blumwald and Poole, 1987; Garbarino and DuPont, 1988 & 1989; DuPont, 1992).

Several studies have provided evidence consistent with the existence of a Na^+/H^+ antiporter in the plasma membrane of a wide range of plant cells. Mennen, *et al.* (1990) looked for plasma membrane Na^+/H^+ antiport activity in 16 crop species and found four (sugar beet, barley, tomato and wheat) which appeared to operate this mechanism. Cooper, *et al.* (1990) found evidence for activity in a halophyte (*Atriplex nummularia*) and glycophyte plasma membranes. Allen, *et al.* (1995) measured activity consistent with a Na^+/H^+ antiport in wheat plasma membrane fractions, and similar results were obtained with barley whole roots (Ratner and Jacoby, 1986).

The transport of Na^+ from the cytosol, *via* the tonoplast antiporter, and its accumulation in the vacoule of the halophytes and salt-tolerant glycophytes is an important mechanism for averting the damaging effects of Na^+ on key biochemical processes in the cytosol. In certain plants the operation of the Na^+/H^+ antiporter at the plasma membrane excretes Na^+ from the cells, and thus acts synergistically with the tonoplast antiporter in maintaining the cytoplasmic Na^+ concentrations (Barkla and Blumwald, 1992; Jacoby, 1993).

1.5.2. Maintenance of Low Cytoplasmic Cl^- Concentration.

Chloride channels are passive anion transport proteins that are present in plasma membranes of most cells and participate in cellular functions such as regulation of cell volume and intracelluar pH. In animals, chloride channels are important for trans-epithelial transport and regulation of excitability of muscle and nerve (Hechenberger, *et al,* 1996).

Chloride is the most abundant anion in plant and animal tissues. Therefore, anion channels are often called chloride channels, even though they may be permeable to other anions as well. In contrast to cations like sodium and especially calcium, the electrochemical gradient of chloride across the animal plasma membrane is close to its electrochemical equilibrium. Thus Cl^- has no established function as a second messenger, and is only rarely involved in electrical excitation (Jentsch and Gunther, 1997). Therefore, the opening of Cl^- channels, like the opening of K^+ channels, generally results in a stabilization of membrane potential (Betz 1991; Rabow, *et al.*, 1995). While the intracellular chloride concentration is close to equilibrium, it can deviate from it by a few tens of mV. While there is no firm evidence for an active chloride-transporting ATPase in mammalian plasma membranes, several transporters use the energy stored in transmembrane gradients of other ions to move chloride against its electrochemical gradient (secondary active transport). Most of these transporters will accumulate intracellular chloride above its equilibrium level (at a membrane voltage of -60 mV and an external chloride concentration of 150 mM, internal chloride would be equilibrium).

In plants, Cl^- has essentially two defined roles. The first role relates to the control of cell volume (turgor) and the second to controlling plasma membrane electrical potential through controlled release through anion channels. The ability of cells to accumulate Cl^- underlines both these functions in plants. Consequently, it is important to understand the mechanism by which intracellular Cl^- levels are regulated and the factors that control Cl^- accumulation (Tyerman, 1992). If chloride is present in the extracellular medium, it is normally accumulated to high levels (in excess of 100 mM) in the vacuole. Thus, the ion can represent a primary component of the internal osmotic pressure, with corresponding importance for the role of Cl^- in turgor generation. This role is most marked in halophytes, where vacuolar Cl^- concentrations of the order 500 mM are not uncommon, but holds true also for many glycophytic species (Cram 1976). Nevertheless, in glycophytes Cl^- is not essentially required as an osmoticum, if Cl^- is not present in the external medium, organic acids are sequestered into the vacuole instead (Osmond, 1976 ; Van Kirk and Raschke, 1978).

1.5.2.1 Chloride Transport

Chloride transport may occur via channels (Serrano, 1985; Hedrich and Schroeder, 1989) or proton co-transport (Serrano, 1985). In the first case, the negative inside membrane potential would drive efflux from the cytoplasm, whereas in the second case, the proton gradient would drive accumulation. The molecular basis for this reaction is not known (Serrano, 1994).

Ion channels with anion selectivity are present in the plasma and vacuolar membranes of plants (Tyerman 1992). Although these channels generally exhibit rather poor selectivity for Cl^- over other anions such as NO_3^- and malate (Hedrich and Jeromin, 1992), it is generally believed that in physiological conditions Cl^- comprises the principal ionic component of currents passing through these channels when open (Sanders, 1994). In glycophytes, the equilibrium potential for Cl^- normally lies more than 200 mV positive of the resting membrane potential, therefore plasma membrane anion channels are viewed to catalyse Cl^- efflux from cells (Sanders, 1994).

Studies of cellular Cl^- release in general, and of anion channels in particular, have led to elucidation of further roles for Cl^- in plant cell biology. It is well established that anion channels, principally carrying Cl^- under most physiological conditions, respond *via* a Ca^{2+} signal cascade, to osmotic shock (Okazaki and Tazawa, 1990). Further, they are involved in volume and turgor regulation in guard cells (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992). The magnitude of the change in cellular Cl^- content during stomatal closure implies co-ordinated release of Cl^- from vacuolar stores. One vacuolar channel which is potentially responsible for mediating this release has been identified as a Ca^{2+}-activated channel which activates slowly in response to an imposed voltage and exhibits weak selectivity between cations and anions (Coyaud, *et al.*, 1987; Sanders, *et al.*, 1990).

Also, electrical signalling in plants might well involve the opening of anion channels through which a major component of the depolarizing current can flow. Chloride currents that flow as a result of the opening of Ca^{2+}-activated anion channel are involved in electrical signalling in the sensitive plant *Mimosa* (Samejima and Sibaoka, 1980), rapid movements associated with prey capture in the insectivorous plants *Drosera* and *Dionaea* (Williams and Pickard, 1972; Hodick and Sievers, 1988), and general wounding reponses (Davies, 1987), all of which are thought to involve signalling *via* action potentials.

Furthermore, chloride release across the plasma membrane also plays an indirect, but important role, in cytosolic pH regulation. Cytosolic acidosis in plants is normally countered by stimulation of the primary H^+-ATPase at the plasma membrane (Kurkdjian and Guern, 1989). This enzyme is electrogenic, and the membrane hyperpolarization that could result from the enzyme enhanced activity is potentially counter-productive: the elevated electrical driving force on H^+ across the plasma membrane would increase H^+ influx back across the plasma membrane. Therefore this tendency for membrane hyperpolarization is off-set by opening of anion channels that facilitate Cl^- release (and hence membrane depolarization) during cytosolic acidosis (Sanders, 1994). Consequently the anion channels appear to open as a result of direct activation by H^+, and through a low pH-mediated increase in Ca^{2+} affinity (Sanders, 1994).

In conclusion, there is little doubt that Cl^- plays a number of important physiological roles in plant cells, but it can also have a deleterious effect when found at high internal or

external concentrations. For example, high levels of Cl^- may interfere with the ability of plants to acquire or assimilate essential nutrient ions. Chloride is known to affect both the uptake and assimilation of nitrate (Gorham, 1992).

1.5.2.2. Classification of Chloride Channels.

Chloride channels are classified into three distinct structural classes (Jentsch, 1997). The first class to be discovered (in 1987) include glycine and GABA receptors (Grenningloh, *et al.*, 1987; Schofield, *et al.*, 1987), which belong to a super-family of ligand-gated receptor channels. The second class may include just one Cl^- channel, CFTR (cystic fibrosis transmembrane conductance regulator), which was cloned in 1989 (Riordan, *et al.*, 1989) which functions as a cyclic AMP-activated Cl^- channel (surprisingly, as it belongs to the family of ATP-binding cassette, or ABC, transporters, which generally function as ATP-driven transporters). Finally, there is the CLC family of Cl^- channels, whose first member was cloned in 1990 (Jentsch, *et al.*, 1990). Members of the CLC family function as voltage-gated channels (Jentsch, *et al.*, 1995), and one particular member is also activated by cell swelling (Grunder, *et al.*, 1992). The CLC channels are all believed to contain 12 membrane spanning domaines (Jentsch and Gunther, 1997).

1.5.2.2.1. The CLC Family of Chloride Channels.

(a). The CLC-0 Family of Voltage-Gated Chloride Channels.

The molecular structure of voltage-gated chloride channels was unknown until late 1990 when the first chloride channel was cloned. A cDNA encoding a voltage-gated chloride channel was isolated from *Torpedo marmorata* electric organ by the transient expression of its corresponding *in vitro* translated mRNA in *Xenopus* oocytes; the clone was identified by an elegant electrophysiological screen (Jentsch, 1990). It encodes a single protein subunit, CLC-0, of about 98 kD and is predicted to have 12 membrane-spanning domains which are considered to be a hallmark of ion channel and other transport proteins (Jentsch, 1990). *In vivo*, CLC-0 appears to function as a double barrel structure with two identical pores which can close and open independently or operate in concert. Therefore,

the CLC-0 channels are believed to exist as homodimers in their native membranes. This channel has been well characterised with respect to its chloride selectivity and voltage-dependence.

Since the cloning of the *Torpedo* electric organ Cl^- channel *clc-0*, it has become clear that it was the first member to be discovered of a large gene family. There are now nine different *clc* genes characterised in mammals (Jentsch, *et al.*, 1995).

(b). The CLC-1 Family of Voltage-Gated Chloride Channels.

Heterologous probe screening with *clc-0* identified the mammalian gene *clc-1* which is specifically expressed in skeletal muscle (Steinmeyer, *et al.*, 1991). Its protein product is functionally similar to *clc0* in that it has a $Cl^- > Br^- > I^-$ selectivity sequence, and its gates open with membrane depolarization. The CLC-1 channel appears to stabilise muscle cell plasma membrane voltage and to modulate muscle excitability. Inactivation of *clc-1* by mutation results in myotonia, an inherited muscle disease caused by an electrical hyper-excitability of the muscle membrane (Steinmeyer, *et al.*, 1991; Koch, *et al.*, 1992).

(c). The CLC-2 Family of Voltage-Gated Chloride Channels.

This class of Cl^- channel is ubiquitous in mammals and is activated by cell swelling and hyper-polarization. The *clc-2* gene was originally cloned from heart and brain cDNA libraries (Thyerman, *et al.*, 1992). Overall protein sequence homology is about 50 % to both *clc-0* and *clc-1* with the highest degree of identity in trans-membrane regions. Expression in *Xenopus* oocytes gave currents which activated very slowly upon strong, a physiological hyper-polarization. Its conductivity sequence is $Cl^- \geq Br > I^-$ Although northern analysis suggested that *clc-2* is expressed ubiquitously (Thiemann, *et al.*, 1992), *in situ* hybridization reveals that *clc-2* is expressed differentially in brain tissues (Smith, *et al.*, 1995). The CLC-2 protein has an important cellular function, that of mediating volume changes (Jentsch, 1997). It has also been proposed that CLC-2 serves to prevent neuronal Cl^- accumulation above equilibrium, thereby modulating the effects of post-synapic GABA receptors (Staley, *et al.*, 1995).

(d). The CLC-3, CLC-4 & CLC-5 Family of Voltage-Gated Chloride Channels.

When deficient, this class of Cl^- channels result in the appearance of kidney stones (Fisher, *et al.*, 1994; Steinmeyer *et al.*, 1995). Based on sequence homology alone, the CLC-5 class includes CLC-3 (Kawasaki, *et al.*, 1994) and CLC-4 (Van, *et al.*, 1994). However, as *clc-4* cannot be expressed functionally, and the reported expression of *clc-3* is controversial (Kawasaki, *et al.*, 1994; Van *et al.*, 1994 and Jentsch, 1995), this grouping has not been confirmed by similarity of function. The CLC-5 channels give rise to chloride currents with the typical $Cl^- > I^-$ conductivity sequences. However, these currents are only detectable at voltage more positive than +20 mV, values that are unlikely to occur *in vivo* (Steinmeyer, *et al.*, 1995; Lioyd,*et al.*, 1996).

(e). The CLC-6 and CLC-7 Family of Voltage-Gated Chloride Channels.

Additional mammalian *clc* genes have been identified but their physiological functions are still unknown. These include the kidney-specific *clc-k* gene (*clc-ka* and *clc-kb* in humans; Uchida, *et al.*, 1993; Kieferle, *et al.*, 1994) and the ubiquitously expressed *clc-6* and *clc-7* genes (Brandt and Jentsch, 1995). Unfortunately, like the CLC-3 and CLC-4 classes, no suitable expression system has been developed to characterise the biophysical properties of these channels (Jentsch, 1995; Kieferle, *et al.*, 1994).

The genome sequencing programs have identified putative prokaryote *clc* genes (in *E. coli,* Fujita, *et al.*; 1994; in *Synechocystis 6803, www.kazusa.or.jp/cyano*); further, a single *clc* homologue has been identified in *S. cerevisiae* (*scclc* originally termed *gef1*) which appear to be closely related to the *clc-6* and *clc-7* family. Since disruption of the yeast gene results in sensitivity to low iron levels in the growth medium (Greene, *et al.*, 1993), *scclc* is believed to be involved in iron metabolism. An alternative hypothesis is that they are members of prokaryote-like class of channels found in the membranes of prokaryotes and cell organelles.

(f). The CLC Family of Chloride Channels in Plants.

In recent years various plant chloride channels have been characterized biophysically, both in plasma membranes and in membranes of different organelles. Despite their roles in various functions, little is known about the molecular structure of plant chloride channels. Recently, using a polymerase chain reaction (PCR) strategy which relied on sequence consensus of the animal and yeast *clc* genes, a putative chloride channel cDNA

(*clc-nt1*) has been cloned from tobacco (Lurin, *et al.*, 1996). The CLC-Nt1 protein displays 24-32 % amino acid identity with animal CLC families. When *clc-nt1* complementary RNA was injected into *Xenopus* oocytes, it elicited slowly activating inward current upon membrane hyper-polarization (more negative than -120 mV). These currents were carried mainly by anions, were modulated by extracellular anions, and became totally blocked by 10 mM extracellular calcium. It was pointed out by Lurin, *et al.*, 1996, that the currents carried by CLC-Nt1 in oocytes show regulatory feature that suggest localisation to the plasma membrane *in planta,* as the voltage activation of CLC-Nt1 in oocytes is closer to the potential of the plant plasma membrane than to that of the tonoplast. The CLC-Nt1 channel might correspond to either one of the anion channels that have been characterised as being activated upon hyperpolarization of the plasma membrane or tonoplast (Hedrich and Becker, 1994).

Information stemming from the *Arabidopsis* genome sequencing program has identified four putative *clc* genes. These have now been cloned using a PCR strategy (Hechenberger, *et al*, 1996). The four genes (*atclca, atclcb, atclc-c* and *atclc-d*) are homologous to *clc-nt1* from tobacco (Lurin, *et al.*, 1996) and are also closely related to the mammalian *clc-6* and *clc-7* gene families. The *atclc* transcripts are broadly expressed in the plant but show some tissue specificity. The strongest expression of *atclc-a,* and *atclc-c* transcripts seem to occur in source leaf, whereas *atclc-b* is most strongly expressed in roots; *atclc-d* is mainly expressed in the fruit. The *atclc-a* and *atclc-b* are highly homologous to each other (~87 % identity at the perceived amino acid level) and ~ 50 % identical to *atclc-c* and *atclc-d.* None of the four cDNAs elicit chloride currents when expressed in *Xenopus* oocytes, either singly or in combination. Only AtCLC-D could functionally substitute for the single yeast SCCLC protein, restoring the iron-limited growth of a strain disrupted for this gene. It has been suggested that AtCLC-D functions as an intracellular chloride channel (Hechenberger, *et al.,* 1996).

1.6. Aim of This Study

The aim of this study is to isolate and characterize genes encoding membrane transporters and channels that may play a central role in conferring resistance to Na^+ and / or Cl^- in salt tolerant plants.

Initially, in 1995 at the start of the project, there was insufficient information available in the data bases to allow putative plant genes to be identified by (BLAST) searches for homology, or by designing PCR primers suitable for cloning purposes. Therefore, it was decided to try to identify plant homologues for cation and anion transporters using heterologous probes to screen a range of salt-resistant plants.

The plants chosen for study were the salt-resistant crops barley (*Hordeum vulgare*) and sugar beet (*Beta vulgaris*), and the halophyte (*Atriplex mummularia*). Genomic libraries of these plants were prepared and these, together with existing cDNA libraries, were used in Southern screening experiments. Early experiments suggested that the *clc-0* probe from *T. marmorata* would be the most fruitful avenue to pursue, and so attention was focused on characterising plant Cl^- channels. However, during this work it became clear that the rapidly expanding data bases were accumulating information at such a rate that a new strategy based on consensus sequences would be more productive. Therefore, sequence alignments of known *clc* channels were made and degenerate PCR primers designed from the emerging consensus sequences. These primers were subsequently used in a series of experiments to try to identify *clc* homologues in salt-resistant plants.

Figure 1.2. Alignment of 17 Putative *CLC*-type Chloride Channels.

In May 1996, the GCG programme STRINGSEARCH was used to identify Cl^- channel sequences in the EMBL database. The sequences were translated and then aligned using GCG's PILEUP programme. The PILEUP alignment was analysed by PRETTY and the output from this is presented. Several of the default parameters of PILEUP were adjusted to derive the best consensus alignment. Only three regions of conserved amino acid sequence were identified using this approach which are marked in bold face type on the CONSENSUS line GKIGPxxH (between position 350 and 360), GVLFxxE (between position 410 and 420) and GEDWIFL (between position 240 and 250). Note, not all sequences contained these conserved motifs. These consensus sequences were used to design degenerate PCR primers for cloning purposes. The sequence accession numbers are as follows: rnmrna, x64139: human placenta CLC-2, s77770: oc15652, chinese hampster CLC-2: *Torpedo californica* CLC-0, tcvgcc: *Torpedo marmorata* CLC-0, tmclchan: human muscle CLC-1, hsclc1mcc: human muscle CLC-1, hsclc1mr: rat skeletal muscle, rrsmcc: rat protein kinase A activated Cl^- channel, rrclck2a: human kidney CLC-2, s80315: human CLC-5, hsclcn5gn: rat CLC-5; rnclc5: human chloride channel protein, hsclcpx: rat CLC-3, rnclc3: human CLC-7, hsclc7mr: rat CLC-7, rnclc7mr: *S. cerevisiae* GEF1, scclcy.

CHAPTER TWO
MATERIALS AND METHODS

2.1. MATERIALS.

2.1.1. Chemicals.

The chemicals used in this study were obtained from BDH (Poole, Dorset) unless indicated otherwise.

2.1.1.1. DNA Modifying Enzymes.

Unless stated otherwise, all restriction enzymes together with their reaction buffers, were purchased from GIBCO-BRL, Paisley, Strathclyde, or Promega Corporation, USA.

2.1.1.2. Oligonucleotide Primers.

Primers for PCR were synthesized by Cruachem Ltd, Glasgow. The primers were supplied desalted, and were resuspended to the appropriate concentration in water before use.

2.1.1.3. Radiochemical.

The radiochemical [α-^{32}P] dCTP used in this study was supplied by Amersham International (UK).

2.1.1.4. Bacteria Growth Media (Liquid and Solid).

One percent (w/v) L-broth (1% bacto-tryptone, 0.5% yeast extract (both supplied by Difco Laboratories, Michigan, USA) and 1% (w/v) NaCl, pH 7.0) plus the appropriate antibiotic was generally used for culturing *E. coli*. For preparation of competent cells, *E. coli* XL1-Blue (Stratagene, Cambridge, UK) was cultured in TYN medium (1 % (w/v) bacto-tryptone, 1 % (w/v) bacto-yeast extract, 0.5 % (w/v) NaCl) plus the appropriate antibiotic. For preparation of *E.coli* XL1-Blue MRA(P2) plating cells (Stratagene) L-broth was used, supplemented with 0.2 % (w/v) maltose and 10 mM $MgSO_4$. For plating out the above bacterial strains, the solid media was prepared by adding 1.5 % (w/v) agar (Difco) to the media prior to sterilization. Top agar was prepared by adding 0.7 % (w/v) agar to L-broth media supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose. Filter-sterilised antibiotics, maltose and $MgSO_4$ were added after autoclaving when the solution had cooled to just below 50°C.

2.1.1.5. Antibiotics, IPTG, and X-Gal.

All antibiotics used were supplied by Sigma Chemical Co. (UK). Ampicillin and kanamycin were dissolved in distilled water (50 mg mL^{-1}) and used at a final concentration of 50 μg mL^{-1}. Chloramphenicol was dissolved in ethanol (30 mg mL^{-1}) and used at a final concentration of 30 μg mL^{-1}. All antibiotics were filter sterilized and stored in sterile bottles before adding to autoclaved media which had been allowed to cool to 50°C.

Isopropyl thiogalactoside (IPTG, Life Technologies, Gaithersburg, USA) was prepared as a 0.1 mM stock in distilled H_2O and used at a final concentration of 50 μM mL^{-1}. X-Gal (5-bromo-4-chloro-3-indolyl β-D galactoside; Life Technologies) was made as a 25 mg mL^{-1} stock in dimethylformamide and used at a final concentration of 50 μg mL^{-1}.

2.1.1.6. Bacterial Strains, Cloning Vectors and Plasmids.

Plasmids were maintained and amplified in *E. coli* XL1-Blue (Stratagene Ltd., Cambridge, UK). *E. coli* XL1-Blue MRA(P2) (Stratagene) was used to plate λ genomic libraries. *E. coli* LE392 was used for the isolation of bacterial genomic DNA, Epicurian Coli XL1-Blue MRF` Kan supercompetent cells (Stratagene) were used as the host strain for transformation with PCR-Script Amp SK(+) cloning vector.

The PCR -Script Amp SK (+) cloning vector (Stratagene) was used to clone and maintain the blunt end PCR products.

Genomic libraries of barley and sugar beet were prepared in the laboratory by construction in λ Dash II *BamH1* vector (Stratagene).

All heterologous probes used in this study are described in Table 1.

2.1.2. General Laboratory Procedures.

2.1.2.1. pH Measurement.

The pH of all solutions other than phenol were measured using a Corning pH meter 220 and a combination electrode (Jenway, pHM6, UK). The pH of phenol-containing solutions was measured using Whatman Narrow Range pH paper pH 6.0-8.0 (Whatman, Maidstone, UK).

2.1.2.2. Autoclaving.

Equipment and solutions were sterilized at 121°C and 0.1MPa pressure for 20 minutes. Large batches were sterilized in a Laboratory Thermal Equipment Autoclave 225E. Small batches were sterilized in a Tower pressure cooker.

2.1.2.3. Filtration.

Heat-labile solutions were sterilized by passing through a sterifil-D GS filter (pore diameter 0.22 μm, Millipore Co.) into a sterile receptacle.

2.1.2.4. Glassware.

Glassware was sterilized by baking in an oven at 180°C for 12 hours or overnight.

2.1.2.5. Solutions and Equipment for RNA Work.

Solutions for RNA work were treated with 0.1 % (v/v) diethyl pyrocarbonate (DEPC, Sigma) overnight as described in Sambrook, *et al.*, (1989).

2.1.3. Plant Material.

2.1.3.1. Growth and Treatment of Plants.

Seeds of barley (*Hordeum vulgare* Var. Golden Promise), were washed in tap water, and then soaked in water for 2 hours. The seeds were germinated in trays containing tap water soaked vermiculite (Silvaperl, William Sinclair Horticulture Limited) at 25°C/ 15°C using a 14 hr/ 10 hr day/night cycle for 7 days. The germinated seeds were then grown hydroponically in 0.5 g L^{-1} Phostrogen for 14 days.

Seeds of sugar beet (*Beta vulgaris* var Saxon) supplied by British Sugar plc were washed in tap water for 2 min and then soaked in tap water overnight. The seeds were then germinated in trays containing tap water soaked vermiculite (Silvaperl, William Sinclair Horticulture Limited) at 24°C/15°C using a 14 hr/ 10 hr day/night cycle for 7 days. The germinated seeds were grown hydroponically for 5 weeks in half-strength Hoagland's solution which was prepared from 0.51 g L^{-1} KNO_3, 0.246 g L^{-1} $Ca(NO_3)_2.4H_2O$,0.115 g L^{-1} $NH_4H_2PO_4$, 0.245 g L^{-1} $MgSO_4.7H_2O$, 1.428 mg L^{-1} H_3BO_3, 0.905 mg L^{-1} $MnCl_2.4H_2O$,

0.04 mg L^{-1} $CuSO_4.5H_2O$, 0.11 mg L^{-1} $ZnSO_4.7H_2O$, 0.045 mg L^{-1} $H_2MoO_4.H_2O$, and then 0.00015% of $FeSO_4$ and 0.00012 % of NaK-Tartrate were freshly added every 5 days.

Cell suspension cultures of red beet (*Beta vulgaris*) were supplied by Prof M. Elliot (De Montford University, Leicester). Forty mL of cells were grown in 500 mL flasks containing 160 mL MSMO media (1 X Murashige and Skoog salts with minimal organics (M6899, Sigma), and 3 % (w/v) sucrose, pH 5.8). Suspension cultures were grown at 22°C in a 24 hour photoperiod (PPFD 5.0 μmol m^{-2} s^{-1}) with constant shaking (140 rpm), and sub-cultured every two weeks using 1/5 dilutions.

Sugar beet cell suspension culture (*Beta vulgaris* var Saxon) was prepared from intact plants in the laboratory (Janet Laird, University of Glasgow) and was grown in MSMO (M 6899, Sigma) containing 3 % (w/v) sucrose, 2.5 μM 2,4D and 0.5 μM kinetin pH 5.8. The cultures were grown in continous light (PPFD 5-20 μmol m^{-2} s^{-1}) at 24°C, shaken at 150 rpm and sub-cultured every 14 days in a laminar flow hood by transferring 10 mL into a sterile flask containing 90 mL of sterile medium.

Seeds of *Atriplex halimus* were obtained from Prof. T. Flowers, University of Sussex. The *A. halimus* cell suspension cultures were prepared in the laboratory (N. Urwin, University of Glasgow) and grown in MSMO (M 6899, Sigma) containing 3 % (w/v) sucrose, 2.5 μM 2,4D and 0.5 μM kinetin pH 5.8). The culture was grown in continous light (PPFD 21 μmol m^{-2} s^{-1}) at 25°C shaken at 150 rpm, and sub-cultured every 14 days in a laminar flow hood by transferring 20 mL into a sterile flask containing 80 mL of sterile medium.

Cell suspension cultures of *Arabidopsis thaliana* were supplied by Prof. C. Leaver, University of Oxford, (May and Leaver, 1993). The suspension culture was grown in MSMO (M 6899, Sigma) containing 3 % (w/v) sucrose, 0.5 mg L^{-1} α-naphthalene acetic acid and 0.05 mg L^{-1} kinetin, pH 5.8. The cultures were grown in continous light (PPFD 18 μmol m^{-2} s^{-1}) at 23°C, constantly shaken at 150 rpm, and sub-cultured every seven days a laminar flow hood by transferring 5 mL into a sterile flask containing 45 mL of sterile medium.

Seeds of *Nicotiana tabaccum* were supplied by Dr J. Milner (Glasgow University). The seeds were surface sterilized by placing them in an envelope made from Whatman No 1 filter paper. The envelopes were then soaked in 25 % (v/v) sodium hypochlorite (purchased from R & J Wood, U.K.) for 30 min. From this point, all manipulations were carried out in a sterile flow hood. The envelope was placed in a pot containing sterile dH_2O for 2 min to remove any bleach. This was repeated 5 times. The envelope of seeds was dried on a petri dish lid in the flow hood for at least 3 h. The seeds were stored in a plate sealed with Micropore tape (3 M Health Care, Loughborough, U.K.) until use. The surface sterilized seeds were germinated at 25°C in trays containing water soaked sterile soil and covered with cling film.

2.1.3.2. Salt Treatment and Harvesting of Plants and Suspension Cultures.

Three-week old hydroponically grown barley plants were treated with 150 mM NaCl for 4 days. Four-week old hydroponically grown sugar beet plants were treated with 300 mM NaCl for 4 days. Red beet and *A. halimus* cell suspension cultures were adapted from non-treated cell suspension cultures by sub-culturing into progressively higher levels of NaCl, starting with 100 mM NaCl and increasing to 200 mM and 300 mM. Sugar beet cell suspension culture (*Beta vulgaris* Var Saxon) was maintained in media containing 200 mM NaCl.

The plants were harvested and quickly frozen in liquid nitrogen prior to use or kept at -80°C until required. The suspension cultures were collected by centrifugation for 10 min at 700 g. The supernatant was removed and the pellet immediately frozen in liquid nitrogen. Samples were then stored at -80°C until required.

2.2. METHODS.

2.2.1. Isolation of Genomic DNA.

2.2.1.1. Isolation of Plant Genomic DNA.

The isolation of high molecular weight (> 25 kb) DNA was carried out according to a modified DNA isolation protocol Puregene (1992).

One gram of leaf tissue or cell suspension culture was quickly frozen in liquid nitrogen and ground to a fine powder in a pre-chilled mortar and pestle. The frozen powder was then transferred with liquid nitrogen into a 30 mL Oak Ridge tube containing 18 mL of Lysis buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.5 % (w/v) SDS) before the sample thawed. The contents of the tube were mixed gently by inversion, incubated at 65°C for 60 minutes and allowed to cool to room temperature. Then 0.1 mL of RNase A (10 mg mL^{-1}) was added to the tube, which was mixed gently, and incubated at 37°C for 15 minutes. Six mL of 5 M ammonium acetate was added, the tube was then shaken gently and incubated at 0°C for 30 minutes. After incubation, the tube was centrifuged at 3000 g in a Beckman J2-HS centrifuge, JA-20 rotor for 10 minutes at 4°C. The supernatant was filtered through one layer of mira cloth filter into a clean 30 mL Oak Ridge centrifuge tube containing 18 mL of iso-propanol and mixed well by inversion. The DNA was pelleted by centrifugation at 3000 g for 5 minutes at 4°C and the supernatant discarded. The tube was then inverted on paper towels for 10 minutes to allow the pellet to dry. After drying the pellet was dissolved in 1mL of TE buffer 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

The DNA solution was transferred to an ultracentrifuge tube and 1 g of caesium chloride per 1 mL of DNA solution was added then mixed by gentle inversion. One mg mL^{-1} ethidium bromide was added to the DNA solution, and left at room temperature for 30 min to allow the CsCl to dissolve. The tube was then capped and centrifuged at 180,000 g for 40 hours at 20°C in a fixed angle rotor (T865.1) of a Sorvall 55B ultracentrifuge. After centrifugation, the DNA band was collected with a sterile plastic pipette and placed into clean 1.5 mL Eppendorf tubes. Ethidium bromide was removed by adding an equal volume of TE saturated *iso*-propyl alcohol. The phases were mixed gently, allowed to separate and the upper phase removed. The extraction procedure was repeated several times until all of the ethidium bromide (pink colour) was removed from the aqueous phase.

The aqueous phase was then dialysed against a 1000 X volume of TE buffer (pH 8.0) using treated dialysis tubing (Sigma) for 2 hours at 4°C, and then overnight against fresh

TE buffer (pH 8.0) at 4°C. The dialysis tubing was prepared by boiling for 5 min in the presence of 0.1 % (w/v) SDS, and allowed to cool prior to use. The DNA solution was transferred from the dialysis tubing into a 1.5 mL Eppendorf tube, the DNA was then precipitated by the addition of a one tenth volume of 3 M sodium acetate and two volumes of ice-cold ethanol, the mixture was then stored at -20°C for 2 hours. The DNA was pelleted in a microfuge at 10,000 g for 3 minutes, washed with 70 % (v/v) ethanol, dried and re-dissolved in TE buffer (pH 8.0). The concentration of the DNA solution was determined as in Section 2.2.6 and finally stored at 4°C until use.

2.2.1.2. Isolation of Bacterial Genomic DNA.

A single *E. coli* colony was used to inoculate 5 mL of L-broth medium in a 20 mL Bijou tube. The culture was incubated overnight at 37°C with constant shaking at 200 rpm. The culture was transferred to a sterile 15 mL centrifuge tube and pelleted at 3000 g for 5 min at 4°C. The supernatant was decanted and the bacterial pellet re-suspended in 1 mL of lysis buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) containing 10 mg mL^{-1} lysozyme (Sigma). The mixture was transferred to a 1.5 mL Eppendorf tube and incubated for 10 min at room temperature. After incubation, 50 µL of 10 % (v/v) SDS was added to the tube and mixed gently at this point, the suspension became transparent signifying the lysis of bacterial cells. An equal volume of phenol/ chloroform *iso*-amyl-alcohol (v/v/v, 50:24:1) was added, mixed thoroughly but gently, and spun in a micro-centrifuge at 10,000 g for 1 min. After centrifugation the upper layer was transferred to a clean tube and an equal volume of chloroform: *iso*-amyl-alcohol (24:1) was added, mixed and centrifuged as before. The upper layer containing the DNA was transferred to a clean tube. The DNA solution was precipitated by adding a one tenth volume of 3 M sodium acetate and two volumes of ice-cold ethanol on ice for 10 min, followed by a short centrifugation at 10,000 g for 10 seconds. The supernatant was aspirated from the pellet, which was then washed in 50 µL 70 % (v/v) ethanol. The pellet was left to air dry for 10 min. The resulting DNA pellet was re-suspended in an appropriate volume of TE buffer (pH 8.0) and stored at -20°C.

2.2.2 Isolation of Total RNA from Plant Material.

2.2.2.1. Preparation of Phenol.

Five hundred grams of phenol (detached crystals) was melted at 65°C for 30 min and 0.1 % (w/w) of 8-hydroxyquinoline was added before the phenol had cooled. The warm solution was then saturated twice with 300 mL of 1M Tris-HCl (pH 8.0) and mixed vigorously for 15 minutes. After allowing the solution to settle, the upper phase was then removed by aspiration and replaced with an equal volume of 100 mM Tris-HCl (pH 8.0). The aspiration of the upper phase was done as before and the pH of the aqueous extract was measured. The aspiration and washing with 100 mM Tris-HCl (pH 8.0) was repeated until the pH of the aqueous extract was greater than 7.8. The upper phase was then replaced with 100 mL of 100 mM Tris-HCl (pH 7.8) and the solution stored in a dark bottle at 4°C.

2.2.2.2 Isolation of Total RNA.

One gram of frozen plant tissue or cell suspension culture was ground to a fine powder under liquid nitrogen with a mortar and pestle. The powder was then transferred to a 15 mL glass Corex tube containing 5 mL of TNT solution [50 mM Tris-HCl (pH 7.8), 10 mM NaCl and 2 % (w/v) Tris-*iso*propylnaphthalene-sulphonic acid] and 5 mL of PCI solution (phenol : chloroform: *iso*-amyl alcohol, v/v/v, 25 : 24 : 1). The solution was mixed vigorously by inversion until an emulsion was formed, and then kept on ice. The tube was then spun at 1000 g for 10 minutes at 4°C in an MSE Mistral 2L centrifuge using an 8 X 50 mL swing out rotor. Following the spin, the upper aqueous phase was removed with a sterile pasteur pipette and transferred to a clean 15 mL Corex tube containing an equal volume of PCI. The sample was mixed and centrifuged as described previously. After centrifugation, the upper aqueous phase was removed to a clean 15 mL Corex tube containing an equal volume of chloroform: *iso*-amyl alcohol (v/v 24:1), mixed and centrifuged as before. The aqueous phase containing RNA was transferred to a 15 mL glass Corex tube and the RNA was precipitated with 2.5 volumes of ice-cold ethanol

and 0.1 volume 3 M sodium acetate (pH 6.0), mixed and incubated at -20°C for 2-12 hours.

After precipitation, the RNA was pelleted by centrifugation at 10,000 g for 15 min in a Beckman J2-HS centrifuge using JA-20 rotor at 4°C. The supernatant was discarded and the pellet washed twice with 80 % (v/v) ice-cold ethanol and re-pelleted after each wash by centrifugation as before. The resulting pellet was dried under a vacuum, dissolved in appropriate volume of DEPC-treated analar water and stored at -80°C.

The purity and the concentration of the RNA were estimated by measuring the AD at 260 nm and 280 nm (Section 2.2.6). A high purity RNA preparation was taken as having a 260/280 nm ratio of 1.8-2.0. The integrity of RNA was examined on a 1.3 % (w/v) agarose mini-gel.

2.2.2.3 Isolation of Mitochondrial and Chloroplast Total RNA.

Twenty grams of plant tissue were homogenized in 80 mL of organelle isolation buffer (300 mM mannitol, 50 mM Tris-HCl, 3 mM EDTA, 0.1 % (w/v) BSA and 1 mM 2-mercaptoethanol), using a pre-chilled Waring blender with two 5 second bursts at medium speed. The homogenate was filtered through eight layers of sterile muslin and transferred to sterile screw capped 250 mL centrifuge bottles. The filtrate was centrifuged at 1000 g for 10 minutes at 4°C in a Beckman J2-HS centrifuge (JA-14 rotor) to collect the nuclear pellet, which was then discarded and the supernatant transferred to a clean centrifuge bottle. The chloroplast pellet was collected by centrifuging the supernatant at 1800 g for 10 minutes at 4°C, the resultant pellet was kept on ice and the supernatant was transferred to a clean centrifuge tube. This supernatant was centrifuged at 10,000 g for 10 minutes at 4°C and the mitochondrial fraction was collected. The chloroplast and mitochondrial pellets were separately re-suspended in 10 mL of ice-cold organelle isolation buffer and again collected at 1,800 g and 10,000 g respectively for 15 minutes at 4°C.

The chloroplast and mitochondrial pellets were separately re-suspended in 10 mL of RNA extraction buffer and the total RNA was isolated from both organelles (chloroplast and mitochondrial) as before (Section 2.2.2.2).

2.2.2.4 Isolation of Poly $(A)^+$ mRNA.

The isolation of poly $(A)^+$ mRNA from total RNA was carried out using a poly (A) Quik mRNA isolation kit produced by Stratagene (1994).

Poly (A) Quik push columns were allowed to stand upright for 5 minutes. The caps of the column were removed from both ends, and then the push column was attached to the plunger of a 10-mL syringe. The storage buffer was slowly pushed out of the column at a rate of ~ 1 drop every 2 seconds (to ensure uniform column packing). Two hundred µL of high-salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 500 mM NaCl) were applied to the push column and pushed slowly through the column at the same rate of 1 drop every 2 seconds. The previous step was repeated with another 200 µL aliquot of high-salt buffer. The total RNA sample was heated at 65°C for 5 minutes, chilled on ice and diluted with appropriate amount of 10 X sample buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 5 M NaCl) to bring the final concentration to 1 X. The sample was then applied to the column and pushed through the column at a rate of ~ 1 drop every 2 seconds. The RNA sample eluate was reapplied to the push column and pushed through the column at a rate of 1 drop every 2 seconds. Two hundred µL of high-salt buffer were applied to the push column and pushed through the column at a rate of 1 drop per second. The high-salt wash was repeated with one additional 200 µL aliquot of high-salt buffer. The push column was then washed three times using 200 µL aliquots of low-salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 100 mM NaCl) and the buffer was pushed through the column at a rate of 1 drop every second. The mRNA was eluted using four 200 µL aliquots of the pre-heated elution buffer (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, heated at 65°C) and pushed through the column into a 1.5 mL sterile Eppendorf tube, at a rate of 1 drop per second.

The mRNA was precipitated with 2.5 volumes of ice-cold 100 % ethanol at -20°C overnight. After pelleting the poly $(A)^+$ RNA, the pellet was washed with 70 % (v/v) ethanol, dried, resuspended in DEPC-treated water and stored at -80°C.

The concentration and the purity of mRNA was estimated as described in Section 2.2.6.

2.2.3. Amplification and Preparation of Plasmid DNA.

2.2.3.1. Preparation of Competent Cells.

Competent cells were prepared using the method described by Sambrook *et al.*, (1989).

E. coli cultures were grown overnight in 10 mL TYN medium (see Section 2.1.5) at 37°C with constant shaking. One hundred mL of fresh TYN medium was inoculated with 5 mL of an overnight culture and shaken constantly at 200 rpm, at 37°C until an OD at 550 nm of 0.35 was reached. Fifty mL of the suspension was transferred to two sterile 30 mL centrifuge tubes. The cells were then pelleted by centrifugation at 2,000 g for 5 min at 4°C (in a Beckman J2-HS centrifuge using a JA 20 fixed angle rotor) and the supernatant decanted. The bacterial pellet was re-suspended in a total volume of 10 mL ice-cold TFB 1 (100 mM RbCl (Sigma), 50 mM $MnCl_2$ (Sigma), 30 mM potassium acetate, 10 mM $CaCl_2$, 15 % (v/v) glycerol, pH 5.8) and kept on ice for 90 min. After incubation, the cell suspension was centrifuged as before and resuspended in 2.8 mL ice-cold TFB 2 (10 mM MOPS (Sigma), 10 mM RbCl, 75 mM $CaCl_2$, 15 % (v/v) glycerol, pH 7.0). The cells were then separated into 0.2 mL aliquots in 1.5 mL Eppendorf tubes, frozen in liquid nitrogen and stored at -80°C until use.

2.2.3.2. Transformation.

Two methods were used for transformation of competent cells.

2.2.3.2.1.Transformation of Competent Cells.

Frozen competent cells (prepared as described in Section 2.2.3.1) were thawed on ice. Approximately 50 ng of plasmid DNA in a volume of 5-10 µL was added to the Eppendorf tube containing 0.2 mL of the competent cells and gently shaken before being returned to ice for 20 min. After this period, the cells were heat-shocked at 37°C for 1 min and returned to ice for a further 2 min. TYN medium (0.8 mL) was added to allow

expression of the antibiotic resistance encoded on the plasmid. Cells were then pelleted by centrifugation at 10,000 g for 30 seconds, the supernatant discarded, and the pellet resuspended in 0.1 mL TYN medium. One hundred µL of serial 1/10 dilutions of the cell suspension were plated on agar containing the appropriate antibiotic. The plates (see Section 2.1.5) were allowed to dry and placed at 37°C overnight. As a control, transformed cells were plated on agar containing no antibiotic. A second control was also performed where competent cells were treated, as above, in the absence of plasmid DNA and plated out on antibiotic containing plates.

2.2.3.2.2. Transformation of *E. coli* XL1-Blue MRF` Supercompetent Cells.

Transformation of *E. coli* XL1-Blue MRF` supercompetent cells (supplied by Stratagene) was performed in accordance with the manufacturer's instructions. Frozen competent cells (prepared by Stratagene) were thawed on ice. Forty µL aliquots of competent cells were transferred into pre-chilled 15 mL Falcon (polypropylene) tubes. Then 25 mM of β-mercaptoethanol was added to the tube and gently swirled before being incubated on ice for 10 min. Two µL of the cloning reaction prepared as described in Section 2.2.15.5 was added, gently swirled and incubated on ice for 30 min. After this period, the transformation reaction was heat-shocked at 42°C for 45 seconds and returned to ice for a further 2 min. Then 0.45 mL of SOC medium (2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.05 % (w/v) NaCl, 1 mM $MgSO_4$, 1 mM $MgCl_2$, 0.4 % (w/v) glucose) was added to the transformation reaction and the tube was shaken at 37°C for 1 hour. Fifty, 100, 150, 190 µL of the transformation reactions were then plated on separate L-broth-ampicillin-kanamycin (50 µg/µL) agar plates containing X-gal (50 µM mL^{-1}) and IPTG (50 µM mL^{-1}). Fifty µL of the control transformation reaction (transformed cells with control insert) were plated onto L-broth-chloramphenicol (30 µg mL^{-1}) agar plate. The plates were allowed to dry and placed at 37°C overnight.

2.2.4 Preparation of Plasmid DNA.

2.2.4.1. Small Scale Preparation of Plasmid DNA.

For this method the Wizard Minipreps DNA Purification System (Promega) was used in accordance with the manufacturer's instructions.

A single bacterial colony was used to inoculate 3.0 mL of TYN or L-broth medium, supplemented with the appropriate antibiotic, in a 7 mL Bijou tube. The culture was grown overnight at 37°C with constant shaking at 200 rpm. One mL of the overnight culture was transferred to a 1.5 mL Eppendorf tube and centrifuged at 10,000 g for 5 min in a microcentrifuge. The supernatant was discarded and the bacterial pellet resuspended by vortexing in 200 µL of cell re-suspension solution (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 mg mL^{-1} RNase A). Once fully re-suspended, 200 µL of cell lysis solution (200 mM NaOH, 1 % (w/v) SDS) was added and the solution mixed by gentle inversion. Next, 200 µL neutralization solution (1.32 M potassium acetate, pH 4.8) was added and the solution mixed as before. The tube was centrifuged at 10,000 g for 5 min and the supernatant removed to a 1.5 mL Eppendorf tube. One mL of the Wizard Minipreps DNA purification resin was added to the solution and gently mixed. Meanwhile, a 3 mL disposable syringe was attached to a minicolumn and placed onto a vacuum manifold (Promega). The resin/DNA mixture was then transferred to the syringe and a vacuum applied to pull the slurry into the minicolumn. The vacuum was broken, 2 mL of wash solution (200 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA, 95 % (v/v) ethanol) was added and the vacuum re-applied. Once the wash solution had passed through, the column was left to dry for 30 seconds. The vacuum was broken and the minicolumn removed from the syringe and transferred to a 1.5 mL Eppendorf tube. This was centrifuged at 10,000 g for 2 min to further dry the resin from the column wash solution. The minicolumn was then transferred to a clean 1.5 mL Eppendorf tube, 50 µL of TE buffer (pH 7.6) added and then left at room temperature for 1 min. Plasmid DNA was eluted by centrifugation at 10,000 g for 20 seconds.

2.2.4.2. Preparation of Plasmid DNA Using Qiagen Maxi Plasmid Purification System.

The Qiagen Maxi-Plasmid Purification System (Qiagen Ltd., Sussex, UK) was used in accordance with the manufacturer's instructions.

A single colony of bacteria containing the plasmid of choice was inoculated in 10 mL of L-broth containing the appropriate antibiotic and grown overnight at 37°C with constant shaking. One mL of the overnight culture was inoculated into 100 mL of L-broth containing the appropriate antibiotic and incubated at 37°C with constant shaking (200 rpm) overnight.

The cells were harvested by centrifugation at 2,000 g for 5 minutes in a Beckman J2-HS centrifuge (JA-20 rotor) at 4°C. The supernatant was discarded and the pellet re-suspended in 10 mL of buffer P1 (100 μg mL^{-1} RNAse A, 50 mM Tris-HCl (pH 8.0), 10 mM Na_2EDTA. Then the cells were lysed by adding 10 mL of buffer P2 (200 mM NaOH, 1% (w/v) SDS) and the solution was gently mixed by inverting the tube several times before incubating at room temperature for 5 minutes. Ten mL of P3 buffer (3 M potassium acetate pH 5.5) was added to the lysate solution, mixed immediately by gently inversion and incubated on ice for 20 minutes. The suspension was removed and centrifuged again for 15 minutes to obtain a particle-free clear lysate. Meanwhile, a Qiagen column (tip 500) was equilibrated by adding 10 mL of QBT buffer (750 mM NaCl, 50 mM MOPS, 15 % ethanol, 0.15 % (v/v) Triton X-100 (pH 7.0)) and the column allowed to empty by gravity flow. The supernatant obtained after centrifugation was then applied onto a Qiagen column and allowed to enter the resin by gravity flow. The column was then washed twice with 30 mL of QC buffer (100 mM NaCl, 50 mM MOPS, 15 % (v/v) ethanol, pH 7.0). Plasmid DNA was eluted into a sterile 30 mL centrifuge tube by the addition of 15 mL QF buffer (1.25 M NaCl, 50 mM Tris-HCl (pH 8.5), 15 % (v/v) ethanol. The DNA was precipitated by adding 0.7 volumes of *iso*-propanol, the solution was mixed and immediately spun at 20,000 g for 30 minutes at 4°C. The supernatant was discarded and the DNA pellet washed twice in 70 %(v/v) ice-cold ethanol and centrifuged as before. After centrifugation, the supernatant was removed and the pellet air-dried before re-dissolving in a suitable volume of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM

EDTA). The concentration of DNA solution was determined as described in 2.2.6 and stored at -20°C

2.2.5. Nucleic Acid Precipitation.

Nucleic acids were precipitated by either the ethanol or *iso*-propanol method.

Nucleic acids were precipitated by the addition of a 1/10 volume of 3 M Na acetate (pH 5.2) followed either by the addition of two volumes of ice-cold 100 % ethanol or 0.7 volumes of ice-cold *iso*-propanol. DNA precipitation was enhanced by chilling the samples at -20°C for at least 20 min before centrifugation at 10,000 g for 3 minutes.

Nucleic acid pellets were washed in 70 % (v/v) ethanol, briefly centrifuged and then air dried, and finally resuspended in an appropriate volume of distilled water or TE buffer (pH 8.0).

2.2.6. Quantification of DNA and RNA.

An aliquot of nucleic acid solution to be quantified (usually 5 - 10 µL) was diluted to 70 µL with distilled or DEPC-treated water. The absorbance of the nucleic acid containing solution was measured scanning at wave lengths between 220 nm and 350 nm using distilled water as a blank in a Shimadzu UV-2101 PC scanning spectrophotometer. An absorbance at 260 nm (A_{260}) of 1 was taken to indicate the following concentrations :

Form of nucleic acid	concentration (µg mL^{-1})
Double stranded DNA	50
Single stranded DNA and RNA	38
Oligonucleotide	20

2.2.7. Agarose Gel Electrophoresis.

GIBCO-BRL Life Technologies Ltd, Paisley, U.K. supplied all agarose (ultra PURE).

2.2.7.1. Electrophoresis of DNA.

The appropriate concentration of agarose (0.5-2.0 % (w/v)) was added to an appropriate volume of 1 X TBE buffer (90 mM Tris-borate, 2 mM EDTA) or 1 X TAE (40 mM Tris-acetate (pH 8.0), 1 mM EDTA) required for the gel being cast. The agarose suspension was heated in a microwave oven until all the agarose had completely dissolved. The molten agarose solution was allowed to cool to around 60°C at which point 10 mg mL^{-1} ethidium bromide was added to a final concentration of 0.5 µg mL^{-1}. The molten agarose solution was then poured into the electrophoresis apparatus and allowed to set for 30 minutes at room temperature. Enough 1 X TBE or TAE running buffer was added to just submerge the gel to a depth of approximately 1-2 mm. The DNA samples to be loaded, were mixed with a one-tenth volume of loading dye buffer (50 % (v/v) glycerol, 1 mM EDTA (pH 8.0), 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF) heated and loaded into the submerged gel. The elecrophoresis was carried out at 20-100 volts (constant voltage) at room temperature until the bromophenol lower band had migrated two-thirds of the way down the gel. The gel was visualized under UV light (Spectroline transilluminator, Model TC-312A). The gel was photographed using Polaroid film (Type 665, professional) or with a Bio Gene Image analysis system (Bio Gene Ltd).

2.2.7.2. Non-denaturing Electrophoresis of RNA.

Non-denaturing elecrophoresis of RNA was carried out as in Section 2.4.7.1 for DNA.

2.2.7.3. Denaturing Electrophoresis of RNA.

This method was used for gels that were to be blotted for northern hybridization analysis. The appropriate amount of agarose (1.3 % (w/v)) was added to 80 mL of DEPC-treated

water. The agarose suspension was then heated in a microwave oven until the agarose had dissolved. Once the agarose solution had cooled to 60°C, 10 mL of 10 X MOPS buffer (200 mM MOPS pH 7.0 (Sigma), 50 mM sodium acetate, 10 mM EDTA) and 10 mL of formaldehyde (37 % (v/v), Sigma) were added and the gel was mixed by swirling in a fume hood prior to pouring into the electrophoresis apparatus. The molten gel was then left in the fume hood for 30 minutes to allow the gel to set and then placed into the electrophoresis tank and submerged to a depth of 1-2 mm in 1 X MOPS running buffer. RNA samples (5-20 µg) were prepared in a solution of 50 % (v/v) formamide (Fluka Biochemicals, Gillingham, U.K.), 1 X MOPS, 5.92 % (v/v) formaldehyde and 2 µL of 10 mg mL^{-1} ethidium bromide in a volume no greater than 50 µL. This solution was heated at 65°C for 5 minutes and chilled on ice. A one-fourth volume of loading dye buffer (50 % (v/v) glycerol, 1 mM EDTA pH 8.0, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF) was added and the RNA samples loaded onto the gel. The gel was run at a constant voltage of 80 V at room temperature until the bromophenol blue band (front) had migrated two thirds of the distance down the gel. The gel was then visualized under UV light.

2.2.7.4. Nucleic Acid Markers.

Nucleic acid markers were run with all samples to size the DNA and RNA that was being analysed. For double stranded (ds) DNA fragments, the following markers were used: 0.075 to 12 kb, 1 kb ladder (Gibco-BRL): 0.568 to 23 kb, *HindIII* digested λ phage (GIBCO-BRL). For analysis of single stranded RNA, the RNA ladder (GIBCO-BRL) containing marker bands ranging in size between 0.24-9.5 kb was used.

2.2.8. Digestion of DNA with Restriction Endonucleases.

The DNA to be cut was prepared in the appropriate buffer with 1-20 units of enzyme(s). The reactions were incubated at 37°C for 2 to 24 hours as required. All reactions were monitored by running an aliquot of the completed reaction mixture on an agarose gel with uncut DNA and appropriate DNA markers.

2.2.9. Isolation of DNA Fragments from Agarose Gels.

For this method the QIAquick Gel Extraction Kit (Qiagen Ltd., Sussex, U.K.) was used in accordance with the manufacturer's instructions. The DNA fragment of interest was initially separated from residual DNA fragments by agarose gel elecrophoresis as described in 2.2.7.1. The fragment was then excised from the agarose gel with a clean, sharp scalpel blade and weighed in a 1.5 mL Eppendorf tube. Three volumes of buffer QX were added to 1 volume of gel (100 mg taken as equivalent to 100 μL), incubated at 50°C for 10 min and mixed by vortexing the tube every 2-3 min during the incubation. After the gel slice had dissolved completely, 1 volume of *iso*-propanol was added to the sample and mixed. A QIAquick spin column was placed in a 2 mL collection tube, the sample applied and then microcentrifuged for 1 min, at 10,000 g. The eluate was discarded and the QIAquick column placed back in the collection tube. The column was then washed with 0.75 mL of buffer QX, incubated for 2-5 min and re-centrifuged for 1 min as before. The eluate was again discarded and the QIAquick column centrifuged once again for 1 min as before to remove the residual buffer PE. The QIAquick column was placed into a clean 1.5 mL microfuge tube and 50 μL of sterile water or 10 mM Tris-HCl, pH 8.5 was added to the centre of the QIAquick column to elute the bound DNA. The QIAquick column was incubated for 1 min, centrifuged for 1 min as before, and stored at -20°C.

2.2.10. Blotting of Nucleic Acids.

2.2.10.1. Southern Blotting.

DNA was separated on agarose gels of appropriate composition as described in Section 2.2.7.1. Essentially, blotting was formed as described in Sambrook, *et al.*, (1989) with the following alterations. A wick of Whatman 3 MM paper was placed onto a horizontal support, soaked in 400 mM NaOH and each end dipped into a reservoir of 400 mM NaOH. The gel was placed on the top of the wick with the well side of the gel (top surface) touching it, ensuring that there were no air bubbles between the wick and the gel.

A piece of nylon membrane (Hybond-N+, Amersham) was cut to a size 1 mm larger than the gel in both dimensions and placed on top of the gel. Any air bubbles between the nylon membrane and the gel were removed using a clean plastic pipette. Two pieces of Whatman 3 MM paper were cut to the size of the gel, soaked in 400 mM NaOH and placed on top of the nylon membrane, again ensuring no air bubbles were entrapped . A frame of cling film was placed around the gel sandwiched between the lower wick and the upper 3 MM paper; this ensured that all rising solution passed from the wick through the gel and up into the upper 3 MM paper. A stack of absorbent paper towels was then placed on top of the 3 MM paper, (approximately 5 cm in height when compressed). A glass plate was placed on the top of the stack of the towels with a 500 g weight on top. The blot was left overnight to allow the nucleic acids to transfer to the membrane. Following this, the membrane was marked with a pen to identify the position of the wells. The gel and the membrane were examined under UV light to assess the transfer of DNA from the gel to the membrane. The membrane was then washed carefully in 6 X SSC (3 M NaCl, 300 mM Na_3 Citrate) for 2 minutes to remove any adhering agarose, and left to dry at room temperature.

2.2.10.2 Northern Blotting.

RNA was separated on a denaturing agarose gel as described in 2.2.7.2. The gel was then blotted as in 2.2.10.1 by using 20 X SSC as blotting buffer (3 M NaCl, 300 mM *tri*-sodium citrate, pH 7.0) and nylon membrane (Hybond-N, Amersham). The RNA was fixed onto the membrane by baking in an oven at 80°C for 2 hours after wrapping between two pieces of Whatman 3 MM paper. At this point the transfer of RNA could be assessed as the RNA on the membrane was still stained with ethidium bromide, or as described in Section 2.2.10.1.

2.2.11. Radiolabelling of DNA.

2.2.11.1 Preparation of DNA Probe.

The DNA fragment to be labelled was cut out of its host plasmid using the appropriate restriction enzyme(s) as described in Section 2.2.8 and purified as described in Section 2.2.9. The amount of DNA was estimated by running an aliquot of the fragment out on an agarose gel as described in Section 2.2.7.1 and comparing it to known amounts of *HindIII*-digested DNA standards run out on the same gel.

2.2.11.2 Random Priming of dsDNA.

The DNA was labelled with [α–^{32}P] dCTP using the Rediprime DNA labelling kit (Amersham International plc, UK) which was used in accordance with the manufacturer's instructions. Approximately 25-50 ng of DNA, prepared as described in Section 2.2.11.1 was diluted to a volume of 45 µL with sterile water in a 1.5 mL Eppendorf tube. The DNA was denatured by heating the sample to 95-100°C in a boiling water bath for 5 minutes and then briefly micro-centrifuged at 10,000 g for 20 seconds to bring the contents to the bottom of the tube. The denatured DNA was added to the labelling mix (dATP, dGTP, dTTP, exonuclease-free 'klenow' enzyme and random nonamer primers) and then mixed by gently flicking the tube until the blue colour of the labelling mix was evenly distributed. After brief micro-centrifugation for 20 seconds at 10,000 g, 50 µCi (3,000 Ci mmol^{-1}) of [α–^{32}P] dCTP was added to the reaction mixture and mixed gently by pipetting up and down. After a brief micro-centrifugation as above, the tube was incubated at 37°C for 10 minutes. The reaction was stopped by adding 5 µL of 500 mM Na_2-EDTA and a further 45 µL of sterile Analar water. For use in hybridization analysis, the DNA was denatured by heating to 95-100°C for 5 minutes. The sample was snap-chilled on ice just prior to use. This was carried out after assessing the level of incorporation and removal of un-incorporated radionucleotides as described in the manufacturer's instructions.

2.2.11.3. Generation of Radioactive ssDNA Probes by Asymmetric PCR.

Asymmetric PCR was performed on DNA fragments which had been cloned into plasmid vectors. The reactions were performed in a 50 µL volume with the appropriate amount of template (~20,000 - 100,000 copies per reaction) and the following ingredients: 200 µM dATP, 200 µM dGTP, 200 µM dTTP, 12 µM dCTP, 25 µCi [α-^{32}P] dCTP, 0.5 µM T3 primer, 0.1 µM T7 primer, 1 X *Taq* buffer and 2.5 units of *Taq* DNA polymerase. The thermal cycler was programmed as follows: 94°C for 30 seconds followed by 35 cycles of 94°C for 10 seconds (melting temperature), the appropriate annealing temperatures for 20 - 120 seconds (as required), 72°C for 120 seconds (extension temperature), followed by 3 min at 27°C.

2.2.11.4. Separation of Labelled DNA from Unincorporated Radio-nucleotides using Spin Columns.

Synthesised oligonucleotides were separated from the un-incorporated nucleotide bases by passing through a Sephadex G-50 column equilibrated with TE buffer pH 8.0.

A 1 mL sterile plastic syringe barrel was plugged with sterilized siliconized glass wool and filled with Sephadex G-50 previously made by autoclaving the Sephadex G50 medium (Pharmacia, Milton-Keynes, UK) in a 20-fold volume of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) containing 0.2 % (w/v) Sodium azide (Sigma). The column was spun in a 15 mL Corex tube at 700 g for 4 minutes at 20°C in a Beckman TJ-6 bench centrifuge; the process was repeated until the column volume was 1 mL. Once this column volume was achieved, 100 µL of TE buffer (pH 8.0) was loaded onto the column and centrifuged as before. The solution of labelled DNA and unincorporated nucleotides were loaded onto the column, and a clean screw-capped Eppendorf tube was placed under the column to collect the eluant from the column. The column was then centrifuged as before and the DNA solution in the Eppendorf tube was stored at 4°C until used.

2.2.11.5. Measurement of Incorporation of Radionucleotide into DNA Probes.

The level of incorporation of radioactivity into DNA probes was estimated by removing 2 µL aliquots of the labelled probe solution before and after centrifugation and throughout the Sephadex column procedure described above (Section 2.2.11.4). The 2 µL samples were placed into screw-capped Eppendorf tubes containing 0.5 mL distilled water. These were then inserted into separate scintillation vials and the radio-activity counted by Cerenkov emission (LKB 1209 Rackbeta scintillation counter). The percentage of incorporation could then be determined by comparing the counts of the two radiolabelled sample aliquots before and after the centrifugation step (counts after centrifugation x 100/counts before centrifugation). The specific activity of the labelled probe (cmp incorporated per µg DNA) could then be calculated, given that the amount of radiolabelled DNA and percentage incorporation of the radiolabel were known. A typical Rediprime reaction with an incorporation of 60 % yielded a specific activity of 1.7 X 10^9 cpm μg^{-1} oligonucleotide.

2.2.12. Hybridization Analysis.

2.2.12.1. Hybridization Analysis of DNA.

Nylon membranes (prepared as described in Section 2.2.10.1) were pre-hybridized at 65°C for 4 hours using either sealed plastic bags placed in a shaking water bath, or sealed in a plastic box and placed in a shaking water bath (Sambrook, *et al.,* 1989), or in a hybridization oven (Techne HB-1D) using borosilicate glass tubes. Enough pre-hybridization solution (5 X SSC, 10 X Denhardt's solution (0.1 % (w/v) Ficoll 400, 0.1 % (w/v) PVP 360, 0.1 % BSA, Sigma), 0.1 % (w/v) SDS, 0.1 % (w/v) Na pyrophosphate, 100 µg mL^{-1} denatured sonicated salmon sperm DNA (Sigma)) was used to just cover the filter(s). The 5 X SSC was prepared by dilution of 20 X SSC (3 M NaCl, 300 mM Na citrate, pH 7.4). The radiolabelled DNA probe was denatured by heating at 95-100°C for 3-5 minutes and then snap-chilled on ice. The denatured probe was then quickly added to the pre-hybridization solution. Care was taken to remove any air bubbles that formed on the surface of the membrane. Hybridization was carried out at 50°C overnight.

2.2.12.2. Hybridization Analysis of RNA.

Northern blots were analysed using the method of Sambrook, *et al.*, (1989). Nylon membranes, prepared as described in Section 2.2.10.2 were pre-hybridized at 42°C in a shaking water bath by using a sealed plastic bag, plastic box or in a hybridization oven for 4 hours. Enough pre-hybridization solution (5 X SSC, 50 % formaldehyde (v/v), 5 X Denhardt's solution, 0.1 % (w/v) SDS, 100 μg mL^{-1} denatured, sonicated salmon sperm DNA) was used to just cover the filter(s). The radiolabelled probe was denatured, as described above, and added to the pre-hybridization solution. Hybridization was carried out at 37°C overnight.

2.2.12.3. Washing Membranes.

After hybridization, the hybridization solution was discarded. The membrane(s) were briefly washed twice in 2 X SSC, 0.1 % (w/v) SDS at room temperature (to remove most of remaining probe from the membrane). The membranes were then washed twice for 15 minutes with an excess of the wash solution (1 X SSC, 0.1 % (w/v) SDS) in a shaking water bath at 50-55°C. The membranes were then washed at increasing stringency depending on the amount of radioactivity bound to the membrane. Details of stringency are given in the results Section and the appropriate figure legends. After washing, the membrane(s) were wrapped in cling film and autoradiographed at -80°C.

2.2.12.4. Autoradiography.

Membrane(s) to be autoradiographed were wrapped in cling film and exposed to Fuji X-ray film (Type RX) in a film cassette with an intensifying screen at -80°C for the appropriate length of time.

2.2.12.5. Stripping Membranes of Bound Radioactive Probes and Blocking Agent.

After hybridization and autoradiography, the bound probe was removed by pouring a boiling solution of 0.1 % (w/v) SDS over the membrane(s). The solution was then allowed at cool to room temperature (approximately 5 min.), and the process was repeated twice. The membrane(s) were then wrapped in cling film and autoradiographed overnight to check that the bound probe had been removed. The presence of RNA on the membrane after stripping could then detected as described below (Section 2.2.12.6).

2.2.12.6. Staining of Membrane-bound DNA/RNA with Methylene Blue.

This procedure was carried out as described by Sambrook, *et al.*, (1989). The filter containing the bound nucleotides (RNA or DNA) was placed in 0.04 % (w/v) methylene blue, 200 mM Na acetate (pH 5.2) for 5-10 min. The filter was then washed in distilled water until the RNA/DNA could be visualised as a blue colouration on a white background. The stain was removed by washing the filter in 20 % (v/v) acetic acid until the bands were no longer visible.

2.2.13. Screening Libraries.

2.2.13.1. Preparation of Plating Bacteria.

XL1-blue MRA(P2) was used as a host strain for plating the genomic libraries. A single bacterial colony was picked and used to inoculate 10 mL of L-broth supplemented with 0.2% maltose (w/v) and 10 mM $MgSO_4$. This was grown overnight at 37°C with constant shaking. Five mL of this overnight culture was inoculated into 100 mL of L-broth containing 0.2% maltose (w/v) and 10 mM $MgSO_4$ and incubated as before for 1-2 hours or until the culture density at 600 nm was 0.3-0.5 A.D. units. The cells were then transferred into two sterile 50 mL Falcon tubes, pelleted by centrifugation for 10 min at 5000 g and re-suspended into 10 mM $MgSO_4$. The suspension was diluted to an OD of 2

at 600 nm (~1.6 x 10^9 cells mL^{-1}) using ice-cold 10 mM $MgSO_4$ and stored at 4°C until use (the cells can be stored for up to three weeks).

2.2.13.2. Plating Bacteriophage.

Plaque screening (hybridization) techniques were used for the identification of recombinant clones of interest from genomic libraries constructed in the λ Dash II *BamHI* vector (Stratagene, Ltd Cambridge, UK). Essentially the protocols of Sambrook, *et al.*, (1989) were used.

300 µL aliquots of plating bacteria, were prepared as described in Section 2.2.13.1 and dispensed into sterile 15 mL Falcon tubes, to which 50 µL of an appropriate dilution of bacteriophage library was added. After the tubes had been mixed, they were incubated at 37°C for 15 min. After this incubation, 10 ml of 'top agar' containing 0.2% (w/v) maltose and 10 mM $MgSO_4$ (42°C) was added to each tube and was briefly mixed and immediately poured onto a dry pre-warmed 150 mm diameter plastic petri dish containing 30 ml of solid LB agar supplemented with 10 mM $MgSO_4$ and 0.2% maltose. The plate was swirled gently in order to distribute the top agar evenly over the surface of the plate.

The plates were left to stand on a flat surface for 20 min at room temperature to allow the top agar to solidify. Then, the petri dishes were inverted and incubated at 37°C for 12-16h until the desired plaque size was achieved (typically 0.5 - 1.0 mm dia.)

2.2.13.3. Plaque Lifts.

The method used was based on that of Benton & Davis (1977). Plates with a diameter of 150 mm were prepared as outlined in Section 2.2.13.2 and an appropriate number of phage (1 x 10^5 pfu per 150 mm plate) were plated out. Plates were chilled at 4°C for 30-60 min to allow the top agar to solidify before plaque lifts were attempted. Meanwhile, the appropriate number of nylon membranes (Hybond-N Amersham) were labelled individually and marked asymmetrically in three locations with permanent marker to allow future plate/membrane re-orientation (see Sambrook, *et al.*, 1989).

A membrane was laid carefully onto each plate from the centre outwards, so that it made direct contact with the plaques with no entrapped air bubbles. The positions of the reference points on the membrane were recorded onto the plate and after 1 min the first membrane was peeled off the plate with a pair of clean, sterile blunt-ended forceps. Duplicate lifts were made for each plate, the second membrane was left on each plate for a duration of 2 min.

After its removal from the plate, each membrane was placed DNA side up, onto two sheets of Whatman 3 MM paper soaked in denaturation solution (500 mM NaOH, 1.5 M NaCl) for 5 min. Then, the membrane were carefully drained of excess solution and transferred to two sheets of Whatman 3 MM paper soaked in neutralisation solution (500 mM Tris-HCl (pH 7.5), 1.5 M NaCl) for a further 5 min. Finally, the membranes were rinsed briefly in 2 X SSC and left, DNA side up, on Whatman 3 MM paper to dry. DNA was fixed onto the membrane by baking at 80°C for 2h.

2.2.13.4. Selection of Bacteriophage Plaques.

Hybridization analysis of membranes and washing was the same as described for Southern blots in Section 2.4.9.1. In cases where the ratio of the background to specific signal was high, membranes were washed again at higher stringency (0.1 X SSC, 0.1 % SDS (w/v) at 65°C for 20 min). Membranes were then wrapped in cling film and autoradiographed at 80°C.

The pattern of dots produced on the autoradiograph, by hybridization of plaque DNA with a probe, was aligned with the corresponding positions on the plate with the help of the reference marks on the membrane and plate. Plaques of interest were removed from initial rounds of screening with the wide end of a sterile pasteur pipette. The agar plaque was transferred to a 1.5 mL Eppendorf tube which contained 1 mL of SM phage buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 8 mM $MgSO_4$) and 50 µL of chloroform, vortexed briefly and left at room temperature for 1 h to allow the phage to diffuse out into the solution. The phage suspension was titered by plating out a series of phage dilutions ranging from 10^{-2} - 10^{-7}. The following equation was used to calculate the titer:

[Number of plaques X volume of packaging extract (mL)] / Dilution

= pfu mL^{-1} original packaging extract.

The process of screening was repeated with a lower plaque density per plate until plaque purity was attained. This typically took three to four rounds of screening.

2.2.13.5. Preparation of Plate Lysate Stocks.

An inoculum of 10^5 pfu was used to produce confluent lysis of a bacterial lawn grown on a 150 mm diameter plate. Phage were eluted out of the agarose by the addition of 10 mL of SM phage buffer to each plate, and then gently shaking the plate at 4°C overnight. The bacteriophage suspension was recovered into a sterile 30 mL polypropylene tube and each plate was rinsed with a further 3 mL of SM phage buffer. A volume of 5 % (v/v) chloroform was added to to each suspension, vortexed and incubated at room temperature for 15 min. Bacterial cell debris was removed from the suspension by centrifugation in Beckman J2-HS centrifuge, JA-20 rotor at 200 g for 5 min. at 4°C. Then the supernatant was transferred to a fresh tube, chloroform was added to 0.3 % (v/v) and the lysate was titered. The phage lysates were stored at 4°C.

2.2.13.6. Large-scale Preparation of DNA From Genomic Clones.

Lysed cultures were prepared as outlined in Section 2.2.13.5. Digestion of bacterial nucleic acid was achieved by the addition of 1 µg mL^{-1} DNAase (type VIII) and 1 µg mL^{-1} of RNAase (type I-A) (both from Sigma) to the tube containing the lysed culture, swirled gently, and incubated for 30 min at room temperature. Solid NaCl was added to the culture to a final concentration of 1 M and dissolved by swirling. The culture was incubated for 1 h at room temperature and then centrifuged in a Beckman J2-HS centrifuge, JA-20 rotor at 11,000 g for 10 minutes at 4°C to bring down cellular debris. The supernatant was poured into a fresh flask containing 10 % (w/v) polyethylene glycol 8000 (PEG 8000), swirled very gently at room temperature until all the PEG 8000 had dissolved and then left to stand at 4°C overnight to precipitate the bacteriophage. The

bacteriophage was harvested by centrifugation at 11,000 g in a Beckman J2-HS centrifuge, JA-20 rotor for 10 minutes at 4°C. The supernatant was discarded, and the precipitate was resuspended gently in 8 mL of SM phage buffer. The tubes were then washed with a further 8 mL of phage buffer and both washes combined in a sterile centrifuge tube. An equal volume of chloroform was added to the bacteriophage suspension, vortexed for 30 seconds, and centrifuged at 3000 g for 15 min at 4°C. The aqueous phases which contains the bacteriophage particles were transferred to a fresh centrifuge tube and the phage particles were collected by centrifugation in Sorvall OTD 55B ultracentrifuge at 18,000 g for 2 h at 4°C. The supernatant was poured off, 1-2 mL of SM phage buffer were added to the tube and left overnight at 4°C on a shaking - rocking platform. The following day the solution containing the phage particles and SM buffer was pipetted up and down to ensure that all of the bacteriophage particles had been resuspended and then transferred to sterile tube. To this tube, 50 μg mL^{-1} of proteinase K and SDS to a final concentration of 0.5 % (w/v) were added. The contents of the tube were mixed by inverting several times, and then incubated at 65°C for 1 h. This process removed the protein coat from the bacteriophage DNA. After incubation, the solution was cooled to room temperature and extracted twice with equal volume of phenol (pH 8.0) and once with phenol : chloroform (50 : 50 (v/v)). The aqueous phase was extracted once more with an equal volume of chloroform and finally precipitated with 2 volumes of ethanol. The resulting DNA pellet was washed with 70 % ethanol, air dried and resuspended in 500 μL H_2O and the stored at -20°C until required.

2.2.14. Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

2.2.14.1. Preparation of First Strand cDNA for use as Template in PCR Reactions.

This reaction was carried out using the Stratagene Reverse Transcription System (RT-PCR) Kit. Any enzymes and reagents used were supplied with this kit unless otherwise stated.

Total RNA (5-10 μg) and poly $(A)^+$ mRNA (50- 100 ng) previously prepared as described in Sections 2.2.2.2 and 2.2.2.4 respectively, were used separately in a final volume of 38

μL (adjusted with DEPC-treated water). Three μL of oligo(dT) (100 ng μL^{-1}) were added to the reactions and mixed gently. The reactions were incubated at 65°C for 5 min and cooled slowly to room temperature (approximately 10 min) to allow the primer to anneal to the total RNA or poly $(A)^+$ mRNA. Then 5 μL of 10 X first-strand buffer, 1 μL of RNase Block Ribonuclease Inhibitor (40 units μL^{-1}), 2 μL of 100 mM dNTPs (dATP, dCTP, dGTP, dTTP) and 1 μL (50 units) of MMLV-RT (Moloney Murine Leukemia Reverse Transcriptase) were added in this order to the above reaction tubes. The reactions were mixed gently and incubated at 37°C for one hour. Finally, the reactions were heated at 90°C for 5 min and then stored on ice. First strand cDNA was also prepared from total RNA and poly $(A)^+$ mRNA using the alternative Random Primer method (100 ng μL^{-1}) in place of oligo (dT) (conditions as above).

2.2.15. Polymerase Chain Reaction (PCR).

2.2.15.1. PCR Amplification of Nucleic Acid Sequences.

The polymerase chain reaction was used to amplify DNA sequences from the cDNA library, genomic DNA and first strand cDNA. In general 0.5-2.5 U of *Taq* DNA polymerase (Promega) per reaction was used in 1 X thermophilic *Taq* buffer (50 mM KCl, 10 mM Tris-HCl, (pH 9.0), 0.1 % Triton X-100, (Promega) with 1-4 mM $MgCl_2$, 200 μM dNTPs (dATP, dCTP, dGTP and dTTP) and 50 pmol of each primer (forward and reverse primer). The reactions were performed in a volume of 50 μL in 0.5 mL Eppendorf tubes. The same reagents were used for PCR reactions where *Pfu* polymerase (Stratagene) was used in place of *Taq* polymerase. This required 1 X *Pfu* buffer (20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH)_2SO_4$, 0.1% Triton X-100, 100 μg mL^{-1} nuclease-free BSA, (Stratagene)). All the reagents were mixed gently, two drops of mineral oil (Sigma) were layered on top to reduce evaporation, and the tubes were placed in a Grant Autogene PCR thermal cycler.

Typically, the thermal cycler was programmed to denature the samples for 5 min at 94°C and then complete 35 cycles. Denaturation was performed at 95°C for 5 min and extension at 72°C for 20 - 180 s. The annealing temperature depended on the primers

being used, and was set initially 5°C below the estimated melting temperature of the primer DNA sequence and subsequently adjusted, as required, to achieve optimisation. The melting temperature was estimated by using the formula T_m=(3 x GC) + (2 x AT)°C where GC and AT are the number of GC or AT base pairs in the primer. The PCR products were generally analysed by agarose gel elecrophoresis (Section 2.2.7.1).

2.2.15.2. Purifying the PCR Products.

Two methods were used to purify the PCR product before cloning from excess PCR primer and enzyme. The PCR fragments were isolated and purified from the gel by using the Qiaquick Gel Extraction Kit (Qiagen, Section 2.2.9), or alternatively by selective precipitation (as described by PCR-Script cloning manual, Stratagene).

2.2.15.3. Purifying the PCR Products by Selective Precipitation.

One-tenth of the volume of 10 x STE buffer (1 M NaCl, 200mM Tris-HCl (pH 7.5), 100 mM EDTA) was added to the PCR products followed by addition of an equal volume of 4 M ammonium acetate. Then, 2.5 volumes of 100% (v/v) ethanol, equilibrated to room temperature, were added. The reaction tube was immediately spun in a microcentrifuge at 10,000 g for 20 min at room temperature to pellet the DNA and the supernatant was carefully removed. The DNA pellet was washed with 200 µL of 70% (v/v) ethanol, spun as before for 10 min at room temperature and the ethanol was removed carefully with a pipette. The DNA pellet was then dried, resuspended to the original volume using TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA). The concentration of the DNA was determined as in Section 2.2.6 and stored at 4°C until use.

2.2.15.4. 'Polishing' the Purified PCR Products.

The ends of purified PCR products generated with *Taq* DNA polymerase were polished using the *pfu*-based PCR-Script™ cloning protocol (Stratagene, 1997).

Polishing reactions were prepared by adding the following components in order to a 0.5 mL microcentrifuge tube. Ten µL of the purified PCR product (0.5-1 µg DNA), 1 µL of 10 mM dNTP mix (2.5 mM of each, dATP, dCTP, dGTP and dTTP), 1.3 µL of 10 X polishing buffer and 1 µL of cloned *Pfu* DNA polymerase (0.5 U). The polishing reaction was gently mixed, layered with 20 µL mineral oil and then incubated for 30 min at 72°C. Finally, the polishing reaction was added directly to the ligation reaction or stored at 4°C until use.

2.2.15.5. Cloning PCR Products.

Ligation of PCR Product into PCR-Script™ cloning vector.

The PCR products generated using *Pfu* DNA polymerase could be cloned into a plasmid vector PCR-Script™ (SK+) (Stratagene). Ligation was performed according to the manufacturer's instructions.

The ligations were carried out in 0.5 mL microcentrifuge tubes by adding the following components: 1 µL of the PCR-Script Amp SK(+) cloning vector (10 ng/µL), 1 µL of PCR-Script 10 X reaction buffer, 0.5 µL of 10 mM 'rATP', 2-4 µL of PCR product (40:1 to 100:1 insert-to-vector ratio) or 4 µL of the control PCR insert, 1 µL of *Srf* restriction enzyme (5 U/µL), 1 µL of T4 DNA ligase and distilled water to a final volume of 10 µL. The ligation reaction was mixed gently, and allowed to proceed at room temperature for one hour before heating at 65°C for 10 min . The ligation reaction was stored on ice until transformation into Epicurian Coli XL1-blue MRF' Kan supercompetent cells as described in Section 2.2.3.2.2.

2.2.15.6. PCR Amplification of Plasmid Vector Insert.

The sizes of DNA inserts in plasmid vectors were estimated using PCR amplification with primers that complement the vector multiple cloning site flanking sequences. This allowed the estimation of the length of the insert in the plasmid vector. The DNA insert was amplified directly from individual bacterial colonies. Individual fresh bacterial

colonies were picked with the tip of a sterile pasteur pipette and resuspended into 0.5 mL microcentrifuge tubes containing 10 μL of sterile water. The tubes were heated at 95°C for 5 min to denature the DNA, cooled on ice for another 5 min, and spun briefly in a microcentrifuge (10,000 g for 20 seconds). Forty μL of the PCR reaction solution was added to each tube in the following order: five μL of 1 X thermophilic *Taq* buffer (Promega), 2 mM $MgCl_2$, 100 μM dNTPs (dATP, dCTP, dGTP and dTTP), 0.1 μM of oligonucleotide primers (T3 and T7 as these primers flank the site and can be used to accurately size the cloned fragments) and 1 unit *Taq* DNA polymerase (Promega). All the reagents were mixed gently, the mixture was layered with two drops of mineral oil (Sigma) to reduce evaporation and the tubes were placed in a Hybond OMI Gene PCR thermal cycler.

The thermal cycler was programmed to denature the samples for 5 min at 94°C and then to complete 35 cycles of 94°C for 1 min (denaturation), 45-50°C for 1 min (annealing) and 72°C for 2 min (extension). At the end of the last cycle the sample was heated at 72°C for a further 3 min to ensure full extension of the product and then cooled down to 18°C. PCR products were generally analysed by agarose gel electrophoresis (Section 2.2.7.1).

2.2.16. DNA Sequencing.

DNA Sequencing of the plasmid insert was performed on a Applied Biosystems Model 373-Stretch or 377 Automated DNA Sequencer by the Molecular Biology Support Unit, IBLS, University of Glasgow. DNA sequencing was performed using the dye terminator method with redi-reaction Ampli *Taq* DNA polymerase.

2.2.17. Computer Analysis of DNA Sequence.

Sequence files in EBI-150 format from the Automated sequencing facility (Section 2.2.16) were analysed using the Sequence Navigator program and the GCG program. Further sequence analysis was performed using a range of programs available at the

European Bioinformatics Institute (EBI located at www.ebi.ac.uk; CLUSTALW, BLAST2 and BLITZ), the National Centre for Biotechnology Information (NCBI located at www.ncbi.nih.gov; ENTREZ) or the Human Genome Sequencing Project (HGMP located at www.hgmp.mrc.ac.uk; GRAIL, NIX, *etc.*). Large scale sequencing (*i.e.* primer walking) was performed by MGW Biotech.

CHAPTER THREE
HETEROLOGOUS PROBING & COLONY HYBRIDIZATION.

3.1. Genomic Southern Analysis with Heterologous Probes.

A great deal of effort has been expended on trying to understand how salinity affects plants and how salt-resistant plants survive high NaCl concentrations. However, although large amounts of information have been gathered, most of these studies have been descriptive. Undoubtedly, if the genes responsible for maintaining ionic balance in salt-resistant plants could be identified, this area of plant biology would make significant progress.

One way of identifying putative genes for H^+, Na^+ and Cl^- transporters from salt resistant plants is to use heterologous probes in Southern and Northern blot experiments. Although this is now considered to be a some what out-dated approach, it has proved successful in the past.

Therefore, genomic Southern and Northern blot experiments were carried out in an attempt to identify homologous plant genes for Na^+ and Cl^- transport from plant cells of salt-tolerant crops. A range of probes from different sources were used, these were *sod2*, *adk-1*, *at57*, *ant* (*nhaA*), and *clc-0* (Table 3.1).

Southern blot analysis was performed using heterologous probes (Table 3.1) hybridized to a range of plant genomic DNAs. Genomic DNA was isolated from leaves of barley (*Hordeum vulgare* Var Golden Promise), sugar beet (*Beta vulgaris*, Var Saxon), *Phaseolus vulgaris* (bean), *Arabidopsis thaliana*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. The genomic DNA was digested individually with restriction enzymes *EcoR1*, *BamH1* and *HindIII* (Section 2.2.8). Appropriate amounts of DNA were loaded onto an agarose gel to ensure equivalent amounts of putative target sequences; (see Figs 3.1- 3.5 for details) this is important as the genome size range from

Insert (gene)	Plasmid containing the insert	Enzyme used to excise insert	Insert size (Kb)	Activity of Insert	Source of Isolation
sod2	psod2	*HindIII*	2.3	Na^+/H^+ antiporter	*S. pombe**
adk-1	pRS316	*EcoRI*	2.8	P-type H^+-ATPase	*N. plumbaginifolia**
at57	pat57	*EcoRI*	1.9	V-type H^+-ATPase	*A. thaliana**
clc-0	pCDM8	*XbaI/XhoI*	1.6	Chloride channel	*T. marmorata**
nhaA	pKR323	*HindIII /BamHI*	1.8	Na^+/H^+ antiporter	*E. coli* KR323*

**Schizosaccharomyces pombe* source of *sod2*, supplied by Jia *et al.*, (1992).
Arabidopsis thaliana source of *at57*, supplied by Manolson *et al.*, (1988).
Nicotiana plumbaginifolia source of *adk-1*, supplied by Boutry *et al.*, (1989).
Torpedo marmorata source of *ClC-0*, supplied by Jentsch *et al.*, (1990).
Escherichia coli KR323 source of *nhaA*, supplied by Karpel *et al.*, (1988).

Table 3.1. Probes used to screen barley and sugar beet genomic DNA.

4 x 10^6 bp (*E. coli*) to 3 x 10^9 bp (barley and bean). The digested genomic DNA was separated on agarose gels (Section 2.2.7.1) and blotted onto nylon membranes (Section 2.2.10.1). These Southern blots were then used in the hybridization experiments with the heterologous probes listed in table 3.1 (Section 2.2.12.1), washed and exposed to X-ray film.

3.1.1. Genomic Southern Analysis using p-type H^+-ATPase Sequences.

Figure 3.1 presents the results from a genomic southern blot of *H. vulgare* (barley), *B. vulgaris* (sugar beet), *A. thaliana* , *Phaseolus vulgaris* (bean), *Schizosaccharomyces pombe* and *Schizosaccharomyces cerevisiae* DNA, each cut with the restriction enzymes *EcoR1*, *BamH1* and *HindIII*, and probed with labelled *adk-1*, a *Nicotiana plumbaginifolia* gene coding for a plasma membrane H^+-ATPase (Boutry, *et al.*, 1989). The blot was washed at high stringency (0.1 X SSC at 65°C) and exposed to X-ray film for 3 days.

The results of the hybridization show that there are several strong hybridization bands found in *B. vulgaris* (sugar beet) genomic DNA digested with *EcoR1*(lane 2, at 1.6, 2.3, 4, and 10 kb), *BamH1* (lane 8, at 4, 6.5 and 10 kb) and *HindIII* (lane 14, at 1, 2.1, 2.5, 3.5 and 10 kb). In addition, strong hybridization bands were also observed in all three restriction digests of the *Phaseolus vulgaris* (bean) genome (lanes 3, 9 and 15). The genomes of *H. vulgare* and *A. thaliana* also appear to have sequences homologous to *adk-1* (lanes 1, 7, 13 for *H. vulgare* and lanes 4, 10, 16 for *A. thaliana*). *Saccharomyces cerevisiae* was found to contain *adk-1* homologues (lanes, 6, 12, and 18). In contrast, no homologues of *adk-1* appear to be present in *Schizosaccharomyces pombe* (lanes 5, 11 and 17).

3.1.2. Genomic Southern Analysis using v-type H^+-ATPase Sequences.

Figure 3.2 presents the results from a genomic southern blot of *H. vulgare* (barley) , *B. vulgaris* (sugar beet), *A. thaliana*, *Phaseolus vulgaris* (bean), *Schizosaccharomyces pombe*

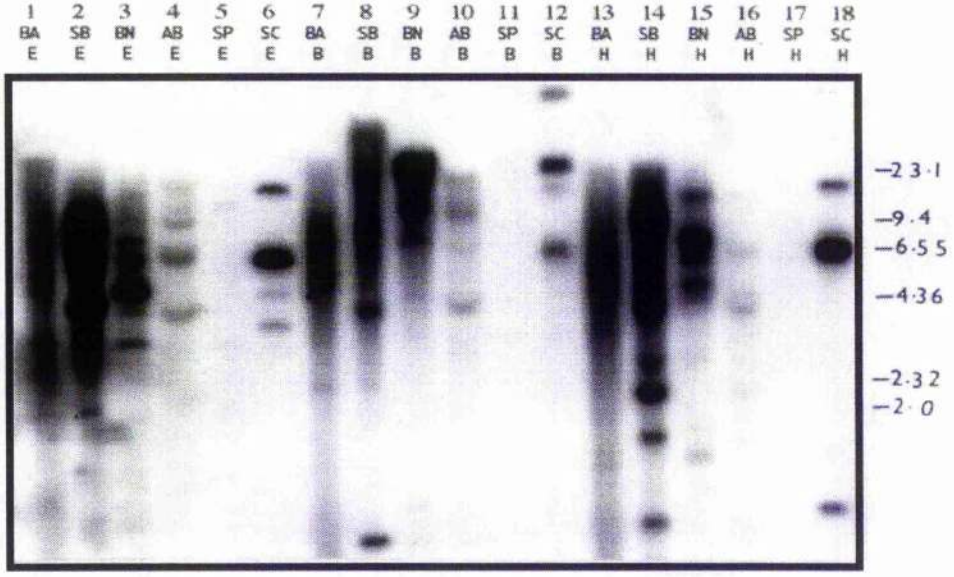


Figure. 3.1. Genomic Southern Blot Analysis of Plant Genomic DNA Hybridized with *adk-1* (encoding a p-type H^+-ATPase).

Genomic DNA was isolated from *H. vulgare* (barley, BA), *B. vulgaris* (sugar beet, SB), *Ph. vulgaris* (bean, BN), *A. thaliana* (AB), *Schizosaccharomyces pombe* (SP) and *Saccharomyces cerevisiae* (SC). Ten μg of genomic DNA from each plant, (1 μg of *A. thaliana*) and 200 ng from each yeast were individually digested with the following restriction enzymes: *EcoRI* (E) in lanes 1-6, *BamHI* (B) in lanes 7-12 and *HindIII* (H) in lanes 13-18. The digested DNA was separated in a 1% (w/v) agarose gel, hybridized with *adk-1* and washed at high stringency (0.1 X SSC at 65°C) then exposed to X-ray film for 3 days at -80°C. The positions of the DNA marker (λ *HindIII*) are indicated.

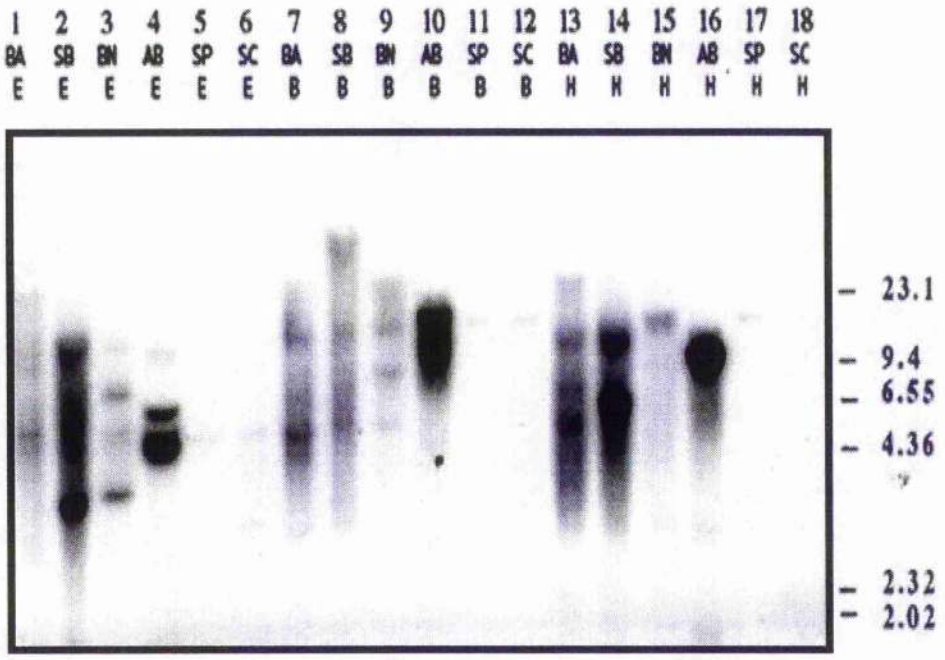


Figure. 3.2. Genomic Southern Blot Analysis of Plant Genomic DNA Hybridized with *at57* (encoding subunit B, of a v- type H^+-ATPase).

Genomic DNA was isolated from *H. vulgare* (barley, BA), *B. vulgaris* (sugar beet, SB), *Ph. vulgaris* (bean, BN), *A. thaliana* (AB), *Schizosaccharomyces pombe* (SP) and *Saccharomyces cerevisiae* (SC). Ten μg of genomic DNA from each plant, (1 μg of *A. thaliana*) and 200 ng from each yeast were individually digested with the following restriction enzymes: *EcoRI* (E) in lanes 1-6, *BamHI* (B) in lanes 7-12 and *HindIII* (H) in lanes 13-18. The digested DNA was separated in a 1% (w/v) agarose gel, hybridized with *at57* and washed at high stringency (0.1 X SSC at 65°C) then exposed to X-ray film for 3 days at -80°C. The positions of the DNA marker (λ *HidIII*) are indicated.

and *Saccharomyces cerevisiae* DNA, each cut with the restriction enzymes *EcoR1* (E), *BamH1* (B) and *HindIII* (H), and probed with *at57*, an *Arabidopsis* gene coding for 57 KDa subunit B of vacuolar membrane H^+-ATPase (Manolson, *et al.*, 1988). The blot was washed at high stringency (0.1 X SSC at 65°C) and exposed to X-ray film overnight. Several hybridizing bands were observed. As would be expected, strong hybridization was found in all three restriction digests of *Arabidopsis* genomic DNA (lanes 4, 10, 16), but it is not possible to determine with any certainly how many copies of *at57* are present in the genome. There is also evidence that homologues of *at57* are found in the *B. vulgaris* (sugar beet) genome (lane 2 at 3, 4.5 and 9.4 kb and lane 14 at 5 and 10 kb). The genomes of *Phaseolus vulgaris* (lanes 3, 9, and 15) and *H. vulgare* (lanes 1, 7, and 13) appear to have sequences with some weak homology to *at57* and this may reflect the fact that these two species are more distantly related to *Arabidopsis* than *B. vulgaris*. Neither *Schizosaccharomyces pombe* nor *Saccharomyces cerevisiae* appear to have sequences that are homologous to *at57*.

3.1.3. Genomic Southern Analysis Using Na^+/H^+ Antiporter Sequences from *Schizosaccharomyces pombe.*

Figure 3.3 shows the results from a genomic southern blot of *H. vulgare* (barley) , *B. vulgaris* (sugar beet), *A. thaliana*, *Phaseolus vulgaris* (bean), *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* DNA, each cut with the restriction enzymes *EcoR1* (E), *BamH1* (B) and *HindIII* (H), and probed with *sod2*, a *Schizosaccharomyces pombe* gene coding for a Na^+/H^+ antiporter (Jia, *et al.*, 1992). The blot was washed at moderate stringency (0.5 X SSC at 65°C) and exposed to X-ray film for 3 days. As would be expected, hybridization signals were observed in all three restriction digests of *Schizosaccharomyces pombe* genomic DNA (lanes 5, 11, and 17). These results suggested the presence of one gene in the *Schizosaccharomyces pombe* genome. A putative homologue to *sod2* was also found in *H. vulgare* (barley), strong hybridization was observed in *HindIII* genomic DNA fragments (lane 13 at 2.2 kb) in addition to some weak hybridization bands (lane 13 at 4.3 kb, 4.7 kb and 5.5 kb). Moreover, the blot shows some weak hybridization bands appearing in *EcoR1* genomic DNA fragments (lane 1 at 3.5kb, 5 kb and 6.5 kb) and *BamH1* fragments (lane 7 at 4, 4.5 and 5 kb). It

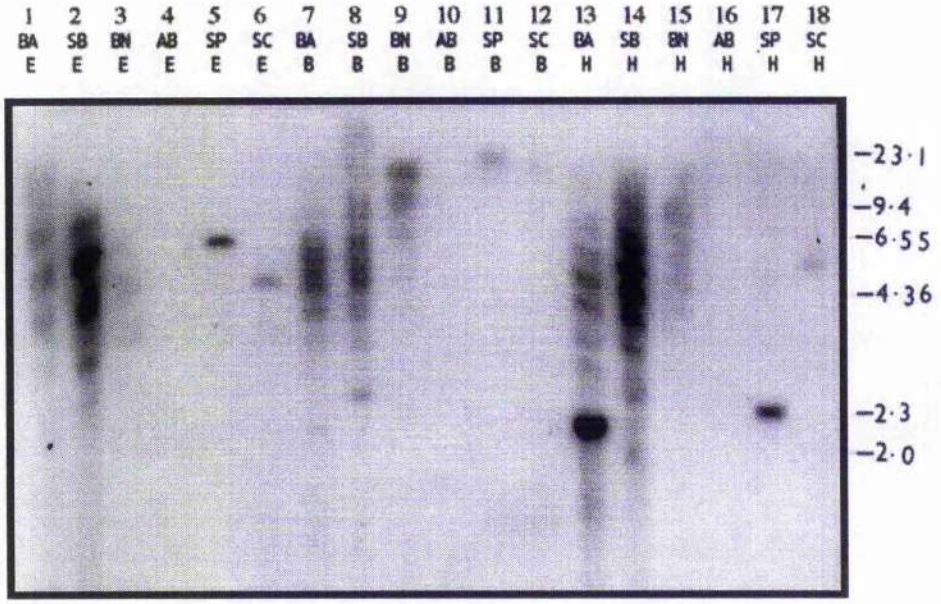


Figure. 3.3. Genomic Southern Blot Analysis of Plant Genomic DNA Hybridized with *sod2* (encoding a Na^+ /H^+ antiporter).

Genomic DNA was isolated from *H. vulgare* (barley, BA), *B. vulgaris* (sugar beet, SB), *Ph. Vulgaris* (bean, BN), *A. thaliana* (AB), *Schizosaccharomyces pombe* (SP) and *Saccharomyces cerevisiae* (SC). Ten µg of genomic DNA from each plant, (1 µg of *A. thaliana*) and 200 ng from each yeast were individually digested with the following restriction enzymes: *EcoRI* (E) in lanes 1-6, *BamHI* (B) in lanes 7-12 and *HindIII* (H) in lanes 13-18. The digested DNA was separated on a 1% (w/v) agarose gel, hybridized with *sod2* and washed at moderate stringency (0.5 X SSC at 65°C) then exposed to X- ray film for 3 days at -80°C. The positions of the DNA marker (λ *HidIII*) are indicated.

was observed also that *B. vulgaris* (sugar beet) has strong hybridization bands in *EcoR1* fragments (lane 2 at 4, 5.5 and 6.6 kb) and in *HindIII* fragments (lane 14 at 3.4, 4, 5, and 5.8 kb), but weak hybridization appeared in *BamH1* fragments (lane 8 at 3.7, 4.4, 6 and 7 kb). The genome of *Saccharomyces cerevisiae* appears to have sequences with weak homology to *sod2* (lanes 6, 11 and 18). Furthermore, the blot shows that there is no evidence that *Ph. vulgaris* (bean) and *A. thaliana* genomes have sequences that are homologous to *sod2* (lanes 3, 9 and 15) and (lanes 4, 10 and 16) respectively.

3.1.4. Genomic Southern Analysis using Na^+/H^+ Sequences from *E. coli*.

Figure 3.4 presents a genomic southern blot of *H. vulgare* (barley), *B. vulgaris* (sugar beet), *A. thaliana* , *Phaseolus vulgaris* (bean), *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* DNA, each cut with the restriction enzymes *EcoR1* (E), *BamH1* (B) and *HindIII* (H), and hybridized with *nhaA*, a clone from *E. coli* coding for a Na^+/H^+ antiporter (Padan, *et al.*, 1989). The blot was washed at moderate stringency (0.5 X SSC at 65°C) and exposed to X-ray film for 3 days. The hybridization resulted in weak hybridization signals, possibly because the membrane had been challenged and stripped several times with other probes. It is concluded that there is some evidence that homologues to *nhaA* are found in *H. vulgare* (barley) genomic DNA (lane 7 at 4 kb and lane 13 at 3.5 kb). The genome of *B. vulgaris* (sugar beet) shows sequence homology to *nhaA* in *HindIII* genomic DNA fragments (lane 14 at 4 kb). The genome of *Phaseolus vulgaris* also appears to have some sequence homology to *nhaA* (lanes 3 and 9). Neither *Saccharomyces cerevisiae* nor *A. thaliana* have sequences that hybridize to *nhaA*. Figure 4b shows the result of the control experiment of a genomic southern blot containing *E. coli* genomic DNA digested with *EcoR1* (E), *BamH1* (B) and *HindIII* (H) and hybridized with *nhaA*. The blot was washed at moderate stringency (0.5 X SSC at 65°C). As expected, strong hybridization was observed in all three restriction digests of *E. coli* genomic DNA (Fig 4b lanes 1, 2 and 3).

3.1.5. Genomic Southern Analysis Using Chloride Channel Sequences.

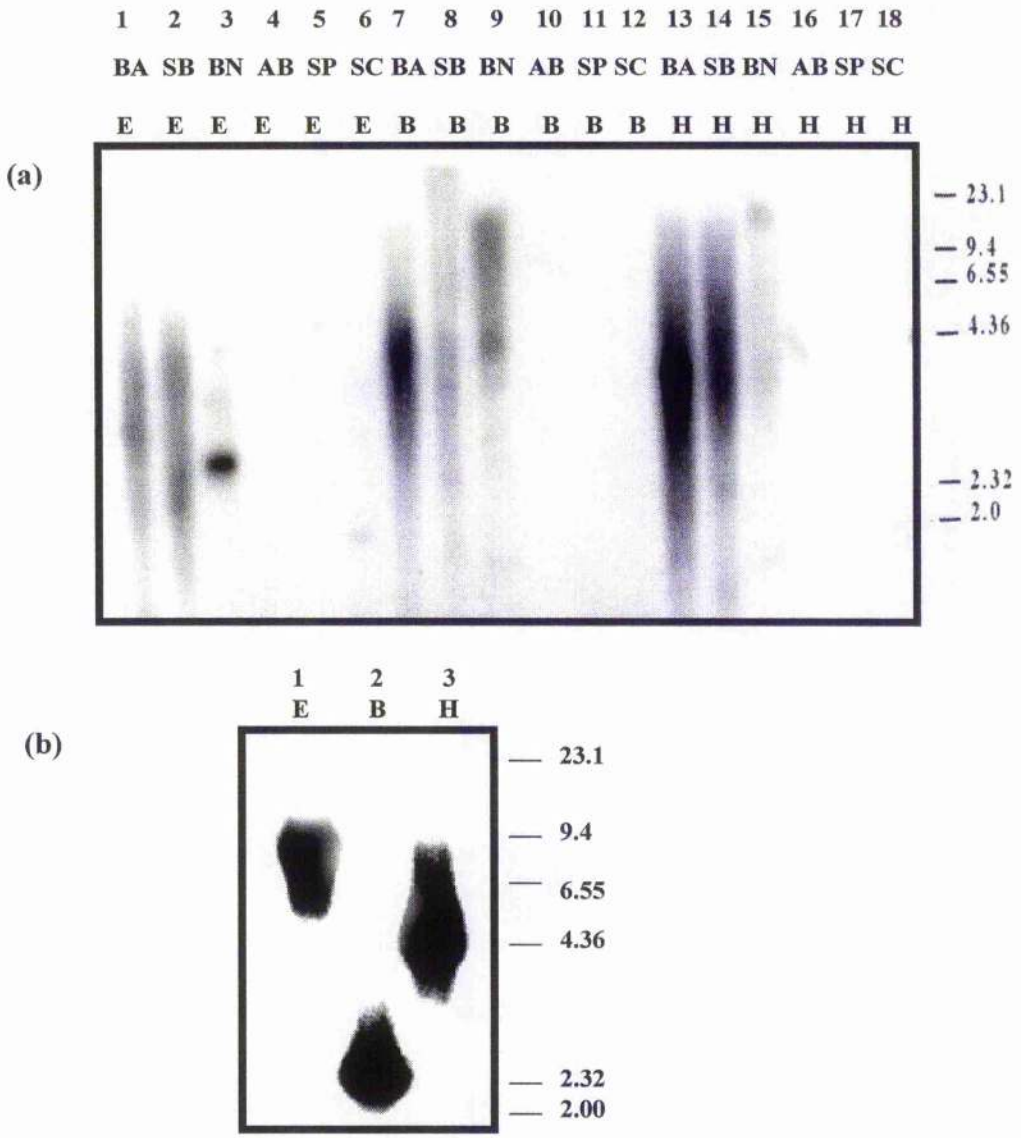


Figure. 3.4. Genomic Southern Blot Analysis of Plant Genomic DNA Hybridized with *nhaA* (encoding a Na^+ /H^+ antiporter from *E. coli*).

(a) Genomic DNA was isolated from *H. vulgare* (barley, BA), *B. vulgaris* (sugar beet, SB), *Ph. vulgaris* (bean, BN), *A. thaliana* (AB), *Schizosaccharomyces pombe* (SP) and *Saccharomyces cerevisiae* (SC). Ten µg of genomic DNA from each plant, (1 µg of *A. Thaliana*) and 200 ng from each yeast were individually digested with the following restriction enzymes: *EcoRI* (E) in lanes 1-6, *BamHI* (B) in lanes 7-12 and *HindIII* (H) in lanes 13-18. The digested DNA was separated in a 1% (w/v) agarose gel, hybridized with *nhaA* and washed at moderate stringency (0.5 X SSC at 65°C) then exposed to X- ray film for 3 days at -80°C. **(b)** Control; Southern blot of 50 ng of *EcoRI, BamH1* and *HindIII* digested *E. coli* genomic DNA hybridized and washed with the filter above. The positions of the DNA marker (λ *HidIII*) are indicated.

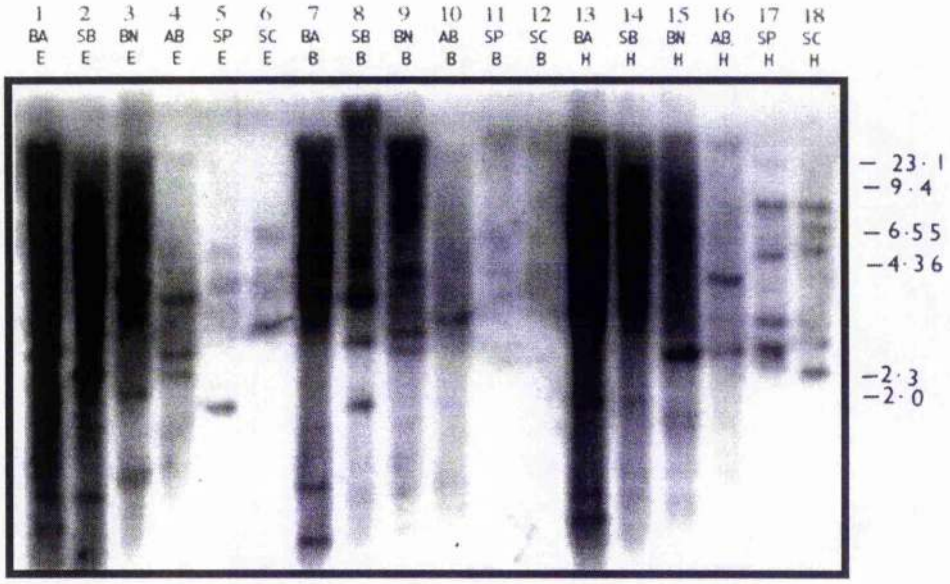


Figure. 3.5. Genomic Southern Blot Analysis of Plant Genomic DNA Hybridized with *clc-0* (encoding a voltage-gated chloride channel from *Torpedo marmorata*).

Genomic DNA was isolated from *H. vulgare* (barley, BA), *B. vulgaris* (sugar beet, SB), *Ph. vulgaris* (bean, BN), *A. thaliana* (AB), *Schizosaccharomyces pombe* (SP) and *Saccharomyces cerevisiae* (SC). Ten µg of genomic DNA from each plant, (1 µg of *A. thaliana*) and 200 ng from each yeast were individually digested with the following restriction enzymes: *EcoRI* (E) in lanes 1-6, *BamHI* (B) in lanes 7-12 and *HindIII* (H) in lanes 13-18. The digested DNA was separated in a 1% (w/v) agarose gel, hybridized with *clc-0* and washed at low stringency (1 X SSC at 50°C) then exposed to X- ray film for 3 days at -80°C. The positions of the DNA marker (λ *HidIII*) are indicated.

Figure 3.5 shows the results from a genomic southern blot of *H. vulgare* (barley), *B. vulgaris* (sugar beet), *A. thaliana*, *Phaseolus vulgaris* (bean), *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* DNA, each cut with the restriction enzymes *EcoR1* (E), *BamH1* (B) and *HindIII*, and hybridized with *clc-0* from *Torpedo marmorata*, a sequence coding for a voltage-gated chloride channel (Jentsch *et al.*, 1990). The blot was washed at low stringency (1 X SSC at 50°C) and exposed to X-ray film for 3 days. The blot shows that the genome of *B. vulgaris* (sugar beet) has sequences that are homologous to *clc-0*. However, hybridization signals were also found in the genomic DNA *EcoR1* fragments (lane 2 at 2, 2.3 kb and larger), *BamH1* fragments (lane 8 at 1.8, 2.7, 3.4 and 4.3 kb) and *HindIII* genomic DNA fragments (lane 13). In addition, several hybridization bands were also observed in all restriction digests of *H. vulgare* (barley) genomic DNA (lane 1 at 1 and 1.5 kb), (lane 7 at 0.5, 1.6 and 2.9 kb) and (lane 13 at 1.5 and 2 kb). The blot also shows that there is evidence that homologues to *clc-0* are found in *Phaseolus vulgaris* and *Arabidopsis thaliana* genomes (lanes 3, 9 and 15) (lanes 4, 10 and 16) respectively. The genomes of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* appear to have sequence homology to *clc-0* (lanes 5, 11 and 17) and (lanes 6, 12 and 18) respectively.

3.2. Northern Analysis with Heterologous Probes.

Southern blot analysis using *sod2*, *adk-1*, *at57*, *ant* (*nhaA*) and *clc-0* revealed the presence of a degree of homology to barley and sugar beet genomic DNA. However, the question now arises, "Are these homologues expressed in the *B. vulgaris* and *H. vulgare?*". Northern blot analysis was performed to study the expression of these heterologous genes (*sod2*, *adk-1*, *at57*, *clc-0*, and *nhaA* on *H. vulgare* (barley) *B. vulgaris* (sugar beet) total RNA.

Ten µg of total RNA was prepared from the roots and leaves of barley grown under 150 mM NaCl, sugar beet grown under 300 mM NaCl, and from controls of both plants (0 mM NaCl, Section 2.2.2.2). RNA was electrophoresed on a 1.3% denaturing agarose gel (Section 2.2.8.3), and blotted onto nylon membrane as described in Materials and Methods Section 2.2.10.2. These northern blots were then hybridized separately with ^{32}P-labelled heterologous probes (*sod2*, *adk-1*, *at57*, *nhaA* and *clc-0*) (Section 2.2.12.2).

All the blots were washed at low stringency (1 X SSC at 55°C) and then exposed to X-ray film.

Unfortunately, northern blot analysis of total RNA isolated from *B. vulgaris* (sugar beet) and *H. vulgare* (barley) and hybridized with *sod2*, *adk-1*, *at57* and *nhaA* revealed that none of the heterologous sequences identified in genomic Southern blots are expressed. However, northern blot analysis using *clc-0* as probe (*T. marmorata* voltage-gated chloride channel) suggested that homologues are expressed in the leaf tissues of *B. vulgaris* (grown in 300 mM NaCl), *H. vulgare* (grown in 150 mM NaCl) and *A. hastata* (grown in 300 mM NaCl). The membrane was washed under moderate stringency (0.5 X SSC at 65°C) and exposed to X-ray film overnight. From Figure 3.6a it is clear that sequences with homology to *clc-0* are weakly expressed in sugar beet (lane 1), moderately expressed in barley (lane 2) and strongly expressed in the halophyte *Atriplex* (lane 3). Consideration of the fluorescent denaturing agarose gel (Fig. 3.6b, lanes 1, 2 and 3) from which this blot (Fig. 3.6a) was prepared, suggested that the signal strength can not be accounted for solely by differences in the amount of total RNA loaded. In each species a band migrating at approximately 1.4 kb was identified. Surprisingly, no *clc-0* hybridization was found to poly $(A)^+$ mRNA prepared from these species (see Fig. 3.6a and 3.6b, lanes 4, 5 and 6). The subsequent challenge of the stripped membrane with a tubuline probe confirmed that poly $(A)^+$ mRNA was present on this membrane, and therefore, it is concluded that the sequences hybridizing to *clc-0* (lanes 1-3) are not extensively poly-adenylated and therefore were not recovered during the preparation of poly $(A)^+$ message by oligo-dT column chromatography. It is conceivable that these putative voltage-gated chloride channel sequences are not encoded in the nuclear genome and therefore, not poly-adenylated.

To assess the cellular location of the message that hybridizes to *clc-0*, a northern hybridization of *clc-0* to total RNA isolated from the leaves and root tissue of *H. vulgare* (grown under 150 mM NaCl) was undertaken. Total RNA was isolated from different organelles of the plant tissues (mitochondria and plastid) and used for northern analysis. The samples were electrophoresed on a 1.3% denaturing agarose gel, transferred to nylon membrane and probed with ^{32}P labelled *clc-0*. The membrane was washed at moderate stringency (0.5 X SSC at 65°C). The northern blot analysis using *clc-0* (Fig. 3.7a) reveals that it was expressed in samples prepared from root tissue mitochondria (lane 2),

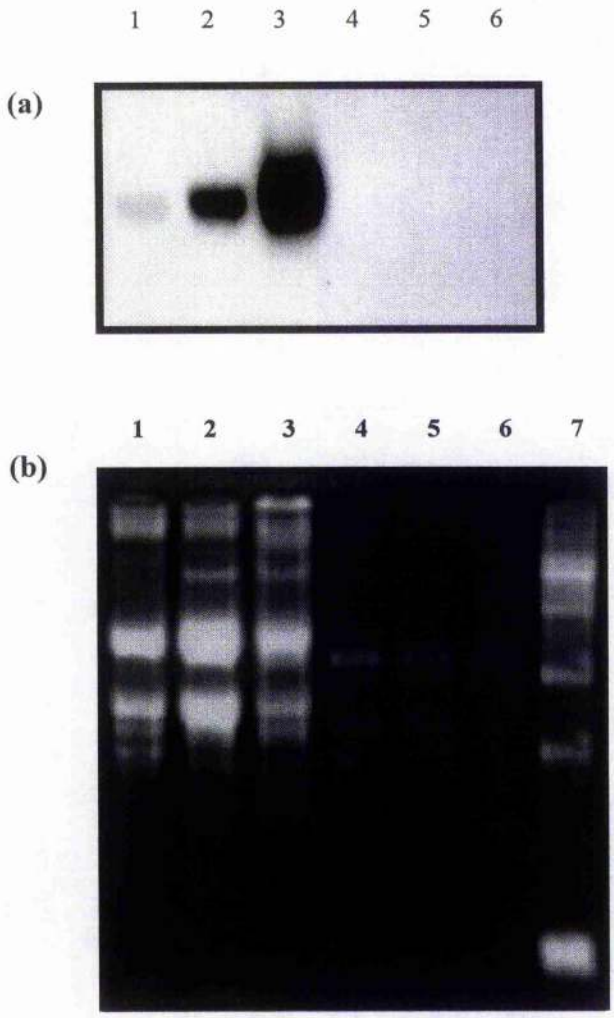


Figure. 3.6. Northern Blot Analysis of Total and Poly (A) mRNA Hybridized with *clc-0* (encoding a voltage-gated chloride channel from *Torpedo marmorata*).

(a) Total RNA (lanes 1-3) and poly $(A)^+$ mRNA (lanes 4-6) were isolated from leaf tissues of *B. vulgaris* (grown in 300 mM NaCl, lanes 1 & 4), *H. vulgare* (grown in 150 mM NaCl, lanes 2 & 5) and *Atriplex hastata* (grown in 300 mM NaCl, lanes 3 & 6). Ten µg of total RNA and 1 µg of poly $(A)^+$ mRNA were separated on a 1.3% (w/v) denaturing agarose gel and blotted onto nylon membrane. The Northern blot was probed with ^{32}P-labelled *clc-0*, washed at moderate stringency (0.5 X SSC at 65°C) and exposed to X-ray film at -80°C overnight. **(b)** Denaturing gel of total and poly $(A)^+$ mRNA that was blotted onto nylon membrane for Northern analysis **(a)**.

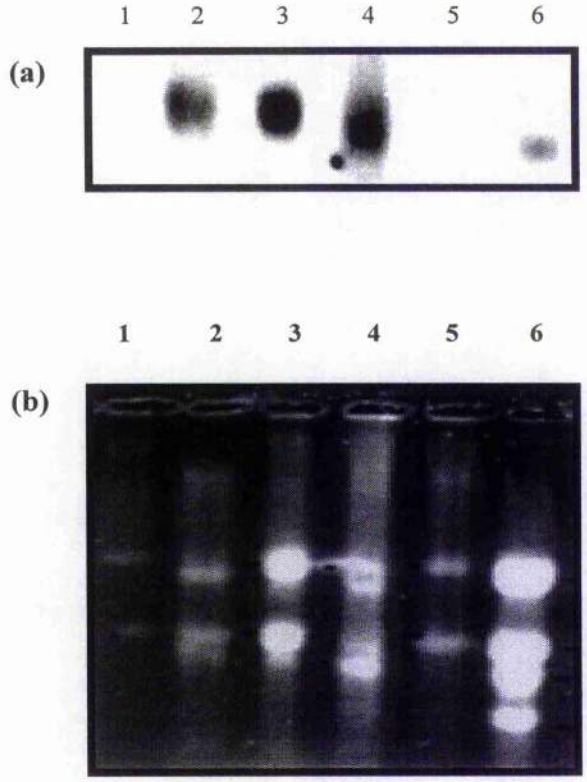


Figure. 3.7. Northern Blot Analysis of *Hordeum vulgare* Total RNA Isolated from Different Organelles and Tissues and Hybridized with *clc-0*.

(a) Northern blot analysis of total RNA isolated from leaf and root tissues of *H. vulgare* grown on 150 mM NaCl. Ten µg of each sample was electrophoresed in a 1.3% (w/v) denaturing agarose gel (figure 3.7b), blotted onto nylon membrane and hybridized with *clc-0* (encoding *Torpedo marmorata* voltage-gated chloride channel) probe. The blot was washed at moderate stringency (0.5 X SSC at 65°C). Lane 1 contains total RNA isolated from root plastids: lane 2, contains total RNA isolated from root mitochondria: lane 3, contains total RNA isolated from root cells: lane 4 contains total RNA isolated from leaf plastids: lane 5 contains total RNA isolated from leaf mitochondria: lane 6. contains RNA isolated from leaf cells. **(b)** Denaturing gel total RNA that was blotted onto nylone membrane.

and root cells (lane 3); no expression was found in root plastid total RNA (lane 1). Expression of *clc-0* was also found in total RNA prepared from leaf plastids (lane 4) and leaf cells (lane 6), however, no expression band was observed in total RNA extracted from leaf mitochondria (lane 5). The expression message of *clc-0* has a size of approximately 1.5 kb. These results suggested that *clc-0* expression was found in the roots and leaves of *H. vulgare*. However, the expression in roots was localized in the mitochondria (lane 2) and in total RNA from the whole cell (lane 3); in contrast, the expression in leaves was found to be localized in the plastid (lane 4). The blot showed different levels of expression between root and leaf organelles and that may be due to the unequal loading quantity of total RNA in the formaldehyde gel shown in Fig. 3.8b.

3.3. Isolation and Characterization of the Genomic Clone.

3.3.1. Introduction.

Northern blot analysis of *clc-0* against barley leaf total RNA (Fig. 3.6a) resulted in a very strong signal. However, this message appears to be poorly poly-adenylated. Consequently, it is unlikely that conventional screening of a barley cDNA library with *clc-0* will identify a homologue. For this reason, it was decided to try to identify a *clc-0* homologue from a barley genomic library. Thus if the full-length genomic clone could be isolated, (it is likely that the entire gene coding for a voltage-gated chloride channel from barley genomic library) and hence the full-length putative protein, could be identified and analysed. In addition there is another good reason for using a genomic library screen. It is well established that in plants, membrane transporters, and in particular ion channels are not highly expressed, thereby making cloning difficult. It can be estimated that the amount of message in 10 µg total RNA is about 2.5 % (i.e ~ 300 ng). It has also been estimated that there are typically 250,000 message molecules expressed at any one time in plant tissues. For a lowly expressed gene (say 0.001 % of total message (see Milner, *et al.*, 1995; Golderg, *et al.*, 1978) 300 ng x 1 x 10^{-6} or 0.3 pg of target will be available for hybridization, with heterologous probes. This is well below the limit of detection. However, for a 1.5 kb gene present as a single copy in a 3 x10^9 bp genome, a 10 µg genomic sample will contain ~ 5 pg of target.

3.3.2. Screening of the Barley Genomic Library.

A barley genomic library was constructed in the λ Dash II vector (Promega) and was screened for genomic clones. The library was constructed by ligating genomic DNA from barley (*Hordeum vulgare* Var Golden Promise) into dephosphorylated Lambda Dash II vector arms.The high molecular weight barley genomic DNA was partially digested with the restriction enzyme *Sau 3A* to produce smaller overlapping fragments. The ends on the DNA fragments were compatible with the ends of the vector arms after the stuffer fragment had been removed by complete digestion with the restriction enzyme *BamH1*. Consequently, the DNA was ligated into the vector arms, packaged and then the titer of the library was determined.

The barley genomic library was screened using *clc-0* as a probe. A total of approximately 3 x 10^5 clones were screened (see Section 2.2.13) using a conventional colony hybridization technique. This was less than the necessary 10^6 recombinants required to represent 99 % of all DNA sequences in the genome (Clarke and Carbon, 1976). This is the number of clones usually screened in order to maximise the chances of isolating the sequence of interest (Sambrook, *et. al.*, 1989).

$$N = \frac{\ln(1-p)}{\ln(1-\frac{x}{y})}$$

where N is the necessary number of recombinants.

p is the desired probability (*i.e.* $p = 0.99$)

x is the size of the insert (20 kb)

y is the haploid genome size (3.5 x 10^9 bp)

Therefore, for barley the required number of clones to screen for 99 % chance of identifying a single clone is:

$$N = \frac{\ln(1-0.99)}{\ln(1-\frac{20x10^3}{3.5x10^9})} = \sim 0.8 \times 10^6$$

In total 3 x 10^5 plaques were screened from an intended of 1 x 10^6 of barley genomic library but no convincing clones were identified.

3.4. Discussion.

This study was undertaken to determine whether genes coding for H^+, Na^+ and Cl^- transporters could be identified by using heterologous probes to barley, sugar beet and *A. hastata* that may confer salt-resistance. Homologues to two H^+ pumps were used, *adk-1* (a *Nicotiana pumbaginifolia* p-type H^+-ATPase) and *at57* (an *Arabidopsis* v-type H^+-ATPase B subunit). In addition, two sequences coding for Na^+/H^+ antiporters were used, *nhaA* (*E. coli*) and *sod2* (*S. pombe*), and also *clc-0*, a sequence coding for a voltage-gated Cl^- channel (from *Torpedo marmorata*). The initial strategy was to determine which if any of the above probes hybridised to sequences in salt-resistant plants, and then to go on to clone the corresponding plant genes and study their expression.

3.4.1. P-Type H^+-ATPase.

Genomic Southern blots (Fig. 3.1) showed that *adk-1* (H^+ATPase) has some homology to sequences in all plants studied but especially in *B. vulgaris* and *H. vulgare*. Moreover, *adk-1* appears to have some homology to sequences in *S. cerevisiae*. These results are not surprising as it is well established that in all plants studied, p-type ATPases have been implicated as the primary pump driving coupled (endergonic) solute transport across the plasma membrane (Harper & Sussman, 1988; Serrano, 1989; Goffeau and Slayman, 1981). However, surprisingly, no *S. pombe* sequences appear to have strong homology to *adk-1* (Fig. 3.1); the reasons for this are not clear.

3.4.2. V-Type H^+-ATPase.

Southern blot analysis using *at57* as probe, show hybridization to barley, sugar beet and *Arabidopsis thaliana* genomic DNA (Fig. 3.2). These results suggest each of these plants possess a v-type ATPase. Again, this is not a surprising result as it is well established that v-type H^+-ATPases play an important role in establishing a driving force for coupled ion and metabolite transport across the tonoplast membrane in all plants studied to date (Sze, *et al.*, 1992). In addition, hybridization of *at57* to *S. cerevisiae* sequences were observed (Fig. 3.2) which probably refelect the homologue coding for the yeast C subunit 160 kDa which has been sequenced (Nelson & Nelson, 1990).

3.4.3. Na^+ / H^+ Antiporters.

Genomic Southern blot experiments using a fragment of the *S. pombe sod2* gene (Fig. 3) suggested that barley, sugar beet and *Atriplex* may have homologues to this class of Na^+/H^+ antiporter. Northern blot analysis was then carried out on root and leaf tissues to determine whether or not the homologous genomic sequences identified by *sod2* Southern blotting were expressed in *B. vulgaris*, *H. vulgare* and *A. hastata*. Unfortunately, no transcripts appeared to hybridize to the *sod2* probe at even moderately low stringency (1 X SSC, at 55°C, data not presented). The inclusion of control *sod2* DNA sequences blotted onto nylon membrane and included in the hybridization and washing procedures suggested that the limit of detection by the ^{32}P probe was ~ 2 pg of target. It is concluded that unless *sod2* homologue transcripts are present at levels below 2 pg / 10 µg total RNA (an estimated abundance of < 0.001 % of transcripts was present), these sequences are not expressed in barley, sugar beet or *Atriplex*.

There is little evidence from genomic Southern blotting experiments that either barley, sugar beet or *Atriplex* contain sequences homologous to the *E. coli nhaA* class of Na^+/H^+ antiporter (Fig. 3.4).

3.4.4. Voltage-gated Cl^- Channels.

Southern blot analysis suggested that homologues of *clc-0* are present in the salt-resistant plants sugar beet and barley (Fig. 3.5). In addition, homologues may also be present in *A. thaliana, Ph. vulgaris, S. cerevisiae* and *S. pombe*. Since these experiments were completed, four putative members of the *clc* family of genes have been identified in the *A. thaliana* genome (Hechenberger, *et al.* 1996), and two in the yeast genome database (Huang, *et al*, 1994; Greene, *et al.*, 1993) although it remains to be established whether these sequences do indeed represent authentic voltage-gated Cl^- channels.

Northern blot experiments using *clc-0* as probe, suggested that the homologues in *B. vulgaris*, *H. vulgare* and *A. hastata* are expressed (at approximately 5, 50 and 250 pg / 10 µg total RNA respectively) and were about 1.4 kb in size. Consideration of the corresponding fluorescence gel from which this northern blot was prepared (Fig. 3.6 b) shows that there was unequal loading of total RNA (lane 1-3), so some adjustments of the relative abundancies should be made.

Surprisingly, no hybridization was found when *clc-0* was used to challenge poly $(A)^+$ mRNA isolated from *B. vulgaris* (lane 4), *H. vulgare* (lane 5) or *A. hastata* suggesting that the *clc-0* message in these plants is not highly poly-adenylated and was lost during the oligo-dT preparation procedure. An alternative explanation for the results presented in Fig. 3.6a is that no homologues of *clc-0* are expressed in these plants and that the probe is binding to the 18S ribosomal band in the total RNA preparations (Fig. 3.6, lanes 1-3). However, this is unlikely for the following reasons. Firstly, consideration of the fluorescence gel (Fig. 3.6b) suggests that lane 3 contained the least 18S RNA whereas the corresponding northern blot shows this lane to have the highest signal; given the highly conserved nature of 18S RNA, this result is difficult to reconcile if *clc-0* did indeed bind to the 18S ribosomal fraction. Secondly, 18S ribosomal RNA has a molecular size of 1.9 kb; careful analysis of the hybridizing band indicated a size of 1.4 kb. Thirdly, when it occurs, non-specific ribosomal hybridization tends to give rather diffuse bands; the bands presented in lanes 1-3 of Fig. 3.6b are 'tight' and characteristic of hybridization to authentic message. Finally, BLAST searches of the *clc-0* probe sequence failed to pick up any ribosomal sequences. As this Northern blot was washed at moderate stringency (0.5 X SSC, at 65°C) it is concluded that the hybridization signals presented in Fig. 3.6a

represent binding between the *clc-0* probe and authentic homologous message in these three salt-resistant plants.

Attempts to isolate a voltage-gated chloride channel from barley genomic λ DASH II library by colony hybridization using *clc-0* as a probe was unsuccessful. This may in part be due to the low copy number of the corresponding gene. However, as only 3×10^5 out of the intended 10^6 clones were screened (for 99 % certainty), it is calculated that there was approximately only an 80 % chance of succeeding with this size of screen. (Clark and Carbon, 1976).

At this stage (April 1996), Maurel and co-workers reported the molecular characterization of the first plant voltage-gated Cl^- channel (Lurin, *et al.*, 1996). This sequence, *clc-nt1* was cloned by RT-PCR from *N. tabaccum* and at that time it seemed that similar approach would provide a rapid and relatively safe way of isolating *clc* homologues from salt-resistant plants. For these reasons, that concerning the ambiguity of the *clc-0* target sequences in barley, and of the reported success with an RT-PCR approach in *N. tabaccum*, it was decided that a more fruitful way forward would be to design degenerate PCR primers and follow the procedures of Lurin, *et al.*, 1996 The details of these RT-PCR experiments are presented in Chapter 4.

CHAPTER FOUR
PCR CLONING

4.1 A PCR Cloning Approach.

Maurel and co-workers success in cloning the first plant chloride channel by RT-PCR (Lurin, *et al.*, 1996) was encouraging and it was decided to adopt a similar approach in this study.

In May 1995 a search of the databases identified only 3 *clc* gene sequences, but these provided insufficient information for designing degenerate PCR primers for cloning plant homologues. However, in December 1995, a similar search by Maurel and co-workers identified 7 putative *clc* gene sequences (C. Maurel, per. comm.), and from this information they went on to clone *clc-nt1*, the first putative voltage-gated chloride channel from tobacco (Lurin, *et al.*, 1996). In May 1996, 17 putative *clc* sequences were deposited on the data bases and the corresponding protein translations of these sequences were subsequently aligned using the PILEUP routine from GCG 7. Three regions of consensus were identified (Fig. 1.2 Chapter 1). Degenerate primers were designed to these conserved motifs using a combination of intuition and the PRIMER routine in GCG 7. Two forward (F1 and F2) and one reverse (R1) primers were designed (see Fig. 4.1) and subsequently used in extensive PCR experiments.

4.1.1. PCR Cloning Using cDNA Libraries as Template.

High salt-grown cDNA libraries had previously been constructed in the laboratory from roots of sugar beet (300 mM NaCl) and barley (150 mM NaCl) plants grown hydroponically. It was decided first to use these cDNA libraries as template.

Forward Primer 1 (F1)

5' 3'
GGWAARGARGGNCCNATGGTNA

Forward Primer 2 (F2)

5' 3'
ATHGARWSNYTNAYTAYGA

Reverse Primer 1 (R1)

5' 3'
AARAGMACWCCNCCNANNGG

Figure. 4.1. Nucleotide Sequences of Degenerate Primers Designed from Voltage-Gated Cl^- Channel Consensus Sequences.

4.1.1.1 *Beta vulgaris.*

Figure 4.2B presents a photograph of the fluorescent gel of the optimized amplified PCR products from the sugar beet cDNA library using the *clc* degenerate primers F1 and R1.

After optimization of conditions, two PCR fragments close to the expected size range (160-300 bp) were identified and named *bv1* (~350bp) and *bv2* (~300bp); see Fig 4.2 B (lane 1 and 2). These two fragments were separated by 0.5 % agarose gel (Section 2.2.7.1), then each fragment cut from the gel separately and purified (Section 2.2.9). The two fragments were subsequently cloned into PCR-Script™ cloning vector (Section 2.2.15.5) and transformed into *E. coli* XL-1 Blue supercompetent cells (Section 2.2.3.2.1).

4.1.1.1.1 Analysis of *bv1*.

Genomic Southern blots were then performed on sugar beet genomic DNA using *bv1* as probe to confirm that it represents an authentic sugar beet sequence. Figure 4.3 shows the results of this genomic Southern analysis; controls of *Hordeum vulgare*, *E. coli* and *S. cerevisiae* genomic DNA were also included. Appropriate amounts of genomic DNA from each organism was prepared and digested with *EcoR1*, *BamH1* and *HindIII* (Section 2.2.8) and electrophoresed in a 1 % (w/v) agarose gel (see Fig. 4.3). The resolved DNA fragments were then blotted onto nylon membrane and hybridized with *bv1* as probe, as described in Materials & Methods (Section 2.2.12.1). The membrane was washed initially at low stringency (1 X SSC at 50°C) and autoradiographed for 3 days at -80°C.

Very strong hybridization was observed in *E. coli* genomic DNA (lanes 2 and 10); no hybridizing bands were found in *E. coli* genomic DNA digested with *BamH1* (lane 6) perhaps due to poor recovery of the digested fragments by ethanol precipitation. The genome of *S. cerevisiae* also appears to have sequences homologous to *bv1* (lanes 1 and 5). However, the blot provides no evidence for higher plant homologues of *bv1* (lanes 3, 4, 7, 8, 11 & 12).

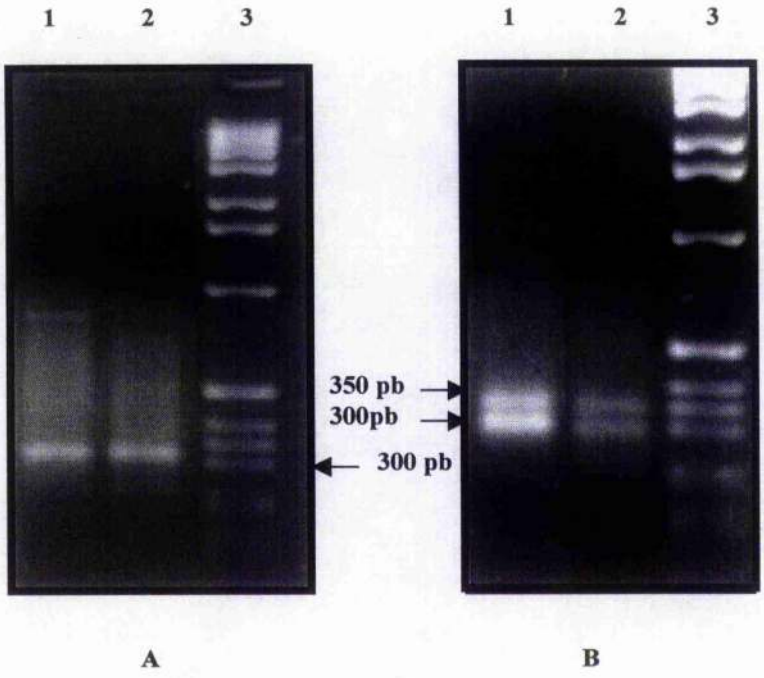


Figure 4.2. Separation of PCR Amplification Products from cDNA Libraries of *H. vulgare* and *B. vulgaris* using Degenerated Primers.

Two degenerate primers F1 and R1 were used with *Taq DNA* polymerase in PCR reactions (35 thermal cycles, denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min) to amplify fragments from *H. vulgare* (barley) cDNA library (**A**, lanes 1 and 2) and *B. vulgaris* (sugar beet) cDNA library (**B**, lanes 1 and 2). The PCR products were separated in a 2 % (w/v) agarose gel, stained with ethidium bromide and visualised under UV light. The PCR reactions resulted in one fragment (*hv1*) of 300 bp from the *H. vulgare* cDNA library (**A,** lanes 1 and 2,) and two fragments (*bv1*) and (*bv2*) of 350 and 300 bp respectively from *B. vulgaris* cDNA library (**B**, lanes 1 and 2). Lane 3 in each gel represents 1 kb ladder markers.

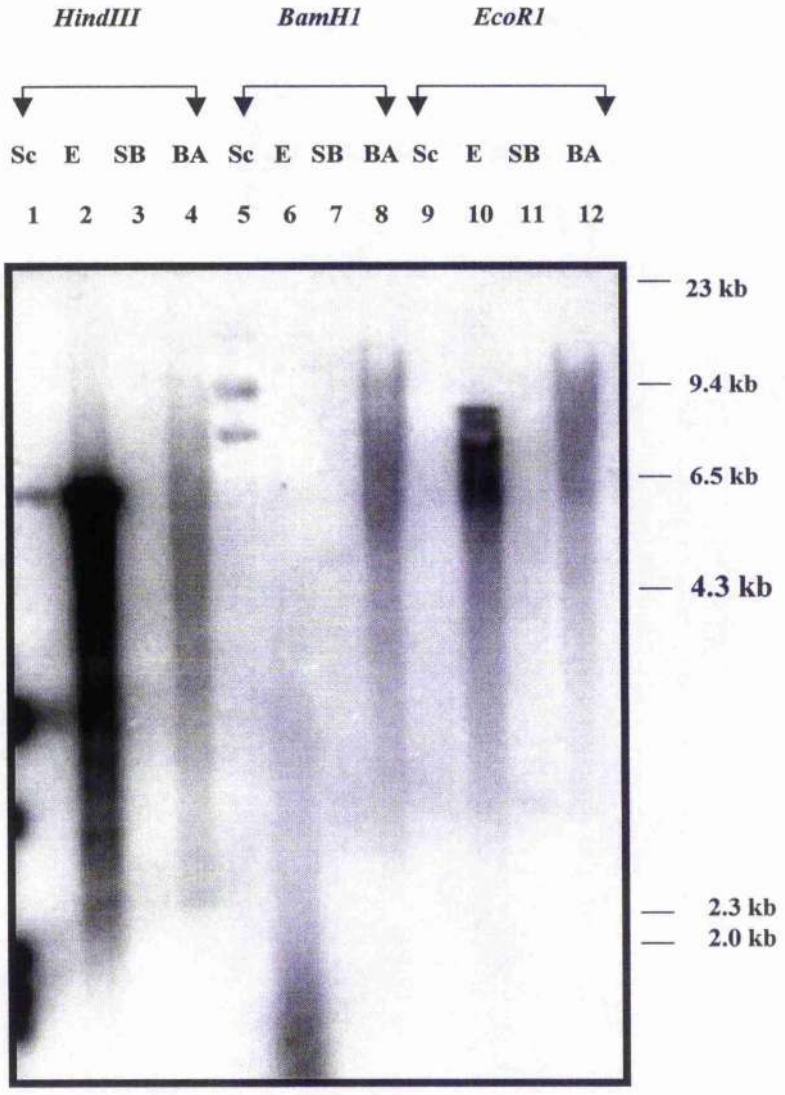


Figure 4.3. Genomic Southern Analysis of Plant Genomic DNA Hybridized to the Sugar Beet PCR Amplified Fragment *bv1*.

Genomic DNA was isolated from *B. vulgaris* (SB), *H. vulgare* (BA), *Saccharomyces cerevisiae* (Sc.) and *E. coli* (E). Ten µg of each plant genomic DNA, 100 ng of *Saccharomyces cerevisiae* and 50 ng of *E. coli* genomic DNA were each digested individually with restriction enzymes *HindIII*, *Bam* and *EcoR1*. The digested samples were electrophoresed in a 1 % (w/v) agarose gel and blotted onto nylon membrane. The blot was hybridized with labelled *bv1* probe, washed at low stringency (1 X SSC at 50°C) and autoradiographed for 3 days at -80°C.

Northern blot analysis was carried out to determine whether *bv1* sequences are expressed in sugar beet plants. Total RNA prepared from roots and leaves of sugar beet and barley plants grown hydroponically in 300 and 150 mM NaCl respectively, and on control plants grown without NaCl additions (Section 2.2.2.2). Total RNA was prepared from each treatment, and electrophoresed on a 1.3 % (w/v) agarose/formaldehyde gel (Section 2.2.8.3), and blotted onto nylon membrane (Section 2.2.10.2). The blot was then hybridized with *bv1* as probe (2.2.12.2), washed at low stringency (1 X SSC at 55°C, see Section 2.2.12.3), and autoradiographed at - 80°C for 4 days. No hybridization of *bv1* to any sample was observed (data not presented).

The cloned *bv1* fragment was also sequenced (Section 2.2.16) and the resulting translated protein information used to search the TREMBL and protein data bases using the BLAST routine from GCG 7 (Fig. 4.4 Section 2.2.17). Neither of the expected protein motifs (GKEGPxxH or GVLFxxE) were found at the ends of the fragment although some homology to the reverse primer could be observed. The BLAST search identified *E. coli* sequence that was 65 % identical to *bv1* and which codes for fructose-phosphatase (Fig. 4.5).

4.1.1.1.2 Analysis of *bv2*.

Figure 4.6 shows the results of a genomic Southern analysis of *Hordeum vulgare, Beta vulgaris, E. coli* and *S. cerevisiae* genomic DNA using *bv2* as probe. Appropriate amounts of genomic DNA from barley, sugar beet, *S. cerevisiae* and *E. coli* were digested with *EcoR1*, *BamH1* and *HindIII* (Section 2.2.8) and electrophoresed in a 1 % (w/v) agarose gel (Section 2.2.7.2). The resolved fragments were then blotted onto nylon membrane (2.2.10.1) and hybridized with *bv2* as probe, as described in Materials & Methods (Section 2.2.12.1). The membrane was washed initially at low stringency (1 X SSC at 50°C) and autoradiographed for 3 days at -80°C.

The genome of *S. cerevisiae* (lanes 1 and 5) and *E. coli* (lane 2) appears to have sequence homology to *bv2*. Sugar beet was found to have sequences with some weak homology to *bv2* (labelled in Fig. 4.6, lane 3 ~ 1.5 kb: lane 7 ~ 6.5 and 3.9 kb: lane 11 ~ 2 kb). Barley may also have some weak sequence homology to *bv2* but due to the high background signal, this result is tenuous (lanes 4, 8 and 12). Washing the filter at

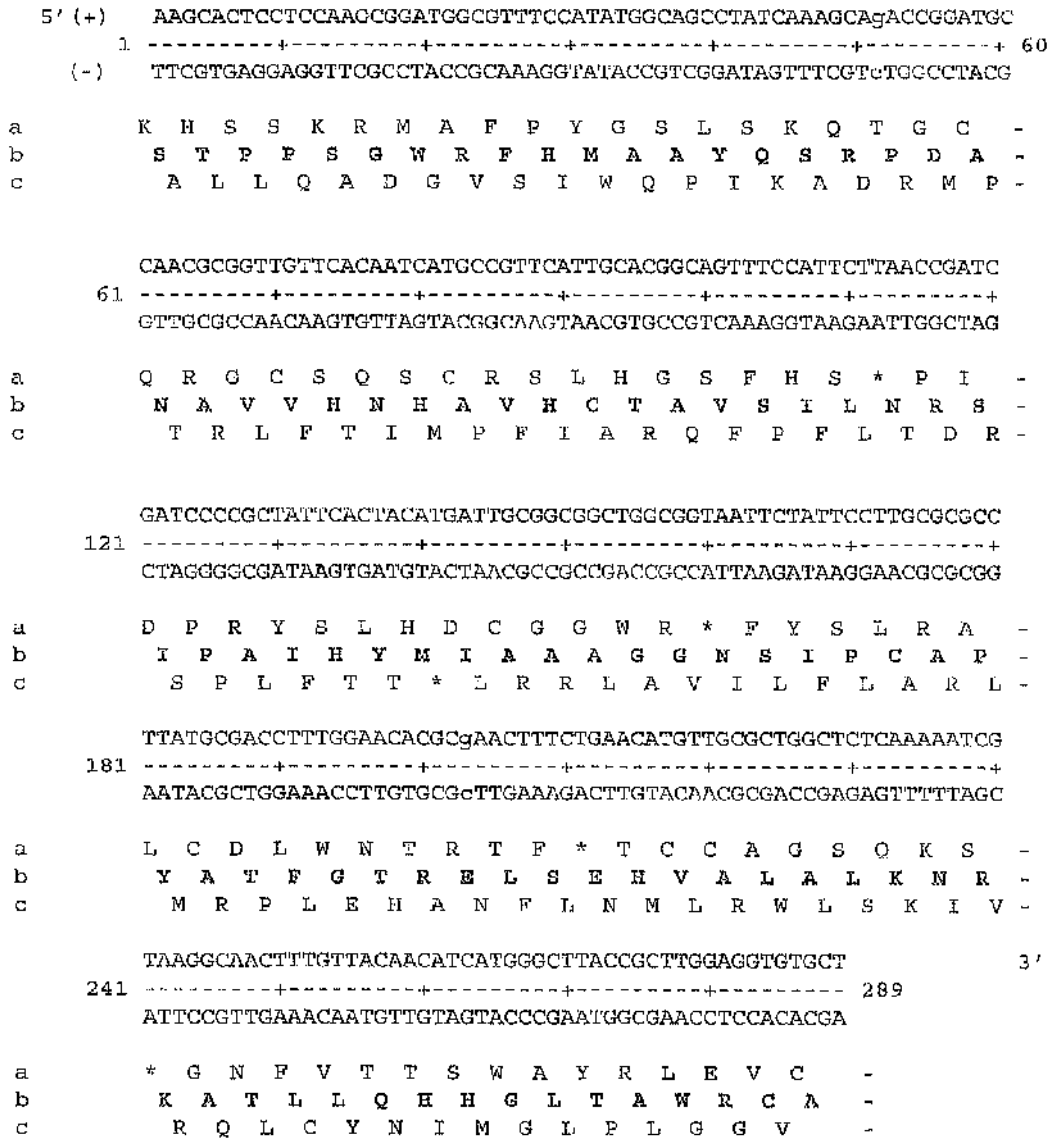


Figure.4.4. Sequence Analysis of *bv1*.

Automated sequencing was performing as described in Materials and Methods (Section 2.2.16). The sequence was translated in all six reading frames using the map program in GCG 7; only the 3 forward frames are shown. The probable reading frame (b) is shown in bold (96 amino acids); this was the only reading frame with no stop or nonsense codons. However, none of the six reading frames contains the expected PGKEGxxxxH (forward) or GVLFxxE (reverse) protein sequence (See Figs. 1.2 and 4.1).

```
Reference:  Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers,
and David J. Lipman (1990).  Basic local alignment search tool.  J. Mol. Biol.
215:403-10.

Query=  /people/pdominy/Genes/Chloride/sb_frags/sb1_t7.pep
        (574 letters)

Database:  trembl
           293,433 sequences; 82,003,986 total letters.

                                                                    Smallest
                                                                      Sum
                                                             High  Probability
Sequences producing High-scoring Segment Pairs:              Score  P(N)       N
..
TR:G1573604 ! U32743_7 FUCULOSE-1-PHOSPHATE ALDOLASE (FUCA).   307  3.8e-38    1
TR:E293135 ! Z84395_46 HYPOTHETICAL 13.1 KD PROTEIN (FRAG...   114  3.9e-08    1
TR:E293135 ! Z84395_46 HYPOTHETICAL 13.1 KD PROTEIN (FRAG...   114  3.9e-08    1
TR:E293135 ! Z84395_46 HYPOTHETICAL 13.1 KD PROTEIN (FRAG...   114  3.9e-08    1


>TR:G1573604 U32743_7 FUCULOSE-1 PHOSPHATE ALDOLASE (FUCA).
  Length = 215

 Score = 307 (152.6 bits), Expect = 3.8e-38, P = 3.8e-38
 Identities = 51/88 (57%), Positives = 68/88 (77%)

Query:    99 PPSGWRFHMAAYQSRPDANAVVHNHAVHCTAVSILNRSIPAIHYMIAAAGGNSIPCAPYA 158
             P S W+FH++ Y +RP+ANAVVHNH++HC  +SIL + IPAIHYM+A +G + IPC PYA
Sbjct:    70 PSSEWQPHLSVYHTRPEANAVVHNHSTHCAGLSILEKPIPAIHYMVAVSGTDHIPCVPYA 129

Query:   159 TFGTRELSEHVALALKNRKATLLQHHGL 186
             TFG+ +L+ +VA  +K  KA LL HHGL
Sbjct:   130 TFGSHKLASYVATGIKESKAILLAHHGL 157
```

Figure 4.5 Blast Output of a *bv1* Search in the TREMBL Data Base.

The frame b translation of the sugar beet sequence *bv1* (see Fig. 4.4) was used to interrogate the TREMBL data base for homology using BLAST from GCG 7. Only one sequence, U32743 from . *E.coli*, showed any strong homology.

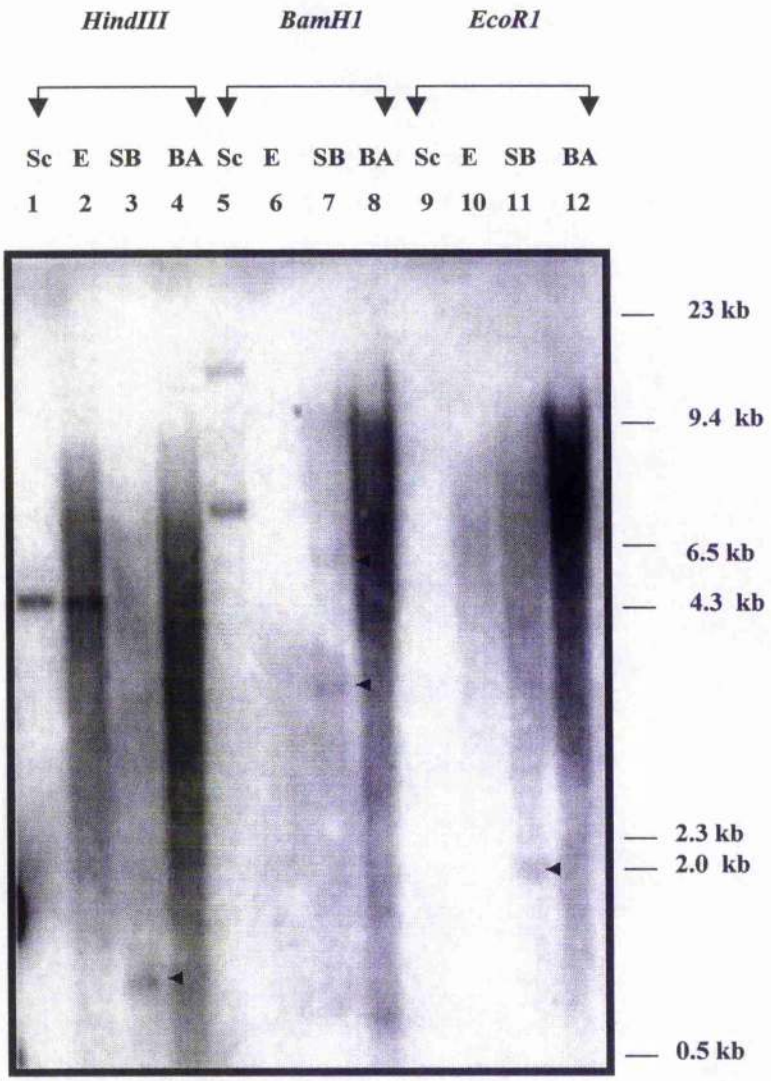


Figure 4.6. Genomic Southern Analysis of Plant Genomic DNA Using *bv2* as Probe.

Genomic DNA was isolated from *B. vulgaris* (SB), *H. vulgare* (BA), *Saccharomyces cerevisiae* (Sc.) and *E. coli* (E). Ten µg of plant genomic DNA, 100 ng of *Saccharomyces cerevisiae* and 50 ng of *E. Coli* genomic DNA were each digested individually with the restriction enzymes *HindIII*, *BamH1* and *EcoR1*. The digested samples were electrophoresed in a 1 % (w/v) agarose gel and blotted onto nylon membrane. The blot was hybridized with labelled *bv2* probe, washed at low stringency (1 X SSC at 50°C) and autoradiographed for 3 days at -80°C.

high stringency (0.1 X SSC at 65°C) removed all of the hybridizing bands (data not presented).

Northern blot analysis was carried out to determine whether *bv2* sequences are expressed in plants. Total RNA was prepared from roots and leaves of sugar beet and barley plants grown hydroponically in 300 and 150 mM NaCl respectively, and from control plants grown without NaCl additions (Section 2.2.2.2). Total RNA was prepared from each treatment, and electrophoresed in a 1.3 % (w/v) agarose / formaldehyde gel (Section 2.2.8.3), and blotted onto nylon membrane (Section 2.2.10.2). The blot was then hybridized with *bv2* as probe (2.2.12.2) washed at low stringency (1 X SSC at 55°C, see Section 2.2.12.3) and then autoradiographed at - 80°C for 4 days. No hybridization of *bv2* to any sample was observed (data not presented).

The cloned *bv2* fragment was sequenced (Section 2.2.16), and the probable protein translation (frame b) information used to search the TREMBL and protein database, using the BLAST routine from GCG 7 (Fig. 4.8; See Section 2.2.17). The BLAST search failed to identify any sequences of strong homology. Again, consideration of Fig. 4.7 reveals that neither of the *clc* conserved protein motifs was present at the ends of the *bv2* fragment (GKEGPxxH or GVLFxxE) although some homology to the primers could be observed.

4.1.1.2. *Hordeum vulgare*.

Figure 4.2B presents the fluorescent gel of the PCR products amplified from the *H. vulgare* cDNA library using the degenerate forward (F1) and reverse (R1) primers shown in Fig. 4.1. After optimisation of conditions, one PCR fragment within the expected size range, *hv1* (300 bp), was observed (Fig. 4.2A, lanes 1 and 2). This fragment was cut from the gel, purified (Section 2.2.9), cloned into PCR-Script™ cloning vector (Section 2.2.15.5), and then transformed into *E. coli* XL-1 Blue supercompetent cells (Section 2.2.3.2.1).

Genomic Southern blot was performed on digested genomic DNA using *hv1* as probe to establish that *hv1* represents an authentic barley sequence. Genomic DNA isolated from *Beta vulgaris*, *E. coli* and *S. cerevisiae* were ran as controls. Appropriate amounts

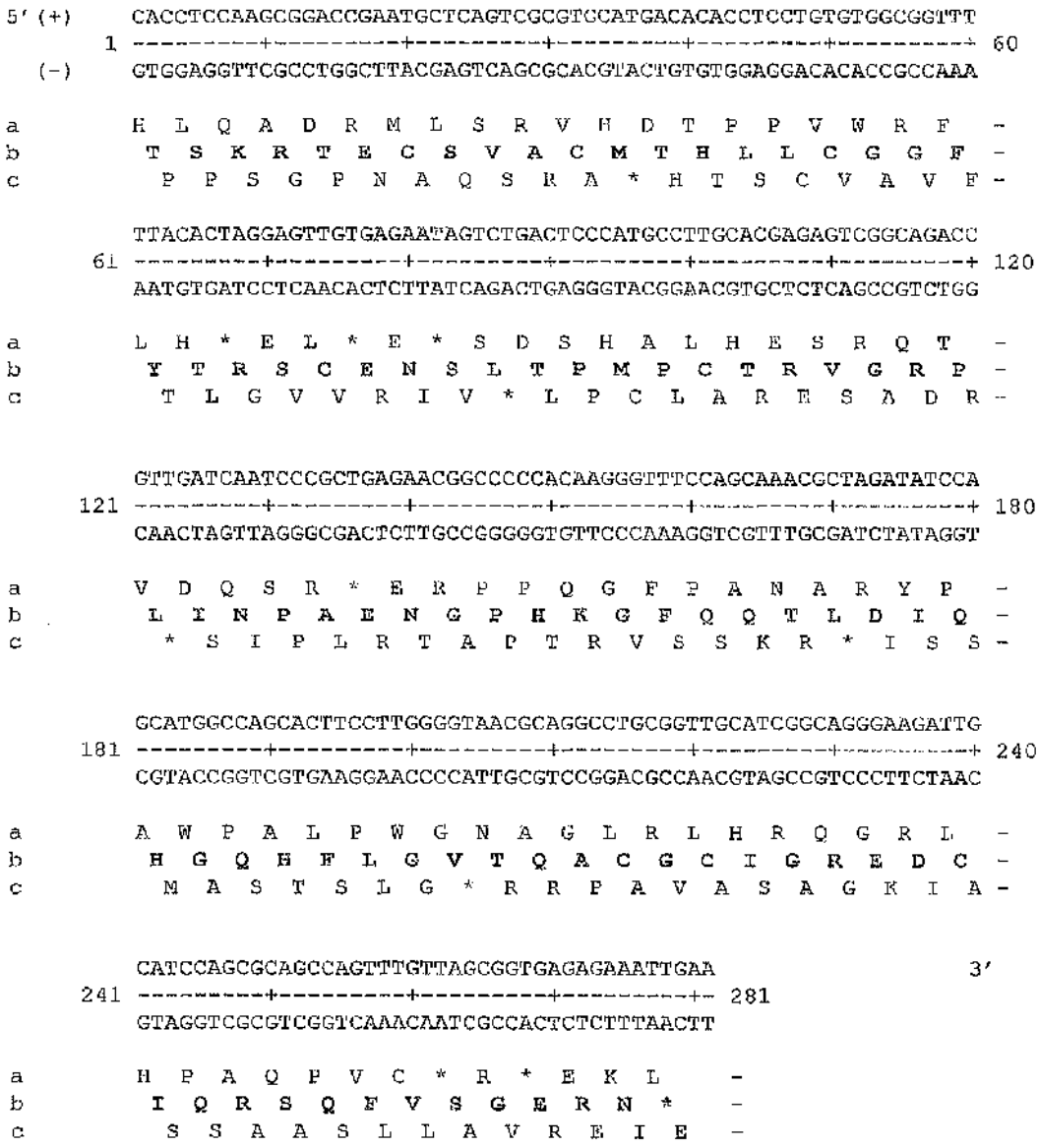


Figure.4.7. Sequence Analysis of *bv2*.

Automated sequencing was performing as described in Materials and Methods (Section 2.2.16). The sequence was translated in all six reading frames using the map program in GCG 7; only the 3 forward frames are shown. The probable reading frame (b) is shown in bold (92 amino acids); this was the only reading frame with no stop or nonsense codons. However, none of the six reading frames contains the expected PGKEGxxxxH (forward) or GVLFxxE (reverse) protein sequence (See Figs. 1.2 and 4.1).

```
Reference:  Gish, Warren (1994-1997).  unpublished.
Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J.
Lipman (1990).  Basic local alignment search tool.  J. Mol. Biol. 215:403-10.

Query=  bv2 or SB2 sequence
, 92 bases, C1A956EA checksum.
        (92 letters)

Database:  swall
           283,052 sequences; 89,381,315 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                      Smallest
                                                                        Sum
                                                              High   Probability
Sequences producing High-scoring Segment Pairs:               Score    P(N)       N

SWALL:O30800   O30800 PMI-LIKE GENE PRODUCT.                    71   0.17       1
SWALL:G1245813 G1245813 CE75-E75A ECDYSTEROID RECEPTOR HO...    51   0.89       1
SWALL:O36403   O36403 ORF53. 1L/98                              58   0.98       1
SWALL:Q51195   Q51195 CLASS 1 OUTER MEMBRANE PROTEIN VARI...    48   0.990      1
SWALL:Q51216   Q51216 CLASS 1 OUTER MEMBRANE PROTEIN VARI...    48   0.990      1


>SWALL:O30800 O30800 PMI-LIKE GENE PRODUCT.
           Length = 469

 Score = 71 (25.0 bits), Expect = 0.19, P = 0.17
 Identities = 23/50 (46%), Positives = 27/50 (54%)

Query:     16 LLCGGFYTRSCENSLTPMPCTRVGRPLINPAENGPHKGFQQTL-DIQGHEF 65
              +LCGG  TR     S T +P    V   PL+      GPH  FQQTL  +QG  F
Sbjct:      9 ILCGGSGTRLWPTSRTSLPKQFV--PLL-----GPHSTFQQTLLRLQGPLF 52


>SWALL:G1245813 G1245813 CE75-E75A ECDYSTEROID RECEPTOR HOMOLOG. 3/98
           Length = 67

 Score = 51 (18.0 bits), Expect = 2.2, P = 0.89
 Identities = 8/20 (40%), Positives = 12/20 (60%)

Query:     18 CGGFYTRSCENSLTPMPCTR 37
              C GF+ RS +   +   PCT+
Sbjct:     21 CKGFFRRSIQQKIQYRPCTK 40


>SWALL:O36403 O36403 ORF53.
           Length = 103

 Score = 58 (20.4 bits), Expect = 3.8, P = 0.98
 Identities = 16/43 (37%), Positives = 21/43 (48%)

Query:     12 CMTHLLCGGFYTRSCENSLTPMPCTRVGRPLINPAEN-GPHKGF 54
              C+  LL     Y  SC-+S TP    T     P++N   N  P  GF
Sbjct:      9 CLWVLLIWYSYTTSCDSSSTPRAVTH---PVLNATSNFNPTAGF 49
```

Figure 4.8 Blast Output of a *bv2* Search in the TREMBL Data Base.

The frame b translation of the sugar beet sequence *bv2* (see Fig. 4.7) was used to interrogate the TREMBL data base for homology using BLAST from GCG 7. No sequences of any significant homology were identified.

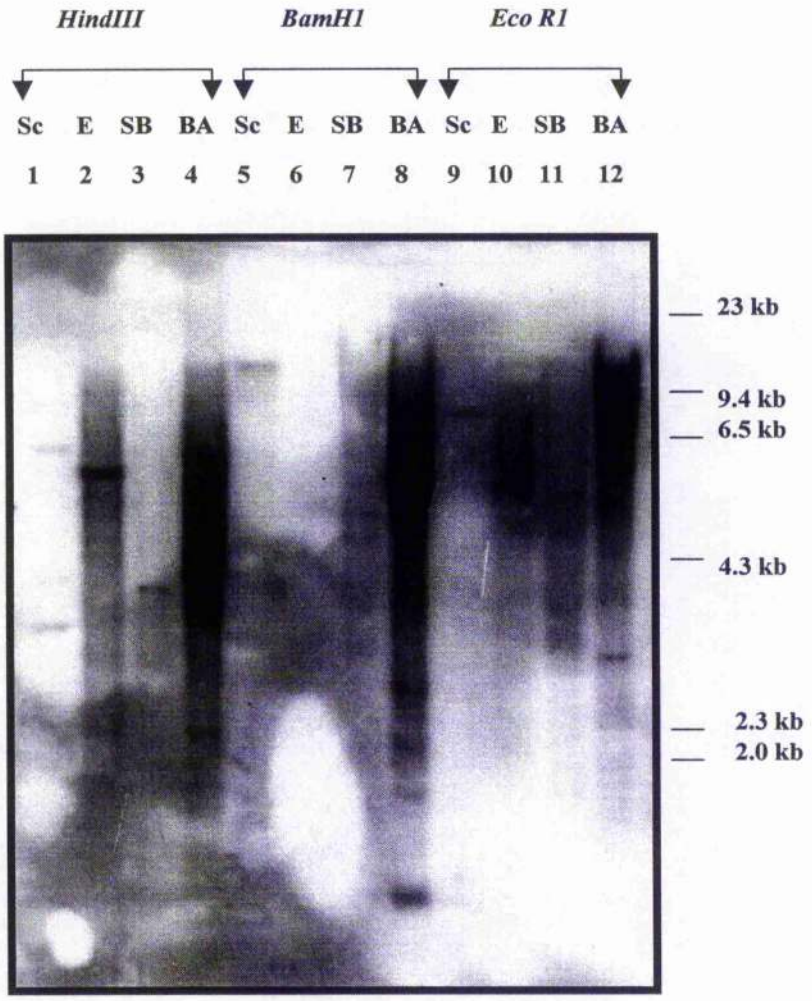


Figure 4.9. Genomic Southern Analysis of Plant Genomic DNA using *hv1* as Probe.

Genomic DNA was isolated from *B. vulgaris* (SB), *H. vulgare* (BA), *Saccharomyces cerevisiae* (Sc.) and *E. coli* (E). Lanes 1-3, 6-8, and 11-13 each contains 10 µg of plant genomic DNA, lanes 4, 9, and 14 each contains 100 ng of *E. coli* genomic DNA and lanes 5 and 10 each contains 10 ng of *E. coli* genomic DNA. Each DNA sample was digested individually with restriction enzymes *HindIII*, *BamH1* and *EcoR1*. The digested samples were electrophoresed in a 1 % (w/v) agarose gel and blotted onto nylon membrane. The blot was hybridized with labelled *hv1* probe (a PCR amplified fragment from *H. vulgare* cDNA library), washed at low stringency (1 X SSC at 50°C) and autoradiographed for 3 days at -80°C.

of DNA from each organism was digested with *EcoR1*, *BamH1* and *HindIII* (Section 2.2.8) and electrophoresed in 1 % (w/v) agarose gel (Section 2.2.7.2). The resolved fragments were then blotted onto nylon membrane (2.2.10.1) and hybridized with *hv1* (Section 2.2.12.1) the membrane washed at low stringency (1 X SSC at 50°C), and then autoradiographed for 3 days at -80°C. Figure 4.9 presents the resulting autoradiogram and shows a strong hybridizing band in *E. coli* (lanes 2 and 10). The genome of *S. cerevisiae* also appears to have sequences with weak homology to the *hv1* probe (lanes 1, 5 and 9). Several hybridizing bands were found in *H. vulgare* genomic DNA digested with the three enzymes (lane 4 ~ 14 kb and ~ 2.3 kb, lane 8, ~1.3 kb ~ 1.6 kb and ~ 4 kb and lane 12 ~ 3 kb and 5 kb). Sugar beet also appears to have some homology to *hv1* (lane 3 ~ 4 kb, lane 7, ~ 4.5 kb and ~ 4.2 kb and lane 11 ~ 5 kb). The results from the Southern blot suggest that *B. vulgaris* and *H. vulgare* have some sequence homology to *hv1*, but when this blot was washed at high stringency (0.1 X SSC at 65°C) all hybridizing bands disappeared (data not presented).

To establish whether or not the *hv1* fragment is part of an expressed barley gene, northern blot analysis was carried out on total RNA prepared from the roots and leaves of *Hordeum vulgare* (barley) plants grown hydroponically in 150 mM NaCl; in addition, total RNA from sugar beet plants grown in 300 mM NaCl was included, as were samples prepared from low-salt grown controls of both plants, and also from *E. coli* (Section 2.2.2.2). Samples of total RNA were electrophoresed in a 1.3 % (w/v) agarose denaturing gel (Section 2.2.8.3), blotted onto nylon membrane (Section 2.2.10.2), hybridized overnight with *hv1* as probe, and washed at low stringency (1 X SSC at 55°C). No hybridizing bands were observed in any of the samples (data not presented).

The cloned fragment *hv1* was sequenced (Fig. 4.10; Section 2.2.16) and the probable translated protein sequence (frame f) used to search the databases using the BLAST routine from GCG 7 (Section 2.2.17). Neither of the expected primer amino acid sequences were observed at the end of the fragment (GKEGPxxH or GVLFxxE, see Fig 1.2 and 4.1) although nucleic acid homology to the reverse primer was found at both ends. The corresponding BLAST search of the protein database suggested that *hv1* is identical to an *E. coli* hypothetical protein (Fig. 4.11).

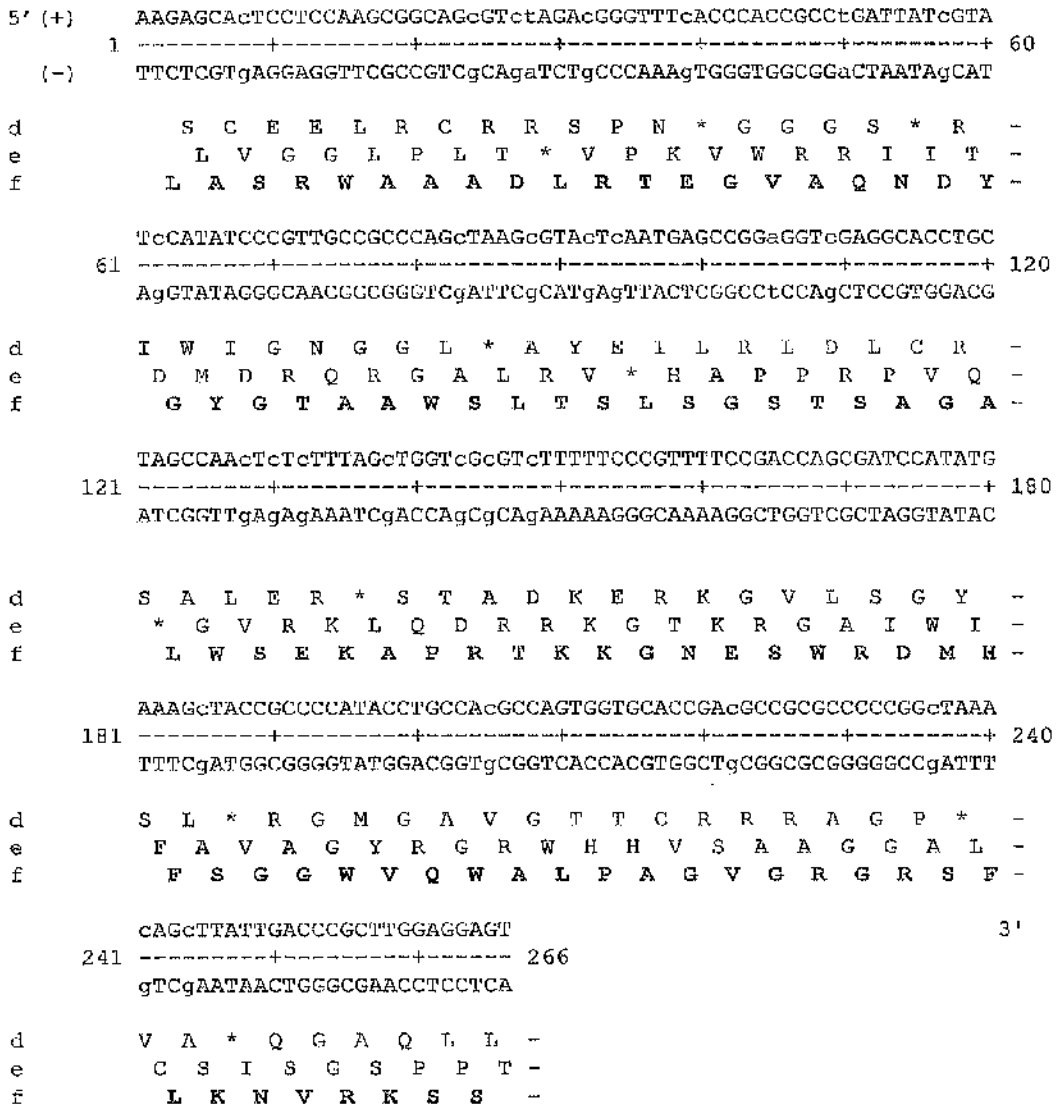


Figure.4.10. Sequence Analysis of *hv1*.

Automated sequencing was performing as described in Materials and Methods (Section 2.2.16). The nucleic acid sequence was translated in all six reading frames using the map program from GCG 7. The probable reading frame (f) is shown in bold (88 amino acids); as this was the only reading frame without stop or nonsense codons. However, neither of the expected primer amino acid sequences were observed (See Figs. 1.2 and 4.1).

```
Reference:  Gish, Warren (1994-1997).  unpublished.
Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J.
Lipman (1990).  Basic local alignment search tool.  J. Mol. Biol. 215:403-10.

Query=  HV1 frame f
, 89 bases, 68860FCC checksum.
        (89 letters)

Database:  swall
           283,052 sequences; 89,381,315 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                    Smallest
                                                                      Sum
                                                             High  Probability
Sequences producing High-scoring Segment Pairs:              Score  P(N)       N

SWALL:YAGX_ECOLI P77802 HYPOTHETICAL 91.2 KD PROTEIN IN I...   405  6.0e-37    1
SWALL:O06206     O06206 HYPOTHETICAL 59.7 KD PROTEIN. 11/98     85  0.0077     1
SWALL:O06304     O06304 SIMILARTO GP|AL009198|MTV004_5 MY...    74  0.54       1
SWALL:O15197     O15197 EPH-FAMILY PROTEIN. 11/98               68  0.62       1
SWALL:Q14526     Q14526 HIC-1 GENE FRAGMENT. 11/98              65  0.63       1


>SWALL:YAGX_ECOLI P77802 HYPOTHETICAL 91.2 KD PROTEIN IN INTF-EAEE INTERGENIC
           REGION PRECURSOR. 11/97
           Length = 841

 Score = 405 (142.6 bits), Expect = 6.0e-37, P = 6.0e-37
 Identities = 76/79 (96%), Positives = 78/79 (98%)

Query:     2 SKRVNKLFSRGRGVGAPLAAWQVWGGSFHMDRWSENGKKTRPAKESWLAGASTSGSLSTL 61
             ++RVNKLFSRGRGVGAPLA WQVWGGSFHMDRWSENGKKTRPAKESWLAGASTSGSLSTL
Sbjct:   345 TQRVNKLFSRGRGVGAPLA-WQVWGGSFHMDRWSENGKKTRPAKESWLAGASTSGSLSTL 403

Query:    62 SWAATGYGYDNQAVGETRL 80
             SWAATGYGYDNQAVGETRL
Sbjct:   404 SWAATGYGYDNQAVGETRL 422


>SWALL:O06206 O06206 HYPOTHETICAL 59.7 KD PROTEIN. 11/98
           Length = 580

 Score = 85 (29.9 bits), Expect = 0.0077, P = 0.0077
 Identities = 28/88 (31%), Positives = 40/88 (45%)

Query:     7 KLFSRGRGVGAPLAAWQVWGG---SFHMDRWSENGKKTRPAKESWLAGAS---TSGSLST 60
             ++F+ G G+G  LAA   W G      H    S     T  A ++W   AS   T  +
Sbjct:    14 RIFA-GAGLGPMLAAASAWDGLAEELHAAAGSFASVTTGLAGDAWHGPASLAMTRAASPY 72

Query:    61 LSWAATGYGYDNQAVGETRLDAAAWRSAL 89
             + W  T  G   QA G+ RL A+A+ + L
Sbjct:    73 VGWLNTAAGQAAQAAGQARLAASAFEATL 101
```

Figure 4.11 Blast Output of a *hv1* Search in the TREMBL Data Base.

The frame f translation of the sugar beet sequence *hv1* (see Fig. 4.10) was used to interrogate the TREMBL data base for homology using BLAST from GCG 7. The high 'High Score' value of 405 to an *E. coli* sequence suggests that *hv1* is probably a bacterial gene cloned from *E. coli* genomic template which contaminated the barley cDNA library.

4.1.2. PCR Cloning Using Genomic DNA as Template.

In view of the difficulties encountered when attempting to amplify *clc* sequences from plant cDNA libraries, it was decided to repeat the experiments using genomic DNA as template.

4.1.2.1 *Beta vulgaris.*

The degenerate forward (F1) and reverse (R1) primers shown in Fig. 4.1 were used with genomic DNA from *B. vulgaris* (red beet) cell suspension cultures and after optimization, the resulting PCR products were resolved by agarose gel electrophoresis (Fig. 4.12). Two products were observed, *bv3* (250 bp) and *bv4* (300 bp); see Fig. 4.12. Both the *bv3* and *bv4* fragments were cloned into the PCR-Script™ cloning vector (Section 2.2.15.5), and then transformed into *E. coli* XL-1 Blue supercompetent cells (Section 2.2.3.2.1).

4.1.2.1.1. Analysis of *bv3.*

To confirm that *bv3* represents an authentic *Beta vulgaris* genomic DNA sequence, genomic Southern blot experiments were performed using *bv3* as probe. Figure 4.13 presents the autoradiogram from appropriate amounts of genomic DNA (sugar beet, red beet, barley and *E.coli*) digested with *EcoR1*, *BamH1* and *HindIII* (Section 2.2.8) and electrophoresed on a 1 % (w/v) agarose gel (Section 2.2.7.2). The gel was blotted onto nylon membrane (2.2.10.1) and hybridized with *bv3* as probe. The membrane was washed at moderate stringency (0.5 X SSC at 65°C) and autoradiographed for 3 days at -80°C. Several hybridization bands are observed. As expected, strong hybridization bands were found in *B. vulgaris* (red beet) genomic DNA digested with *BamH1* (lane 7 ~ 1.5 kb) and *EcoR1* (lane 12 ~ 3.5 kb) but only weak hybridization was found in *HindIII* restriction digest of *B. vulgaris* (red beet) genomic DNA (lane 2 ~ 2 kb). There is also evidence that homologues to *bv3* are found in the sugar beet genome (lane 6 ~ 1.5 kb; lane 11 ~ 3.2 kb; again, only weak homology to *bv3* was found in the *HindIII* genomic DNA digest, lane 1 ~ 2 kb). These results are not unexpected, as red beet and

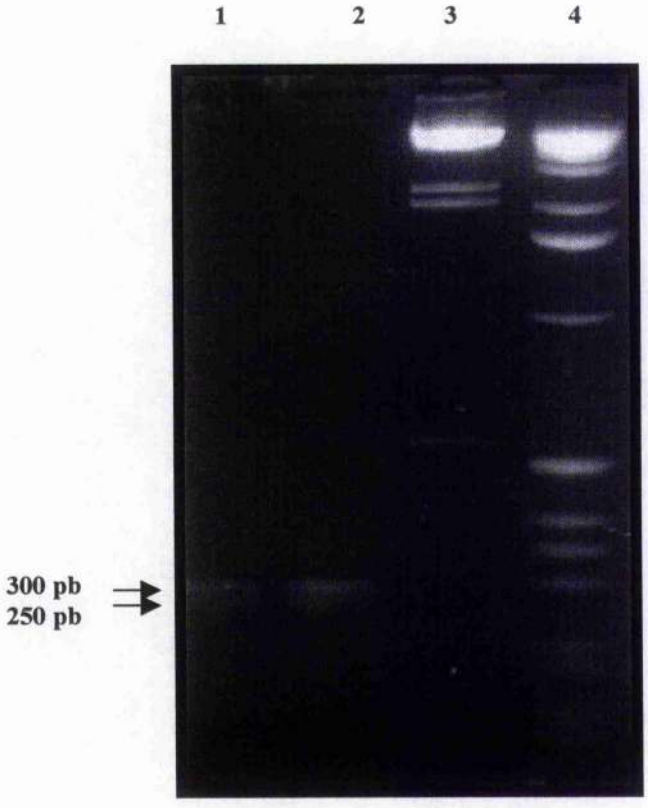


Figure 4.12. Fluorescent Gel of *B. vulgaris* PCR Amplified Fragments *bv3* (250 bp) and *bv4* (300 bp).

Two degenerate primers F1 and R1 were used with *Pfu DNA* polymerase in the PCR reactions to amplify fragments from genomic DNA isolated from a *B. vulgaris* (red beet) cell suspension culture. The PCR reaction proceeded through 35 cycles (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min). PCR products were then resolved in a 2 % (w/v) agarose gel, stained with ethidium bromide and visualized under UV light. The PCR reactions produced two fragments (*bv3*) and (*bv4*) with sizes of 250 and 300 bp (lanes 1 and 2) respectively. Lanes 3 and 4 represent the molecular weight markers λ *HindIII* and 1 kb ladder respectively.

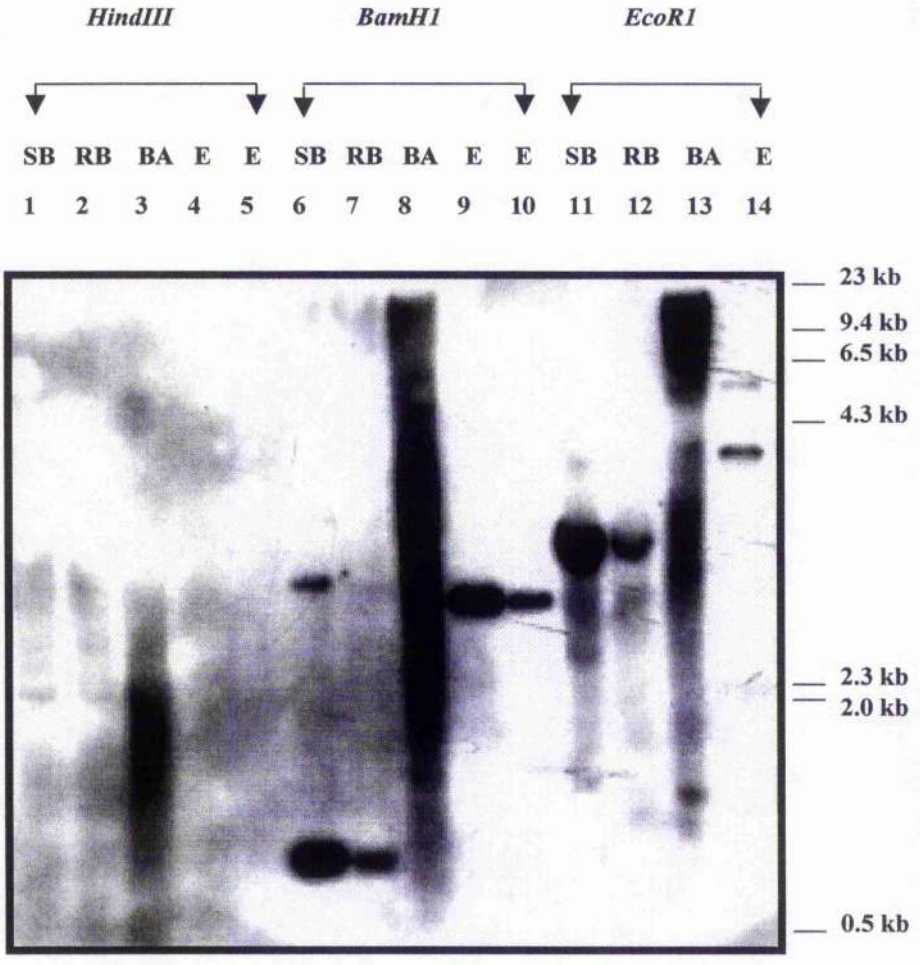


Figure 4.13. Genomic Southern Analysis of Plant Genomic DNA Using *bv3* as Probe.

Genomic DNA was isolated from *B. vulgaris* (sugar beet, SB), *B. vulgaris* (red beet, RB) cell suspension culture, *H. vulgare* (barley, BA) and *E. coli* (E). Lanes 1-3, 6-8, and 11-13 each contains 10 µg of plant genomic DNA, lanes 4, 9, and 14 each contains 100 ng of *E. coli* genomic DNA and lanes 5 and 10 each contains 10 ng of *E. coli* genomic DNA. Each DNA sample was digested individually using *HindIII, BamH1* and *EcoR1*. The DNA samples were electrophoresed in a 1 % (w/v) agarose gel and blotted onto nylon membrane. The blot was probed with labelled *bv3* (250 bp), a PCR fragment of genomic DNA isolated from *B. vulgaris* (red beet) suspension culture, washed at moderate stringency (0.5 X SSC at 65°C) and autoradiographed at - 80°C for 3 days.

sugar beet are both varieties of the species *Beta vulgaris*. Finally, the genome of *E. coli* also appears to have strong sequence homology to *bv3* (lane 9, 10 ~3 kb; and lane 14 ~4 kb).

Northern blot analysis was then carried out to confirm whether or not *bv3* sequences are expressed in *B. vulgaris*. Total RNA and poly $(A)^+$ mRNA was isolated from the following high-salt treated samples: sugar beet leaves and roots grown in 300 mM NaCl: red beet cell suspension cultures grown in 200 mM NaCl: barley leaves and roots grown in 150 mM NaCl. Samples of total RNA and poly $(A)^+$ mRNA were electrophoresed in a 1.3 % (w/v) agarose/formaldehyde gel and the resolved RNA fragments blotted onto nylon membrane. The blot was hybridized with *bv3* as probe, washed at low stringency (1 X SSC at 55°C) and autoradiographed for 4 days. No evidence was found of *bv3* expression in total RNA or poly $(A)^+$ mRNA isolated from any of these samples (data not presented).

4.1.2.1.2. Analysis of *bv4*.

Figure 4.14 presents the autoradiogram from a genomic Southern analysis of sugar beet, red beet, barley and *E. coli*. The experiment was undertaken to confirm whether or not *bv4* represented an authentic red beet sequence. Appropriate amounts of genomic DNA from each organism was prepared and digested with *EcoR1*, *BamH1* or *HindIII* (Section 2.2.8) and electrophoresed in a 1 % (w/v) agarose gel (Section 2.2.7.2). The resolved fragments were then blotted onto nylon membrane (2.2.10.1) and hybridized with *bv4* as probe (Section 2.2.12.1). The membrane was then washed at moderate stringency (0.5 X SSC at 65°C) and autoradiographed for 3 days at -80°C. The resulting autoradiogram provided no evidence of sequence homology to *bv4* in either sugar beet, red beet or barley although some homology to sequences in *E. coli* were apparent (lanes 4, 8, 9 and 14).

The expression of *bv4* was examined by northern blot analysis. Total RNA and poly $(A)^+$ mRNA was isolated from the following salt-stressed samples: sugar beet leaves and roots grown in 300 mM NaCl: red beet cell suspension culture grown in 200 mM NaCl: barley leaves and roots grown in 150 mM NaCl. The blot was washed at low

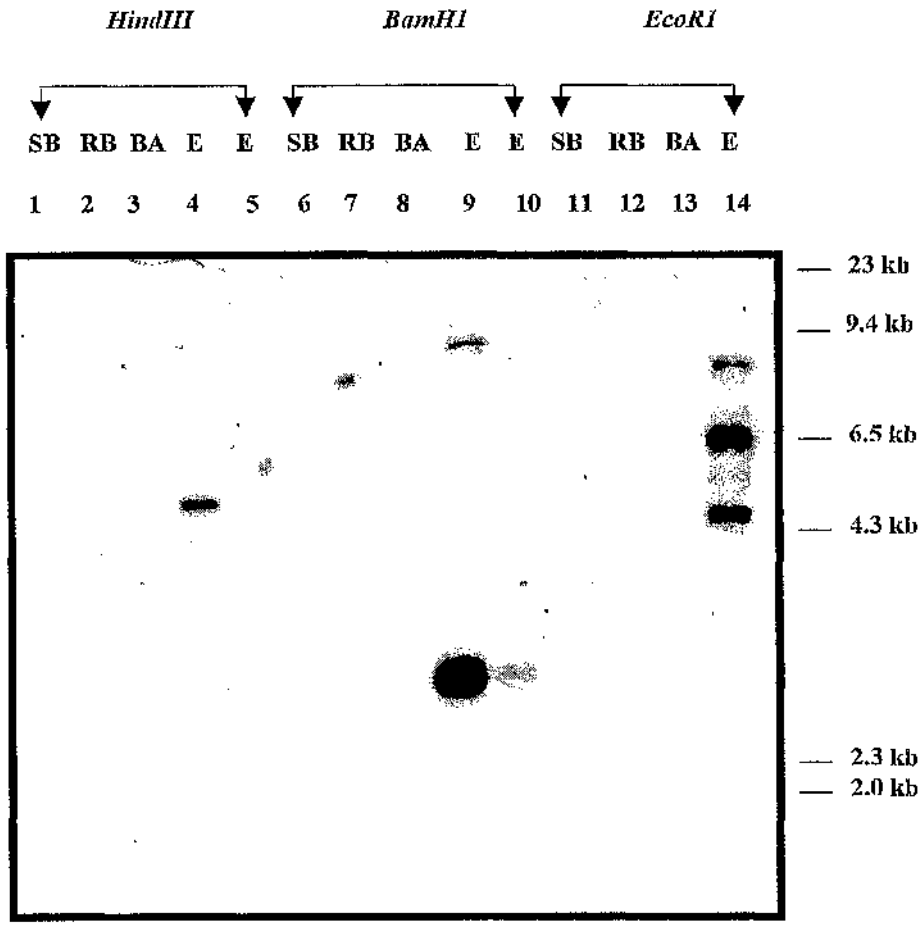


Figure 4.14. Genomic Southern Analysis of Plant Genomic DNA using *bv4* as Probe.

Genomic DNA was isolated from *B. vulgaris* (sugar beet, SB), *B. vulgaris* (RB) cell suspension culture, *H. vulgare* (BA) and *E. coli* (E). Lanes 1-3, 6-8, and 11-13 each contains 10 µg of plant genomic DNA, lanes 4, 9 and 14 each contains 100 ng of *E. coli* genomic DNA and lanes 5 and 10 each contains 10 ng of *E. coli* genomic DNA. Each DNA sample was digested individually with *HindIII, BamHI* and *EcoRI*. The DNA samples were electrophoresed in a 1 % (w/v) agarose gel and blotted onto nylon membrane. The blot was probed with labelled *bv4* (300 bp) a PCR fragment of genomic DNA isolated from *B. vulgaris* (red beet) cell suspension culture, washed at moderate stringency (0.5 X SSC at 65°C) and autoradiographed at - 80°C for 3 days.

stringency (1 X SSC at 55°C). No evidence for the expression of *hv4* was found in any of the samples (data not presented).

4.2. RT-PCR Cloning Approach.

The failure to amplify any authentic plant *clc* homologues using cDNA libraries or genomic DNA as template was disappointing and so it was decided to try a RT-PCR approach. RT-PCR was performed on the following tissues: leaves of 300 mM NaCl-grown sterile sugar beet plants, roots of 300 mM NaCl-grown sterile sugar beet plants, leaves of 150 mM NaCl-grown sterile barley plants, roots of 150 mM NaCl-grown sterile barley plants and cells from a sterile red beet suspension culture containing 200 mM NaCl. Both oligo dT and random primer first strand cDNA synthesis methods were used on each of the above preparations. After first strand synthesis, both F1/R1 and F2/R1 primer pairs were used on each of the cDNA samples (*i.e.* 20 experiments).

4.2.1 RT-PCR using Oligo dT Primer First Strand Synthesis.

Several bands were generated from the different preparations. However, the F1/R1 primer pair failed to generate any fragments of the expected size (150-300 bp) although some larger products were observed. In contrast, after optimization, the F2/R1 primer pair (see Fig. 4.1) resulted in the amplification of a single PCR fragment from leaf tissues with a size of approximately 600 bp (Fig. 4.15, lanes 1 and 2), exactly as predicted from the consensus of aligned CLC sequences (Fig. 1.2). No PCR fragments were produced from the F2/R1 PCR reactions using root RNA as template (Fig. 4.15, lanes 4 and 5) or from red beet or barley preparations. However, when the F2 primer was used on its own with sugar beet leaf poly $(A)^+$ mRNA as template, an unexpected 600 bp fragment was amplified (lane 3). This suggests that the F2 consensus sequence on the sense strand has homologous sequences on the nonsense strand 600 bases downstream.

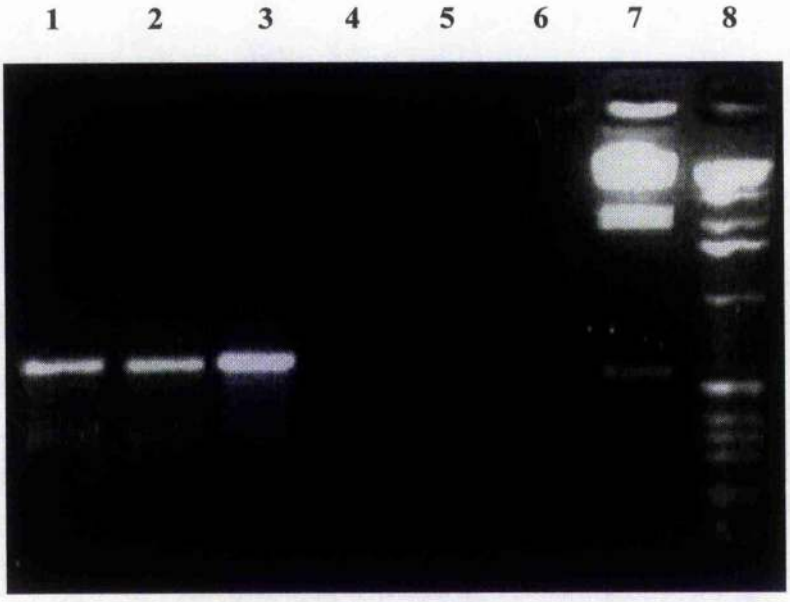


Figure 4.15. Agarose Gel of RT-PCR Amplified Fragment from Sugar Beet using *clc* Degenerate Primers.

RT-PCR was performed on the first strand cDNA prepared from high-salt-grown sugar beet plants. Poly $(A)^+$ mRNA was prepared from leaf and root tissues. The first strand cDNA was then prepared using MMLV reverse transcriptase and oligo (dT) primer. The resulting cDNAs of sugar beet were amplified through 35 thermal cycles using F2, R1, and *taq* DNA polymerase with the corresponding buffers. The PCR products were analysed in a 1 % (w/v) agarose gel and stained with ethidium bromide. The gel contains the PCR products amplified from leaf tissue (lanes 1 and 2) and from root tissue (lanes 5 and 6). Lane 3 contains the PCR product when the F2 primer only was used with leaf and root cDNA as template. Molecular weight markers (λ *HindIII* and 1 kb ladder) are in lanes 7 and 8 respectively.

Despite this concern, the resultant 600 bp fragment (*bv600*) was cloned into PCR-Script™ cloning vector (Section 2.2.15.5), and transformed into the host *E. coli* strain XL-1 blue MRF (Section 2.2.3.2.1) for further analysis.

Genomic Southern blot analysis was performed to confirm whether or not *bv600* represented an authentic *B. vulgaris* sequence. Figure 4.16 presents the autoradiogram from a blot of sugar beet, red beet, barley and *E. coli* genomic DNA. Appropriate amounts of genomic DNA from each organism were digested with either *EcoR1*, *BamH1* or *HindIII* (Section 2.2.8), electrophoresed in a 1 % (w/v) agarose gel (Section 2.2.7.2), blotted onto nylon membrane (Section 2.2.10.1) and hybridized with *bv600* as probe (Section 2.2.12.1). The membrane was washed at moderate stringency (0.5 X SSC at 65°C) and exposed to photographic film for 2 days at -80°C. The experiment confirmed that homologues to *bv600* are present in both the sugar beet and red beet genomes with almost identical patterns observed in corresponding lanes (*cf.* lanes 1 & 2 ~ 3 kb: lanes 6 & 7 ~ 11 kb: lanes 11 & 12 ~ 3 kb and ~ 2 kb). Neither *H. vulgare* nor *E. coli* appear to have sequences that are homologous to *bv600*.

The expression of *bv600* was analysed by northern hybridization. Figure 4.17(A) presents the autoradiogram from a northern blot made from the denaturing agarose gel shown in Fig. 4.17B. Total RNA and poly $(A)^+$ mRNA was prepared from leaf and root tissues of high-salt-grown (300 mM NaCl) sugar beet plants, and from high-salt grown (200 mM NaCl) cell suspension cultures of red beet (Sections 2.2.2.2 and 2.2.2.4 respectively). Total RNA (10 µg) and poly $(A)^+$ mRNA (1µg) samples were separated on 1.3 % agarose denaturing gel (Section 2.2.8.3) and the resolved fragments (Fig. 4.17 B) blotted onto nylon membrane (Section 2.2.10.2). The blot was then hybridized with labelled *bv600* and washed at low stringency (1 X SSC at 55°C, see Section 2.2.12.3) and autoradiographed at -80°C for 5 days. Hybridization to a band of about 1.4 kbp was observed in sugar beet leaf (lane 1) and root (lane 2) total RNA; similarly, a hybridizing band of about 1.4 kbp was found in a red beet cell suspension total RNA (lane 3). The 16S ribosomal band from plastids also migrates at approximately 1.4 kbp on agarose gels and so poly $(A)^+$ mRNA was prepared from each of these samples and electrophoresed (Fig. 4.17A & B, lanes 4-6). Hybridizing bands at about 1.4 kbp were found from sugar beet leaf (lane 4) and red beet suspension cells (lane 6). No signal was detected in sugar beet roots (lane 5).

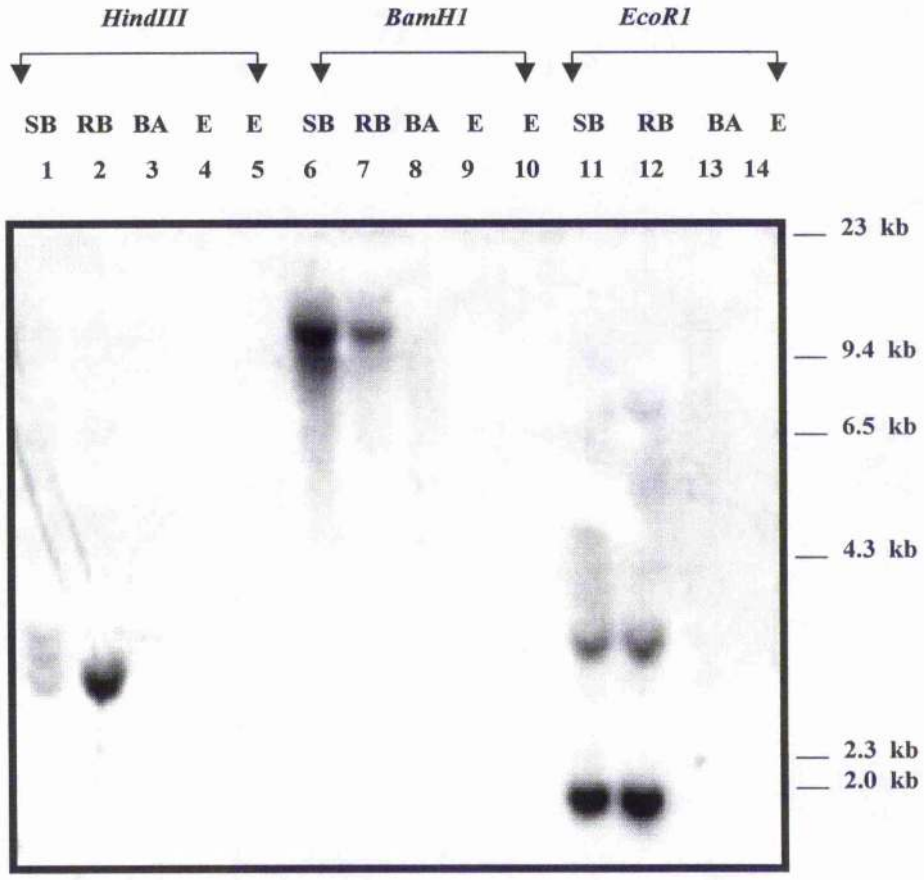


Figure 4.16. Genomic Southern Aanalysis of Plant Genomic DNA using *bv600* as Probe.

Genomic DNA was isolated from sugar beet (SB), red beet (RB) cell suspension culture, barley (BA) and *E. coli* (E). Lanes 1-3, 6-8, and 11-13 each contains 10 µg of plant genomic DNA, lanes 4, 9 and 14 each contains 100 ng of *E. coli* genomic DNA and lanes 5 and 10 each contains 10 ng of *E. coli* genomic DNA. Each DNA sample was digested individually with *HindIII, BamH1* and *EcoR1*. The DNA samples were electrophoresed in a 1 % (w/v) agarose gel and blotted onto nylon membrane. The blot was probed with labelled *bv600* a RT-PCR fragment of *B. vulgaris* (sugar beet) leaf tissue, washed at moderate stringency (0.5 X SSC at 65°C) and autoradiographed at - 80°C for 2 days.

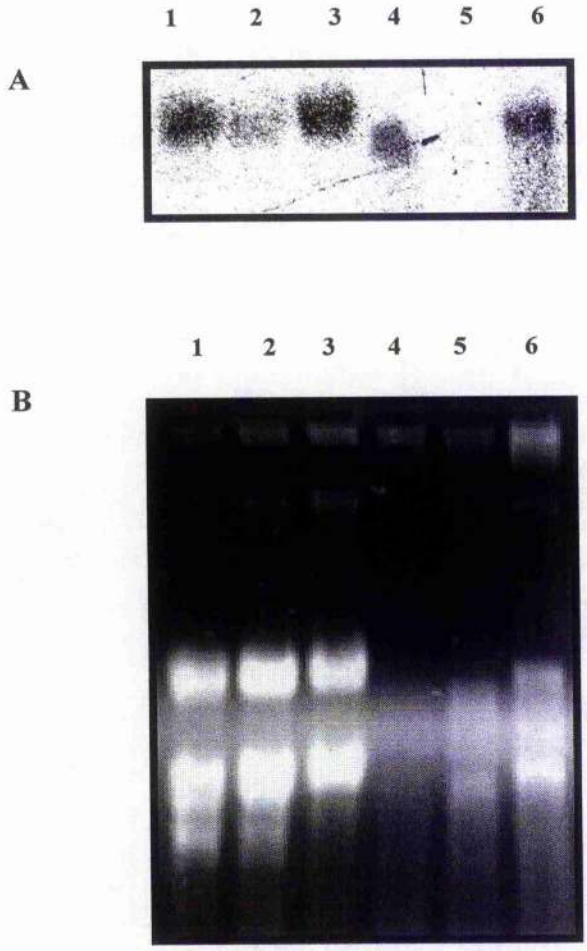


Figure 4.17. Northern Blot Analysis of *bv600* Expression in *B. vulgaris*.

(A) Ten μg of total RNA (1-3) and 1 μg of poly $(A)^+$ mRNA (4-6) were isolated from leaf (lanes 1 and 4 respectively) and root (lanes 2 and 5 respectively) tissues of high-salt-grown sugar beet and from high-salt- grown red beet cell suspension culture (lanes 3 and 6 respectively). The samples were electrophoresed in a 1.3 % (w/v) agarose gel containing formaldehyde shown in **(B),** blotted onto a nylon membrane and probed with radiolabelled *bv600*. The membrane was washed at low stringency (1 X SSC at 55°C) and autoradiographed for 5 days at -80°C.

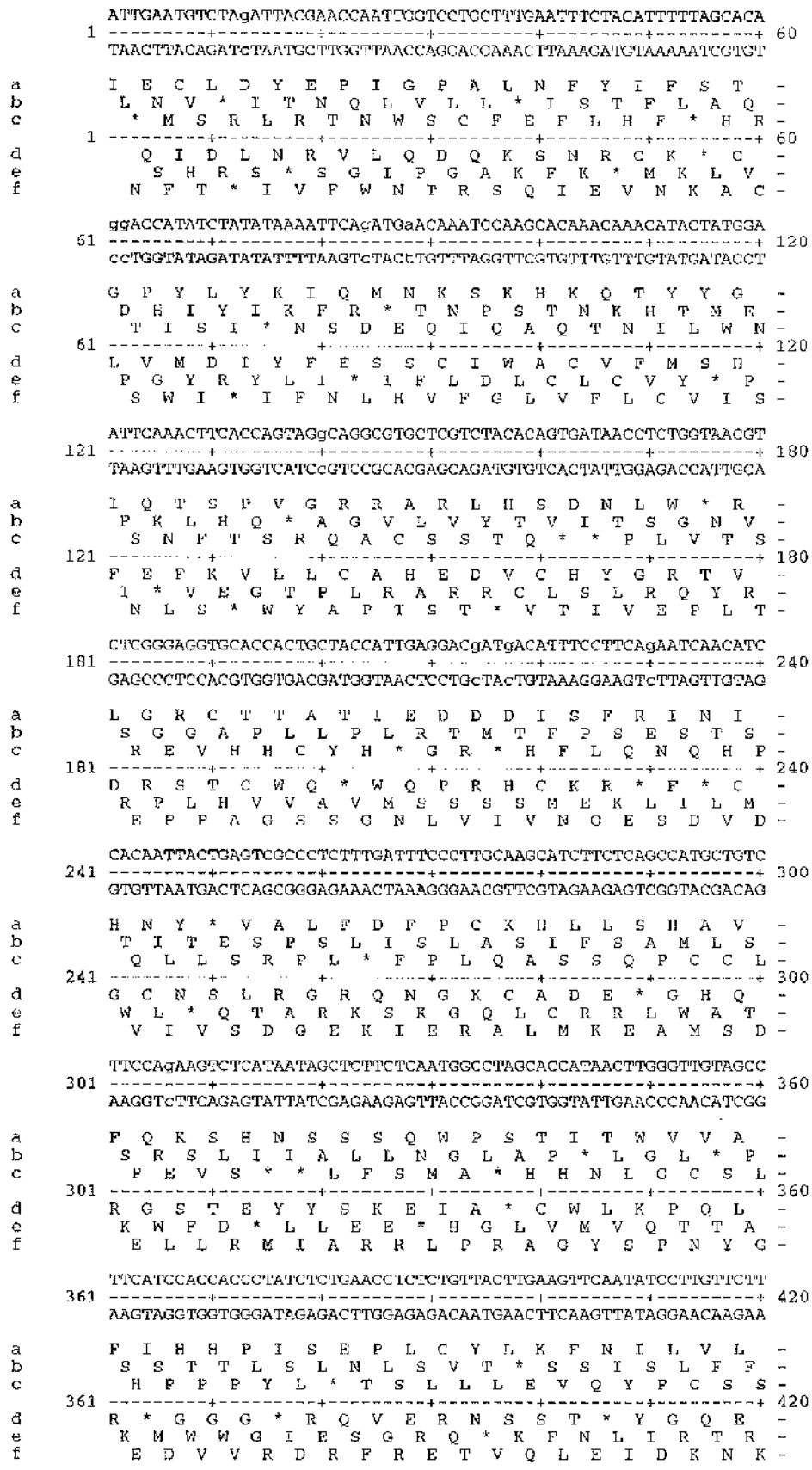
The *bv600* fragment was sequenced in both directions and the resulting nucleic acid information analysed by the MAP programme in GCG 7 to determine the correct reading frame. Figure 4.18 presents the corresponding MAP output and shows stop or nonsense codons present in each of the 3 forward and 3 reverse reading frames. Clearly, this should not occur in RT-PCR cloned fragments and so sequencing errors were suspected; consideration of the raw sequencing data did not resolve these potential errors. However, despite these errors, the nucleic acid sequence data was used to interrogate the EMBL database using the BLAST routine from GCG 7. Good homology (Fig. 4.19 >6 % identity) was found to tomato cDNAs encoding proteases, and this implies that the *bv600* sequence does not represent a ClC homologue. This contention is supported by translation from each of the reading frames containing the GKEGPxxH conserved motif (see Fig. 1.2 and 4.18).

4.2.2 RT-PCR using Random Primer First Strand cDNA Synthesis.

For two reasons, the oligo dT primer RT-PCR strategy used in Section 4.2.1, can be unsatisfactory and therefore, it was decided to use an alternative random primer method. First, the efficiency of first strand cDNA synthesis decreases markedly with increasing distance from the 3' poly $(A)^+$ sequences used in the oligo d'T' method; the *clc* consensus sequences identified in Section 4.2 (Fig. 4.1) occur at the 5' end of the *clc* homologues. Second, there is some evidence from heterologous probing experiments using the *T. marmorata clc-0* sequence as probe, that plant homologues may not be extensively poly-adenylated (Section 3.4.4).

RT-PCR was performed on samples prepared from high-salt grown (200 mM NaCl) red beet cell suspension cultures. Total RNA was prepared (Materials and Methods, Section 2.2.2.2) and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase and random primers (Section 2.2.14) were used to produce first strand cDNA.

Figure 4.20 presents a photograph of a 1% (w/v) agarose gel used to separate the F1/R1 products of RT-PCR. Two PCR products were resolved with approximate sizes of 190 and 250 bp, very close to the predicted size from the consensus sequences from aligned *clc* genes (Fig. 1.2).



CONTINUED/

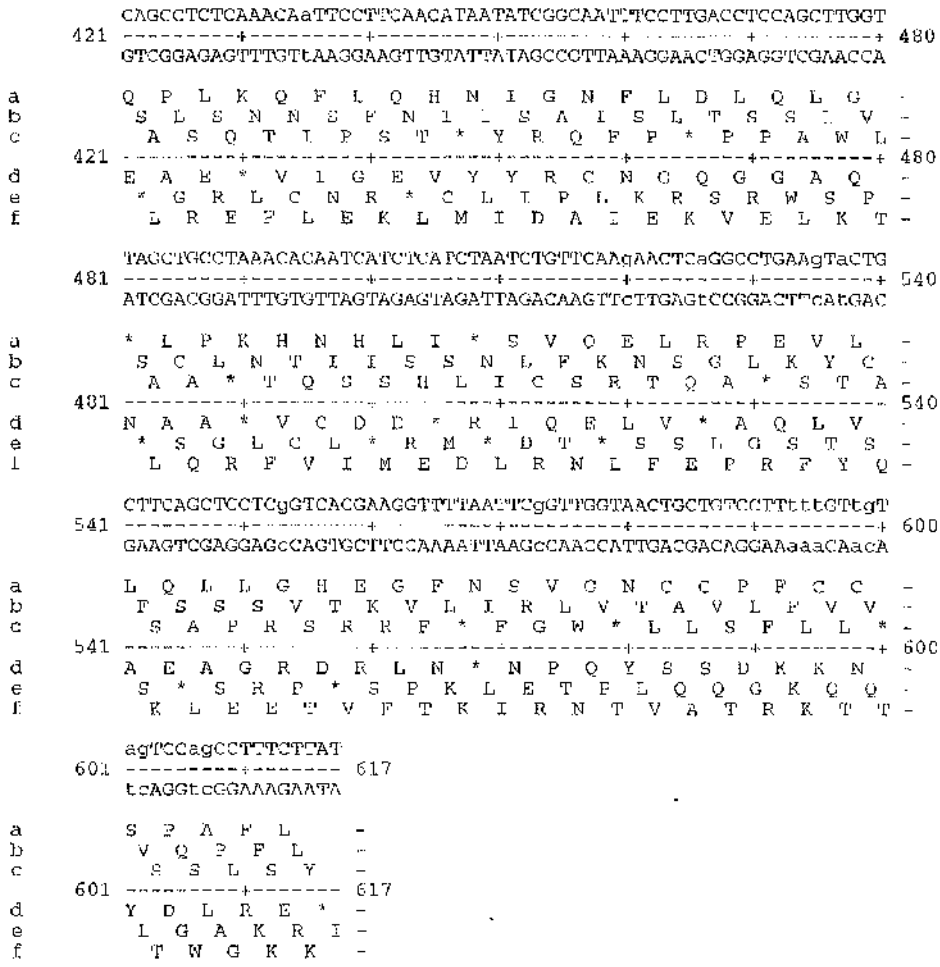


Figure 4.18 Sequence Analysis of *bv600*.

Automated sequencing was performed on the sugar beet sequence *bv 600* generated using RT-PCR and the F2 / R1 primer pair (see Fig. 4.1). The nucleic acid sequence was translated in all 6 reading frames using the MAP program from GCG 7. Due to the apperance of stop or nonsense codons in all 6 reading frames, it is not possible to determine the corresponding protein sequence. However, homology to the F2 (position 1) and R1 (position 617) primers can be seen.

```
Reference:  Gish, Warren (1994-1997).  unpublished.
Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J.
Lipman (1990).  Basic local alignment search tool.  J. Mol. Biol. 215:403-10.

Notice:  this program and its default parameter settings are optimized to find
nearly identical sequences rapidly.  To identify weak similarities encoded in
nucleic acid, use BLASTX, TBLASTN or TBLASTX.

Query=  Sugar beet fragment.  600 bp
, 617 bases, E341EDCB checksum.
        (617 letters)

Database:  embl
           884,419 sequences; 1,224,343,988 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                    Smallest
                                                                      Sum
                                                              High  Probability
Sequences producing High-scoring Segment Pairs:               Score  P(N)        N

EM_PL:LECD4A     M32603 Tomato ATP-dependent protease (CD...  1456  6.8e-59     1
EM_PL:LECD4B     M32604 Tomato ATP-dependent protease (CD...  1362  1.3e-54     1
EM_PL:PSPCLP     L09547 Pisum sativum (clone pCLp) nuclea...  1327  2.5e-53     1
EM_PL:BNCLPA     X75328 B.napus (Topas) clpA mRNA. 7/94       1306  1.5e-52     1
EM_PL:AF022909   Af022909 Arabidopsis thaliana ClpC mRNA,...  1281  2.4e-51     1
EM_HTG:AB017063  Ab017063 Arabidopsis thaliana genomic DN...  1290  3.9e-51     1
EM_PL:AF053562   Af053562 Mesembryanthemum crystallinum A...  1023  5.7e-40     1
EM_PL:OSYK4      D29692 Rice mRNA, partial homologous to ...   753  1.4e-27     1
EM_BA:SS16134    U16134 Synechococcus sp. Clp protease pu...   684  5.6e-24     1
EM_BA:SSSLRB     D64000 Synechocystis sp. PCC6803 complet...   623  5.3e-21     1
EM_BA:CJY13333   Y13333 Campylobacter jejuni clpB gene. 4/98   372  9.4e-10     1


>EM_PL:LECD4A M32603 Tomato ATP-dependent protease (CD4A) gene, complete cds.
           4/92
           Length = 5429

  Minus Strand HSPs:

 Score = 1456 (218.5 bits), Expect = 6.8e-59, P = 6.8e-59
 Identities = 408/531 (76%), Positives = 408/531 (76%), Strand = Minus / Plus

Query:   607 CTGGACTACAACAAAAAACGACAGCAGTTACCAACCGAATTAAAACCTTCGTGACCGAGGA 548
            CT GA T   AC A AAGGA AG AGTTAC AACCG AT AA A CTT GTGAC GACGA
Sbjct:  4857 CTAGATTTTGACGAGAAGGATAGTAGTTAC-AACCGTATCAAGAGCTTGGTGACTGAGGA 4915

Query:   547 GCTGAAGCAGTACTTCAGGCCTGAGTTCTTGAACAGATTAGA-TGAGATGATTGTGTTTA 489
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Sbjct:  4916 GTTGAAACAGTACTTCAGGCCAGAGTTTTTAAACAGATT-GAGTGAGATGATTGTATTCC 4974

Query:   488 GGCAGCTAACCAAGCTGGAGGTCAAGGAAATTGCCGATATTATGTTGAAGGAATTGTTTG 429
            G CAGCT AC AAG TGGAGGT AAGGA ATTGCCGATAT ATG T AAGGA  T TTTG
Sbjct:  4975 GTCAGCTCACTAAGTTGGAGGTGAAGGAGATTGCCGATATCATGCTTAAGGAGGTCTTTG 5034

Query:   428 AGAGGCTGAAGAACAAGGATATTGAACTTCAAGTAACAGAGAGGTTCAGAGATAGGGTGG 369
             GAGG TGAAGAA AAGGA AT GAACT CAAGT ACAGAGAGGTT AGAGATAGGGT G
Sbjct:  5035 TGAGGTTGAAGAATAAGGAGATAGAACTCCAAGTGACAGAGACGTTTAGAGATAGGGTAG 5094

Query:   368 TGGATGAAGGCTACAACCCAAGTTATGGTGCTAGGCCATTGAGAAGAGCTATTATGAGAC 309
            T GATGAAGG TA AACCCAAG TATGG GCTAG CCATTGAG AGAGCTATTATGAGAC
Sbjct:  5095 TTGATGAAGGATATAACCCAAGCTATGGAGCTAGACCATTGAGGAGAGCTATTATGAGAC 5154

Query:   308 TTCTGGAAGACAGCATGGCTGAGAAGATGCTTGCAAGGGAAATCAAAGAGGCGCACTCAG 249
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Sbjct:  5155 TGCTAGAGGATAGTATGGCCCAGAAGATGCTTGCAGGTGAGATCAAAGAAGGTGATTCAG 5214
```

Figure 4.19 Blast Output of a *bv600* Search in the EMBL Data Base.

Part of the BLAST output from the *bv600* nucleotide sequence interrogation on the EMBL data base. Good homology was found at the DNA level (76% identity) to tomato CD4A ATP-dependent protease.

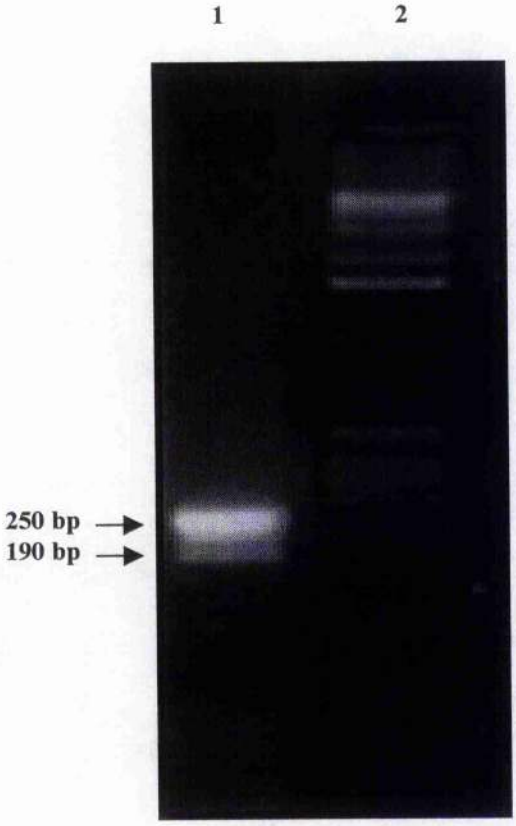


Figure 4.20. Agarose Gel of RT-PCR Product from *B. vulgaris* Cell Suspension Culture.

Two degenerate primers F1 and R1 were used with *Taq* DNA polymerase in the PCR reactions, to amplify sequences from first strand cDNA made from total RNA isolated from red beet cell suspension culture. The PCR products were separated in a 2 % (w/v) agarose gel, stained with ethidium bromide and visualised under UV light. The PCR reactions resulted in two fragments which have apparent sizes of 190 bp and 250 bp (lane 1), these two fragments are referred to as *bv160* and *bv163* respectively. The molecular weight marker (1 kb ladder) is in lane 2.

The two fragments produced from the RT-PCR reaction were separated (Section 2.2.7.1) and purified from the gel (Section 2.2.9), cloned into PCR-Script™ cloning vector (Section 2.2.15.5), and then transformed into *E. coli* XL-1 Blue supercompetent cells (Section 2.2.3.2.1). The two clones were hereafter designated as *bv160* and *bv163*.

Genomic Southern blots were performed to confirm whether or not sequences homologous to *bv160* and *bv163* are found in the *B. vulgaris* genome. Figure 4.21 presents the result of a genomic Southern analysis on sugar beet, red beet, barley and *E. coli*. Appropriate amounts of genomic DNA from each organism was digested with either *EcoR1*, *BamH1* or *HindIII* (Section 2.2.8), electrophoresed on a 1 % (w/v) agarose gel (Section 2.2.7.2), blotted onto nylon membrane (Section 2.2.10.1) and probed with *bv160* as probe. The membrane was subsequently washed at moderate stringency (0.1 X SSC at 65°C) and exposed to autoradiographic film overnight at -80°C. Several hybridizing bands were observed. Strong hybridization of *bv160* to genomic DNA from both sugar beet and red beet was found regardless of the restriction enzyme used (lanes 1 & 2, ~1.5 and ~2 kb: lanes 6 & 7, 4 and 10 kb: lanes 11 & 12 ~2-7 kb fragments). No hybridizing bands were found in the barley or *E. coli* genomic samples.

In order to confirm that *bv160* sequences are expressed in *B. vulgaris*, northern blot analysis was performed. Total RNA was isolated from a range of high and low-salt grown cells and electrophoresed in a 1.3 % (w/v) agarose/formaldehyde gel, blotted onto a nylon membrane and hybridized with *bv160* as probe. The membrane was then washed at high stringency (1 X SSC at 65°C) and autoradiographed for 3 days. Initially, signal strength was poor and so the experiment was repeated, as before except that the *bv160* probe was synthesised using asymmetric PCR (primed from the T3 and the T7 vector sequences) in an attempt to improve sensitivity (see Section 2.2.11.3.).

Figure 4.22A presents a photograph of the resulting autoradiogram prepared from the denaturing agarose gel shown in Fig. 4.22B. The northern blot shows a hybridizing band of approximately 1 kb in total RNA prepared from high-salt grown sugar beet and barley leaves (lanes 2 & 4 respectively), but not from the corresponding root tissues (lanes 3 & 5 respectively). A similar pattern was observed from low-salt grown sugar beet and barley leaves (lanes 9 & 11), but not from their roots (lanes 10 & 12). Control

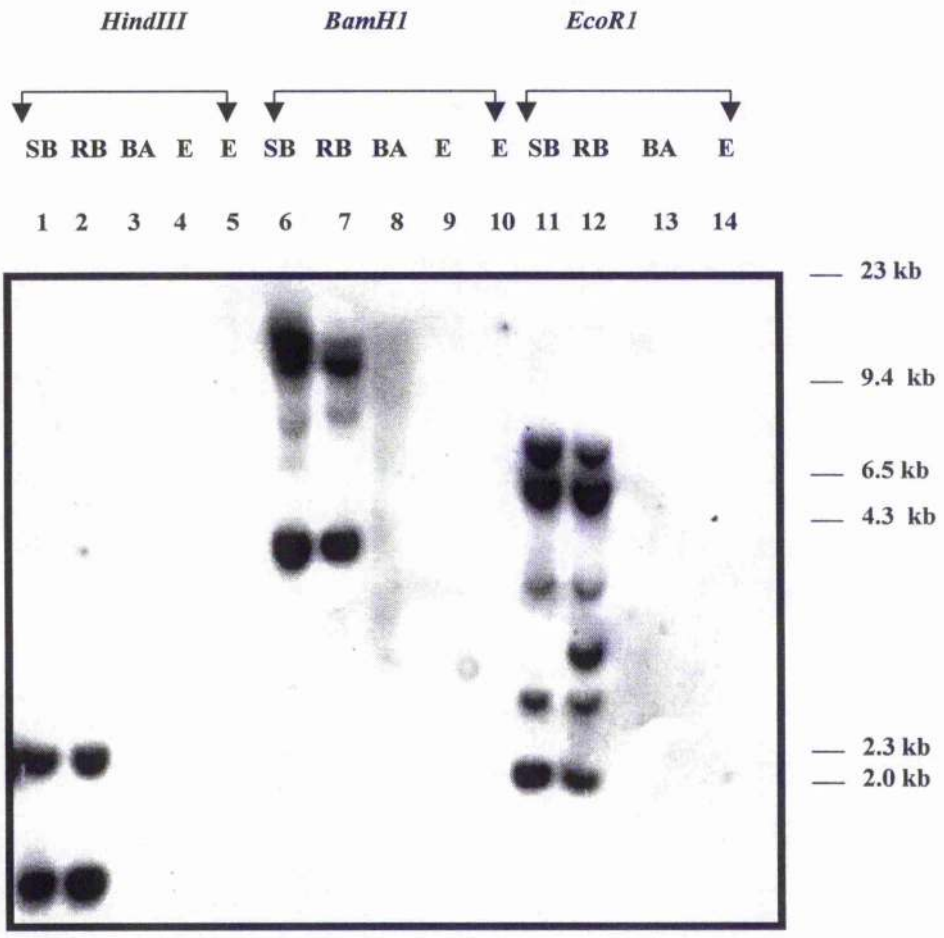


Figure 4.21. Southern Blot Analysis of Plant Genomic DNA using *bv160* as Probe.

Genomic DNA was isolated from sugar beet (SB), red beet cell suspension culture (RB), barley (BA) and *E. coli* (E). Lanes 1-3, 6-8, and 11-13 each contains 10 μg of plant genomic DNA, lanes 4, 9, and 14 each contains 100 ng of *E. coli* genomic DNA and lanes 5 and 10 each contains 10 ng of *E. coli* genomic DNA. Each DNA sample was digested individually with *HindIII, BamH1* and *EcoR1*. The DNA samples were electrophoresed in a 1 % (w/v) agarose gel and blotted onto nylon membrane. The blot was probed with labelled *bv160* (160 bp), washed at high stringency (0.1 X SSC at 65°C) and autoradiographed at -80C overnight.

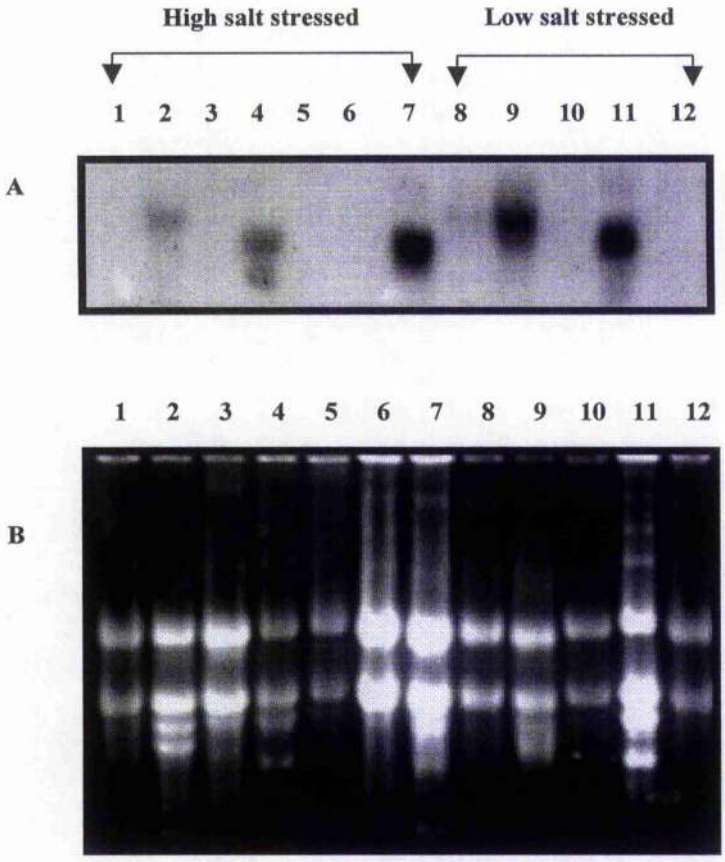


Figure 4.22. Northern Blot Analysis of Plant Total RNA Hybridized with *bv160*.

Total RNA was isolated from the following salt-stressed plant tissues: 200 mM NaCl-grown red beet cell suspension culture (lane 1): 300 mM NaCl-grown sugar beet leaf (lane 2) and root tissues (lane 3): 150 mM NaCl-grown barley leaf (lane 4) and root tissues (lane 5) and 300 mM NaCl -grown *A. halimus* cell suspension culture (lane 6). From low salt-stressed:(control) *N. tabaccum* (lane 7), red beet cell suspension culture (lane 8), sugar beet leaf (lane 9) and root tissues (lane 10), barley leaf (lane 11) and root tissues (lane 12). The Total RNA samples were electrophoresed in a 1.3 % (w/v) agarose gel containing formaldehyde shown in **(B)**, blotted onto a nylon membrane and hybridized with radiolabelled bv160 (RT-PCR amplified fragment of red beet cell suspension culture). The membrane was washed at high stringency (0.1 X SSC at 65°C) and autoradiographed for 3 days at -80°C.

(low-salt-grown) *N. tabaccum* leaf tissue also appeared to contain *bv160*-like sequences (lane 7), although the halophyte *A. halimus* does not (lane 6).

The cloned red beet fragments *bv160* and *bv163* were sequenced (Section 2.2.16) and found to have actual sizes of 160 and 163 bp respectively. The nucleic acid sequence of *bv160* and *bv163* were translated in all 6 reading frames using the MAP program from GCG 7 (Figs. 4.23 & 4.24 respectively). The two deduced sequences were found to be > 95% identical at the amino acid level. Two possible open reading frames exist for *bv160* (Fig. 4.23, frames b & d) as only these contained no stop or nonsense codons; for the same reason, frames c and d are the only possible reading frames for *bv163* (Fig. 4.24). However, in both cases, the reverse strand translation (frame d) gave the expected GKEGPxxII peptide motif (see Fig. 1.2), suggesting that this was the correct reading frame. None-the-less, both the forward and reverse reading frame translations of *bv160* and *bv163* (b & d in Fig. 4.23, and c & d in Fig. 4.24) were used in BLAST homology searches. Figure 4.25 presents part of the BLAST output using *bv160* frame b (forward, i.e. KGQWYT *etc.,*) as the query sequence, and some weak, local homology was to mammalian blood proteins; similar results were obtained when the *bv163* frame b sequence was used. Figure 4.26 presents part of the BLAST output when *bv160* frame d (reverse, i.e. GKEGPMV *etc.,)* was used as the query sequence. Again, some weak localized homology was found to several classes of protein, including an *Arabidopsis* putative reverse transcriptase.

4.3. Discussion.

Essentially, two PCR approaches have been used in this study to identify and clone plant *clc* genes, conventional PCR and RT-PCR. An alignment of 17 *clc* genes identified three regions of conserved protein sequence that were suitable for designing PCR primers. Consequently, two forward (F1 & F2) and one reverse (R1) primer were synthesized and subsequently used (see Figs. 1.2 & 4.1). Two of these primers, F1 and R1, are similar (but not identical) to those used by Maurel and co-workers to isolate *clc-ntl* (Lurin, *et al.,* 1996).

Figure 4.23. Sequence Analysis of *bv160*.

Automated sequencing was carried out as described in Materials & Methods (Section 2.2.16). The resulting nucleic acid sequence was translated in all 6 reading frames using the MAP program in GCG 7. Only frames b and d contained no stop or nonsense codons, but frame d contains the expected PGKEGP motif (reading backwards from position 159).

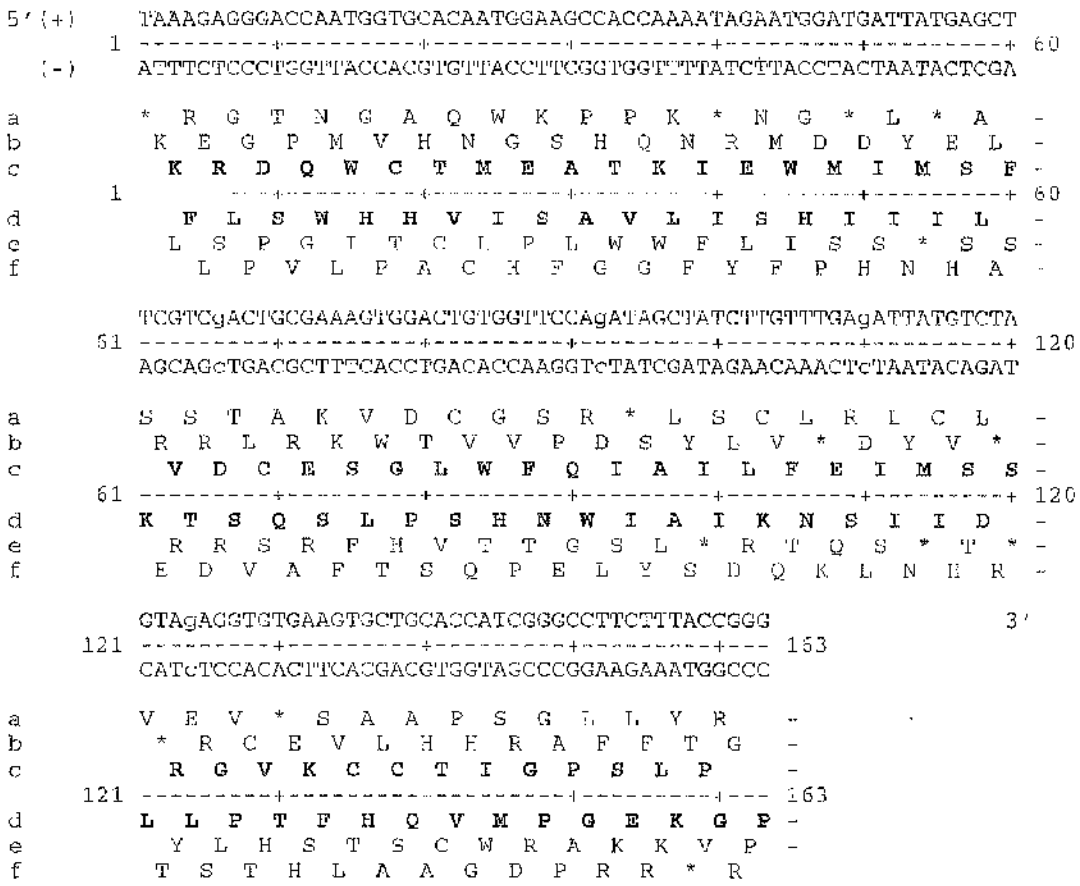


Figure 4.24. Sequence Analysis of *bv163*.

Automated sequencing was carried out as described in Materials & Methods (Section 2.2.16). The resulting nucleic acid sequence was translated in all 6 reading frames using the MAP program in GCG 7. Only frames c and d contained no stop or nonsense codons, but frame d contains the expected PGKEGP motif (reading backwards from position 163).

```
Reference:  Gish, Warren (1994-1997).  unpublished.
"
Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J.
"
Lipman (1990).  Basic local alignment search tool.  J. Mol. Biol. 215:403-10.

Query=  BV160 FRAME B
, 52 bases, 3C9484EF checksum.
        (52 letters)

Database:  swall
           284,064 sequences; 89,768,165 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                    Smallest
                                                                      Sum
                                                             High   Probability
Sequences producing High-scoring Segment Pairs:              Score  P(N)        N

SWALL:O46871      O46871 HLA-DQB1*06X (FRAGMENT). 6/98         69  0.071       1
SWALL:O19724      O19724 HUM MHC CLASS II HLA-DR-BETA-1 CH...  60  0.22        1
SWALL:Q61369      Q61369 ALPHA-1 TYPE III COLLAGEN (FRAGME...  43  0.23        2
SWALL:Q29826      Q29826 DMA, DMB, HLA-Z1, IFP2, LMP2, TAP...  65  0.32        1
SWALL:HB2X_HUMAN  P05538 HLA CLASS II HISTOCOMPATIBILITY A...  65  0.33        1
SWALL:Q29877      Q29877 MHC CLASS II (FRAGMENT). 11/98        64  0.33        1
SWALL:Q30084      Q30084 MHC CLASS II HLA-DQ-BETA-1 (FRAGM...  64  0.33        1
SWALL:O19764      O19764 HUMAN MHC CLASS II HLA-DQ (FRAGME...  64  0.37        1
SWALL:Q30093      Q30093 MHC CLASS II DQ-BETA ASSOCIATED W...  64  0.39        1
SWALL:Q29884      Q29884 HUMAN LEUKOCYTE ANTIGEN-DQ BETA C...  64  0.39        1


>SWALL:O46871 O46871 HLA-DQB1*06X (FRAGMENT). 6/98

            Length = 183

 Score = 69 (24.3 bits), Expect = 0.074, P = 0.071

 Identities = 18/53 (33%), Positives = 30/53 (56%)

Query:      1 KGQWYTNEATKIAWMIMSFVDCESGLW-FQIAILFEIMSSRG-VKCCTIG-PSL 51
              K QW+ N+   + A +- + +     +G W FQI ++ EI    RG +  C +  PSL
Sbjct:    123 KVQWFRNDQEETAGVVSTSL-IRNGDWTFQILVMLEITPQRGDIYTCQVEHPSL 175


>SWALL:O19724 O19724 HUM MHC CLASS II HLA-DR-BETA-1 CHAIN (FRAGMENT). 11/98
            Length = 83

 Score = 60 (21.1 bits), Expect = 0.25, P = 0.22
 Identities = 17/53 (32%), Positives = 30/53 (56%)

Query:      1 KGQWYTNEATKIAWMIMSFVDCESGLW-FQIAILFEIMSSRG-VKCCTIG-PSL 51
              K +W+ N+   + A ++ + +     +G W FQI ++ E+    RG V  C +  PSL
Sbjct:     26 KVRWFRNDQEETAGVVSTPL-IRNGDWTFQILVMLEMTPQRGDVYTCHVEHPSL 78
```

Figure 4.25 Blast Output of a *bv160* Search in the EMBL Data Base.

Part of the BLAST output from the *bv160* protein sequence deduced from frame b (see Fig. 4.23) interrogation on the EMBL database. Some weak homology was found (33% identity, 53% similarity) to mammalian blood serum proteins.

```
Reference:  Gish, Warren (1994-1997).  unpublished.
Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J.
Lipman (1990).  Basic local alignment search tool.  J. Mol. Biol. 215:403-10.

Query=  BV160 FRAME D
, 53 bases, 26C2B75F checksum.
        (52 letters)

Database:  swall
           284,064 sequences; 89,768,165 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                    Smallest
                                                                      Sum
                                                             High  Probability
Sequences producing High-scoring Segment Pairs:              Score  P(N)       N

SWALL:Q50095      Q50095 U1740AK. 11/96                        58  0.41       1
SWALL:G3461840    G3461840 PUTATIVE REVERSE TRANSCRIPTASE....  72  0.44       1
SWALL:O15218      O15218 G-PROTEIN COUPLED RECEPTOR. 11/98     62  0.77       1
SWALL:G3738337    G3738337 PUTATIVE REVERSE TRANSCRIPTASE....  66  0.86       1
SWALL:G1839579    G1839579 VACUOLAR INVERTASE 2, GIN2. 3/98    40  0.94       3
SWALL:Q87929      Q87929 GLYCOPROTEIN 120 (FRAGMENT). 11/98    50  0.94       1


>SWALL:Q50095 Q50095 U1740AK. 11/96
           Length = 122

 Score = 58 (20.4 bits), Expect = 0.52, P = 0.41
 Identities = 15/44 (34%), Positives = 24/44 (54%)

Query:      8 VQHFTPLLDIISNKIAIWNHSPLSQSTKLIIIHSILVASI-VYHW 51
              V H+ P LD+I N    +W  SPL  ++  +     +LV  + + HW
Sbjct:      3 VYHY-PELDLIVNAPTVWT-SPLLPYSDKLADRYLLVTGLPLAHW 45


>SWALL:G3461840 G3461840 PUTATIVE REVERSE TRANSCRIPTASE. 10/98
           Length = 1529

 Score = 72 (25.3 bits), Expect = 0.57, P = 0.44
 Identities = 13/41 (31%), Positives = 26/41 (63%)

Query:     11 FTPLLDIISNKIAIWNHSPLSQSTKLIIIHSILVASIVYHW 51
              ++PL++ +  KI+ W    LS + +L +++S++V SI   W
Sbjct:   1083 YSPLIEAVKTKISSWTARSLSYAGRLALLNSVIV-SIANFW 1122


>SWALL:O15218 O15218 G-PROTEIN COUPLED RECEPTOR. 11/98
           Length = 404

 Score = 62 (21.8 bits), Expect = 1.5, P = 0.77
 Identities = 13/41 (31%), Positives = 27/41 (65%)

Query:      8 VQHFTPLLDIISNKIAIWNHSPLSQSTKLIIIHSILVASIV 48
              + ++T LLD+ ++ ++    H  LSQSTK +++ ++ +A  V
Sbjct:     26 THNWTELLDLFNHTLSEC-HVELSQSTKRVVLFALYLAMFV 65
```

Figure 4.26 Blast Output of a *bv160* Search in the EMBL Data Base.

Part of the BLAST output from the *bv160* protein sequence deduced from frame d (see Fig. 4.23) interrogation of the EMBL data base. Some local weak homology was found (34% identity, 54% similarity) to putative *Arabidopsis* reverse transcriptases.

4.3.1. Conventional PCR Approach

Initially, attempts were made to use F1 / R1 and F2 / R1 primer pairs to generate PCR products from high-salt-grown barley and sugar beet cDNA libraries that had been generated in our laboratory. The F2 / R1 primer pair failed to amplify any fragments from either the sugar beet or barley cDNA libraries. However, the F1 / R1 primer pair generated two fragments from the sugar beet library, bv1 (289 bp) and *bv2* (281 bp), and a single product, *hv1* (266 bp) from the barley library. These three fragments were cloned but subsequent genomic Southern analysis (Figs. 4.3, 4.6 & 4.9) suggested that these sequences might have originated from *E. coli*. The three fragments were sequenced and BLAST homology searches performed on each. Strong homology was found to *bv1* (Fig. 4.5) and *hv1* (Fig. 4.11), but there appears to be no known proteins with strong homology to *bv2*. However, the proteins with the highest similarity to *bv1* (score of >300) was an *E. coli* fructose -1-phosphate aldolase and therefore *bv1* was probably generated from *E. coli* and not sugar beet template. Similarly, *hv1* was almost identical to an *E. coli* 91.2 kDa hypothetical protein of unknown function, possibly a chloride channel; more analysis of the sequence is required to clarify this point.

Clearly, there appears to be sufficient amounts of *E. coli* (host cell) DNA present in our barley (and possibly our sugar beet) library to thwart attempts to clone *clc* homologues by PCR. For this reason, it was decided to use freshly prepared genomic DNA as template, as this should be free from contaminating sequences. This approach seemed sensible given the generally acknowledged low abundance of ion channel transcripts in plant cells and the availability of sugar beet and barley genomic libraries in our laboratory.

Two fragments from *B. vulgaris* genomic DNA were amplified, *bv3* and *bv4* using the F1/R1 primer pair; the F2/R1 primer pair failed to generate any product. No products were generated using either primer pair when barley genomic DNA was used as template. Genomic Southern and northern blot analysis of *bv3* and *bv4* was undertaken but the results caused concern. It appears that although *bv3* and *bv4* were generated from sterile cell cultures of red beet, *E. coli* appears to have homologous sequences to both fragments. Further, in the case of *bv4*, no homologous plant sequences were

identified (Fig. 4.14). These results were extremely worrying, and therefore it was decided not to proceed with this approach.

4.3.2 RT-PCR Approach.

RT-PCR was performed on the following tissues: leaves of 300 mM NaCl-grown sugar beet plants, roots of 300 mM NaCl-grown sugar beet plants, leaves of 150 mM NaCl-grown barley plants, roots of 150 mM NaCl-grown barley plants and cells from a red beet suspension culture containing 200 mM NaCl. Both oligo d'T and random primer first strand cDNA synthesis methods were used on each of the above preparations.

No PCR products of the expected size (150-300 bp) were generated from any of the samples when the F1/R1 primer pair was used with oligo dT-synthesised cDNA. However, when the F2/R1 primer pair was used, a single product of the expected size, *bv600* (600 bp) was generated from leaf tissue of 300 mM NaCl-grown sugar beet plants. Genomic Southern analysis confirmed that *bv600* is an authentic *B. vulgaris* sequence with homologues present in both red beet and sugar beet (Fig. 4.16). Northern analysis confirmed the expression of *bv600* primarily in the leaf of sugar beet; the transcript size was 1.4 kb. The *bv600* DNA fragment was sequenced but it's corresponding protein sequence could not be determined as stop or nonsense codons were present in each reading frame (Fig. 4.18). Presumably, sequencing or 'base-call' errors account for these anomalies. However, consideration of the sequencing electrophoretograms could not resolve these discrepancies; clearly *bv600* requires re-sequencing before its primary structure can be confirmed. Not withstanding these sequencing errors, the nucleic acid sequence was used in a BLASTN search of the EMBL database. Disappointingly, it appears that *bv600* codes for an ATP-dependent protease (Fig. 4.19), and as such, was not worth persuing.

When used with cDNA synthesized with random primers from barley sugar beet and red beet tissues, the F1/R1 primer pair generated several products with a range of sizes. However, the red beet cell culture sample generated two bands (*bv160* & *bv163*) of the expected size (150-300 bp) and these were chosen for further study. Genomic Southern analysis demonstrated clearly that *bv160* is an authentic *B. vulgaris* sequence (Fig. 4.21) which is expressed primarily in leaf tissue of several plants, although its expression

appears to be down regulated in response to high NaCl; curiously, no expression was detected in samples from red beet cells, the source of the fragment.

Both *bv160* and *bv163* were sequenced and found to be >95% identical at the amino acid level; two reading frames were feasible but BLASTP searches of the TREMBL and SWISSPROT databases using both translations failed to identify any proteins with significant homology (Fig. 4.25 & 4.26). Careful consideration of the peptide sequence between the conserved GKEGPxxH and GVLFSI motifs shows no consensus between the 17 CLC homologues presented in Fig. 1.2, apart from a conserved 8H and semi-conserved 16L 17L. Despite the fact that salt appears to down-regulate *bv160* expression, the presence of 9H and two I residues at positions 17 and 18 was encouraging. Although tenuous, it was decided to proceed, and use the *bv160* sequence to identify a full-length clone from *B. vulgaris*.

CHAPTER FIVE ISOLATION & CHARACTERIZATION OF GENOMIC CLONES.

5.1 Introduction.

Identification of the fragments *bv160* and *bv163* by RT-PCR from high-salt grown red beet suspension cells provided several strategies for identifying full-length clones of putative Cl^- channel genes. These strategies include cDNA library screening using *bv160* as probe, genomic library screening using *bv160* as probe, and a PCR approach using *bv160* sequences as primers for identifying 5' and 3' ends, *i.e.* RACE (rapid amplification of cDNA ends).

Due to time constraints on the duration of this project, it was decided to use a genomic library screening strategy as considerable experience had already been gained with this approach (see Section 4.22), and a good quality sugar beet genomic library was already available in the host laboratory. In contrast, no experience had been gained with RACE, and the available sugar beet cDNA library was prepared from root tissues (*bv160*) is not highly expressed in roots, see Fig. 4.17, Section 4.22).

5.2 Screening the Sugar Beet Genomic Library with *bv160* as Probe.

It was felt that the similarity between the two *B. vulgaris* varieties (sugar beet and red beet), and their similar Southern analysis patterns using *bv160* as probe (see Fig. 4.21, Section 4.22), warranted the use of the red beet *bv160* fragment as a probe for screening the existing sugar beet λ-DASH II genomic library.

5.2.1 Direct Sequencing from the Genomic Library using PCR.

As the sequence of *bv160* was known, it is possible to obtain 5' and 3' sequence information flanking *bv160* using the PCR. The rationale of this approach is that in

some λ-DASH II clones the *bv160* sequence may lie close to the vector's T3 or T7 site that flank the 20 kb insert. Therefore, if suitable forward and reverse primer regions can be identified in *bv160* (*i.e.* internal sites), it may be possible to use a combination of these along with T3 or T7 primers to generate PCR products that can be used in sequencing reactions.

The PRIME program from GCG 7 was used to identify suitable primer sites in *bv160* (see Fig. 5.1) and forward (F) and reverse (R) oligonucleotides synthesised. Four PCR reactions were run using the following primer pair combinations: F + T3; F + T7; R + T3; R + T7. These combinations should allow PCR products to form, regardless of insert orientation, as long as the *bv160* internal primer sequence is < 3 kb from either one or the other end of the 20 kb insert.

Many attempts were made to optimize these reactions and Figure 5.2 presents the results from one of these experiments. Surprisingly, when the internal forward primer (F) was used with either the T3 (lane 1) or T7 (lane 2) primer, an identical pattern of fragments was obtained, with a major product observed at approximately 1.5 kb. When the internal reverse primer (R) was used either with the T3 (lane 3) or T7 (lane 4) primer, 1kb fragment was obtained, here two major products of less than 300 bp were generated.

Further PCR experiments were conducted where each of the four primers were used on their own. These confirmed that each of the *bv160* internal primers were capable of generating the above mentioned products on their own (data not presented). These findings suggest that within 1.5 kb of the *bv160* complementary sequences there are several inverted sequences that are homologous to the *bv160* internal primer sites (F & R). The possibility that these are identical inverted repeats can not be ruled out.

Alternatively, it is conceivable that the *bv160* sequence does not lie within 3 kb of the end of any of the 20 kb inserts in the λ-DASH II library. Therefore, the PCR products shown in Fig. 5.2 might have arisen from other loci in the sugar beet genome where homologous inverted sequences are found (Fig. 5.2).

The results from these experiments were disappointing, and therefore it was decided to proceed with a different approach, conventional genomic library screening using *bv160* as probe.

Figure 5.1. Sequence *bv160* Showing Internal Primer Sites.

The *bv160* sequence was analysed by the PRIME programme from GCG 7. The best primer sequences identified by PRIME (shown in bold / underline) in the forward and reverse direction were synthesisied and these oligonucleotides used in PCR experiments with the λ-DASH sugar beet library to generate 5' and 3' sequences that flank *bv160* .

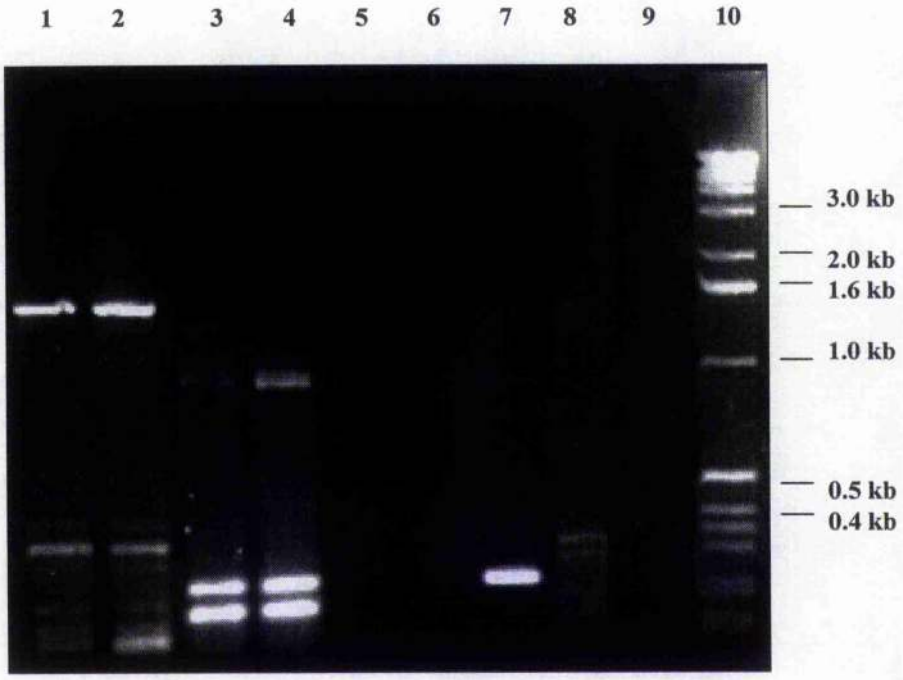


Figure 5.2. PCR Amlification of Sugar beet λ- DNA Clone Ends using *bv160* Sequences.

An internal sequence in the red beet *bv160* fragment was identified by GCG 7's PRIME programe as suitable PCR primer site. Forward and reverse primers were synthesised (see Fig. 5.1) as internal primer site. Each internal primer was then used in combination with primers compatible with either T3 or T7 sequence which flank the vector multiple cloning site. *Pfu* DNA polymerase was used in the PCR reaction during 35 thermal cycles (54°C, 72°C and 94°C). The PCR products were analysed by a 1 % (w/v) agarose gel electrophoresis, stained with ethidium bromide and visualised under the UV light. The PCR products were loaded on the gel as follows: forward and T3 primer (lane 1): forward and T7 primer (lane 2): reverse and T3 primer (lane 3): reverse and T7 primer (lane 4). Five controls were set for the PCR reaction (lanes 5-9). These were complete reaction with the followin primersg: lane 5, T3 primer alone: lane 6, T7 primer alone: lane 7, reverse primer alone: lane 8, forward primer alone: lane 9 no primer addition. Lane 10, 1 kb ladder molecular weight markers.

5.2.2 Conventional λ-Library Screening Using *bv160* as Probe.

Fresh phage was prepared from the sugar beet λ-DASH II library and about 15,000 plaques generated on each of 20 150 mm diameter plates. The plaques were then transferred onto duplicate nylon membrane filters (*i.e.* duplicate plaque lifts, see Section 2.13.3). ^{32}P-labelled *bv160* probe was prepared using the Amersham RediPrime kit and this was then used to screen the filters at low stringency (1 X SSC, 55°C). Therefore, a total of about 300,000 clones were screened which corresponds to a probability of p = 0.82 (see Section 3.3.2) that at least one of the clones contains the *bv160* complementary sequence. However, no positives were identified from this screen. Controls were run with all hybridization reactions which included dilutions of target sequence (*i.e. bv160*) spotted directly onto strips of test nylon membrane which were hybridized and washed in parallel with the plaque filters. From these controls, it was estimated that *bv160* could detect target sequence down to a limit of 1-2 pg which corresponds to about 30 10^{-18} mol (1 10^{-12} g / [660 g x 160 bp]), which is comparable to the manufacturer's stated sensitivity. However, each plaque is estimated to contain ~10^6 phage particles (Sambrook *et al.*, 1989), and therefore, a positive plaque containing an insert with the *bv160* sequence would contain only about 175 fg (~ 1.7 10^{-18} mol) of target sequence, well below the experimentally determined detection limit.

It was decided to try to improve the sensitivity of the hybridization using asymmetric PCR to generate the *bv160* probe. The rationale here is to make the probe using either an excess of either the forward (F) or reverse (R) primer in F /R primed PCR reactions using *bv160* as template. This approach should generate an excess of single-stranded probe that can only hybridize to target sequence on the plaque filters (see Section 2.15). However, despite exhaustive attempts, tests showed that only modest improvements in sensitivity were achieved (~ 0.5 pg detection limit). Further, generation of probe using just the forward or reverse primer and either the Klenow fragment or *Pfu* as the polymerase (as opposed to *Taq*) did not give a significant improvement in the sensitivity of hybridization (data not presented). The reasons for this failure are not clear.

Consequently, an alternative approach was used to improve the sensitivity of the *bv160* / target DNA hybridizations. It had been observed previously that the 1.6 kb *Torpedo*

marmorata clc-0 sequence did not hybridize to sugar beet genomic DNA when washed at high stringency (0.1 x SSC, 65°C; see Section 3.2). Therefore, it was decided to ligate *bv160* to *clc-0* to give a 1.8 kb chimera and to use this as a probe. After high stringency washing, only the *bv160* part of the chimera should bind to authentic *bv160* target sequence on the filter, but the increased label incorporated into non-hybridizing the *clc-0* fragment should increase signal strength. Preliminary tests with dilutions of the *bv160* sequence spotted onto nylon membrane suggested that the *bv160 / clc-0* probe could detect target DNA down to below 100 fg.

A second library screen was performed using the *bv160 / clc-0* chimera as probe. Duplicate plaque lifts were made from 25 150 mm diameter plates, each containing approximately 15,000 plaques. These filters were then hybridized with probe and washed at high stringency (0.1x SSC, 65°C), and the filters were then autoradiographed (see Section 2.11). Thirteen putative positive plaques were identified, and five were selected for further analysis. Each of the 5 'positives' was plated out at lower density (*ca.* 200 plaques / 65 mm diameter plate), and duplicate filter lifts taken. The filters were subsequently baked to fix the DNA onto the membrane, and then hybridized with the *bv160 / clc-0* chimera probe, washed at high stringency and autoradiographed, as described above. A third (and where necessary a fourth) screen was performed on these plaques to ensure that each contained only one clone. Of the original 5 'positive' plaques selected, Southern blotting suggested that only two were true positives, and hereafter these are referred to as *bvFAS1* and *bvFAS2*.

5.2.3 Analysis of the λ-DASH *bvFAS1* and *bvFAS2* Genomic Clones.

The genomic clones *bvFAS1* and *bvFAS2* were digested with a range of restriction enzymes and then subjected to Southern analysis using *bv160 / clc-0* as probe. Figure 5.3 presents the resulting ethidium bromide stained fluorescent agarose gel (A) and the corresponding Southern blot (B) of *bvFAS1* and *bvFAS2*. The difference in the resulting pattern in the Southern analysis (Fig. 5.3 B) suggests that *bvFAS1* (lanes 1-6) and *bvFAS2* (lanes 7-12) represent different genomic clones. Restriction enzyme digests with *XbaI* (lanes 1 and 7, ~8 kb), *EcoRI* (lanes 2 and 8, ~ 4 kb) and *BamHI* (lanes 3 and 9, ~ 9 kb), produce fragments that hybridize strongly to *bv160 / clc-0*. However,

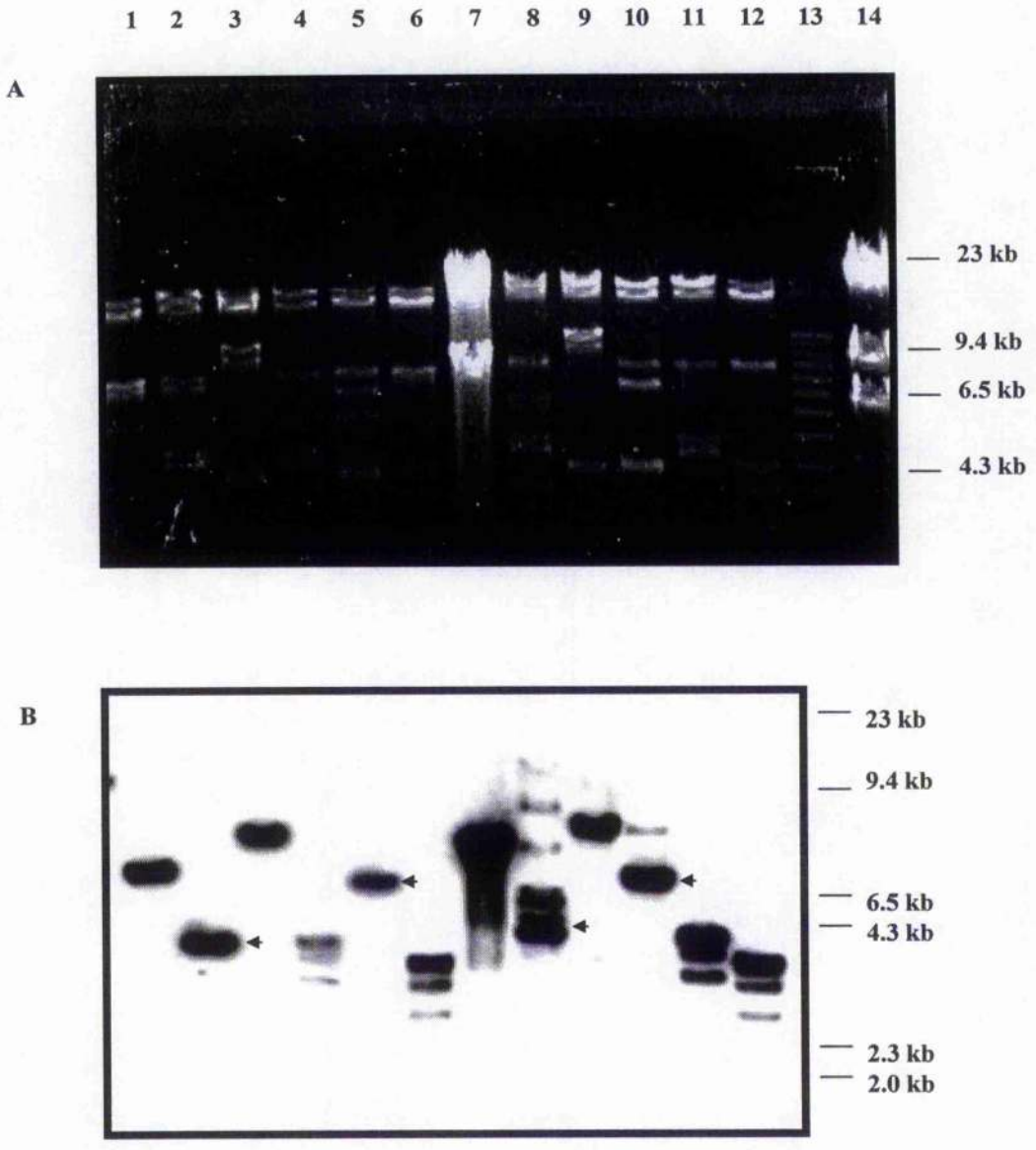


Figure 5.3. Southern Analysis of Sugar beet λ-DNA Clones *bvFAS1* and *bvFAS2*.

A. DNA was prepared from isolated λ-DNA Phage (*bvFAS1* lane 1-6 and *bvFAS2* lane 7-12) and digested with *Xba1* (lane 1 and 7), *EcoR1* (lane 2 and 8) and *BamH1* (lane 3 and 9). Double digests were also performed: *Xba1/EcoR1* (lane 4 and 11), *Xba1/BamH1*(lane 5 and 10) and *EcoR1/Bam*H1*:* (lane 6 and 12). Two µg of each sample was separated in a 0.5 % (w/v) agarose gel, stained with ethidium bromide and visualized under the UV light. One kb ladder (lane 13) and λ *HindIII* markers (lane 14) are shown.

B. An autoradiograph of the Southern blot of the same gel (A) hybridized with *bv160/clc-0*. The membrane was washed at high stringency (0.1 X SSC at 65°C) and autoradiographed at -80°C overnight.

due to their similarity in size to the 9 kb vector arm, the *Xba1* and *BamH1* fragments were considered unsuitable for sub-cloning. Double digests of *bvFAS1 and bvFAS2* suggested that the *Xba1 / BamH1* combination produced a strongly hybridizing fragment of ~7 kb (lanes 5 and 10), that could easily be separated from the vector arms (9 and 20 kb). Therefore, *bvFAS1* and *bvFAS2* were subsequently digested with either *EcoR1* alone, or with *Xba1* and *BamH1* in combination, to generate fragments of ~4 kb and ~7 kb respectively, and fragments resolved in a 1% (w/v) agarose gel. The resulting bands were excised from the gel, purified (Section 2.15.2) and then sub-cloned into pBluescript II SK (+/-) ™ vector previously digested with the appropriate enzymes (Section 2.2.15.5). Host cells (DH5α) were transformed with the ligated vector (Sections 2.2.3.2.1) and plated out. Positive clones were identified by antibiotic resistance and β-galactosidase (blue / white colour) selection (Section 2.2.3.2.1), and subsequently confirmed by colony PCR (Section 2.2.15.6) and Southern analysis using the *bv160 / clc-0* chimera as probe. The resulting sub-clones are hereafter referred to as *bvFAS1.7* and *bvFAS2.7* (~7 kb fragment inserts), and *bvFAS1.4* and *bvFAS2.4* (~4 kb fragment inserts).

The Southern analysis of these four sub-clones shows that *bv160 / clc-0* hybridized strongly to the *bvFAS1.7* and *bvFAS2.7* (Fig. 5.4 A, lanes 1 & 2). Only weak hybridization was found between *bvFAS1.4* and the *bv160 / clc-0* chimera probe (Fig. 5.4 A, lane 3), but *bvFAS2.4* did not hybridize at all (Fig. 5.4 A, lane 4). Although the Southern blot shown in Fig. 5.4 A was washed at high stringency (0.1 X SSC, 65°C), some hybridization at 3 kb was observed which presumably is vector sequence.

Therefore, to confirm the specificity of the above hybridizations to the target *bv160* sequence, the label was washed off the filter shown in Fig. 5.4 A (Section 2.2.12.5), and then re-hybridized with *bv160* as probe. This filter was subsequently washed at high stringency (0.1 x SSC, 65°C) and autoradiographed. Strong signals were obtained that confirmed the presence of *bv160* sequences in the *bvFAS1.7* and *bvFAS2.7* sub-clones (Fig. 5.4 B, lanes 1 & 2). However, again, only a weak signal at ~4 kb was observed in *bvFAS1.4* (Fig. 5.4 B, lane 3), and no signal was observed in *bvFAS2.4* (lane 4). The presence of the weak signals observed at ~ 3 kb (assumed above to be vector) were greatly reduced.

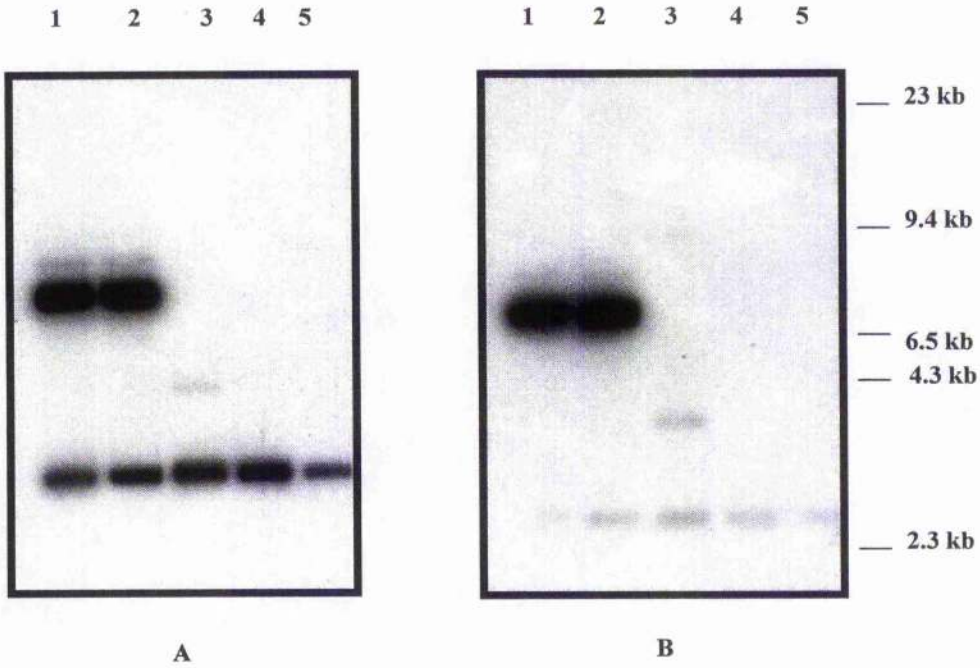


Figure 5.4. Southern Analysis of Sub-clones *bvFAS1* and *FAS2*.

The *EcoR1* fragment (4 kb) and *Xba1/BamH1* fragment (7 kb) were subcloned into pBlue-Script SK (+/-) and transformed to *E. coli.* The cloned plasmid were isolated and digested with *Xba1/BamH1*(lane 1 and 2) to produce 7 kb fragment and with *EcoR1*(lane 3 and 4) to produce 4 kb fragment. The digested DNA (25 ng) was separated in a 1 % (w/v) agarose gel, and blotted onto nylon membrane. Blot A was hybridized with *bv160/clc-0* and blot B with *bv160*. In both cases, high stringency washing was performed (0.1 X SSC at 65°C

From the Southern analysis of these sub-clones it is concluded that *bvFAS1.7* and *bvFAS2.7* probably represent identical 7 kb fragments of the sugar beet genome. Therefore it was decided to sequence the entire *bvFAS1.7* sub-clone in an attempt to identify the corresponding full-length gene which strongly hybridized to *bv160.*

5.3 Sequence Analysis of the Sugar Beet Sub-Clone *bvFAS1.7.*

Due to time constraints on the duration of this project, it was decided not to attempt further sub-cloning of *bvFAS1.7* but to use a 'primer walking' sequencing strategy to characterize the putative Cl^- channel gene. The rationale of this method is to generate 600-800 bp fragments for sequencing by extending inwards from the insert ends using the vector's T3 (5') and T7 (3') primer sites. After sequencing each of the resulting 600 – 800 bp products, new primers can be designed that 'walk' further into the middle of the insert; the process is repeated until the 5' and 3' 'walks' overlap in the middle of the insert. Although it was feasible to perform this at Glasgow University, time constraints on the duration of this study necessitated the work was undertaken commercially. Therefore, purified vector (p*bvFAS1.7*) was dispatched to MWG-Biotech (Ebersberg, Germany) for sequencing by primer walking.

The full sequence of *bvFAS1.7* is presented in Fig. A1 (see Appendix) along with the contig map of the overlapping fragments. The insert was 7751 bp in length and required overlapping fragments to obtain the full sequence.

5.3.1 Sequence Analysis of *bvFAS1.7.*

The *bvFAS1.7* clone was first analysed to establish where the *bv160* complementary sequences were located. This region was found to be between position 2431 and 2587 (see Fig. A1, see Appendix). Absolute matches between the complementary *bv160* and *bvFAS1.7* sequences are shown in bold type, discrepancies are shown in normal face type. As might be expected, the ends of *bv160,* which are determined by the sequence of the degenerate primers used to amplify the fragment (underlined in Fig. A1, see Appendix), are not exact matches of the corresponding *bvFAS1.7* sequence, although there is a near-perfect match between these two primer regions. The few inconsistencies that are apparent between the primer regions may be due to either *bv160*

or *bvFAS1.7* sequencing errors; however, careful consideration of the three original sequencing electrophoretograms (forward and reverse strand of *bv160* and forward strand of *bvFAS1.7*) did not resolve these discrepancies (data not presented). Alternatively, the discrepancies may be due to genuine varietal differences between *bv160* (red beet clone) and *bvFAS1.7* (sugar beet clone).

The entire *bvFAS1.7* genomic clone was analysed further using the NIX series of computer programmes available at the MRC's Human Genome Mapping Project site (Section 2.2.17). The first analysis performed was a scan of the entire sequence by the REPEATMASKER programme which looks for, and then masks from subsequent analysis, any repetitive sequences. However, no repetitive sequences were found in *bvFAS1.7*. Next, programmes were run to identify any known vector (BLAST/VECTOR), tRNA (t-RNASCAN-SE), or *E. coli* (BLAST/ECOLI) sequences that might occur in *bvFAS1.7*; again, none was found.

The remaining analysis can be considered to have four component parts, *viz.* looking for putative promoter sites (CATT and TATA boxes), looking for putative 3' polyadenylation signals, looking for intron/exon borders to establish ORFs, and finally performing BLAST searches in the databases. There are several programmes available at the HGMP site that can perform each of the four tasks, but the rationale behind NIX is to provide facilities for finding consensus between the different programmes. Therefore, several programmes were run to analyse both the (+) and (-) strand of *bvFAS1.7* for each features (promoter sites, poly A sites and ORFs) so that where consensus was reached, some measure of confidence can be attributed.

5.3.2 Analysis of *bvFAS1.7* for Promoter Sites.

Four programmes were run to search for putative promoter sites, FGENE's 'Promoter', GENESCAN's 'Promoter', TSSW's 'Promotor' and GRAIL's 'PolII Promoter'. The putative promoter sites identified by each of these programmes are shown as green triangles in Fig. 5.5. Three putative promoter sites were identified on the (+) strand but no consensus was reached by these programmes, and therefore confidence in any of the three is not high. Four putative promoter sites were identified on the (-) strand, but only

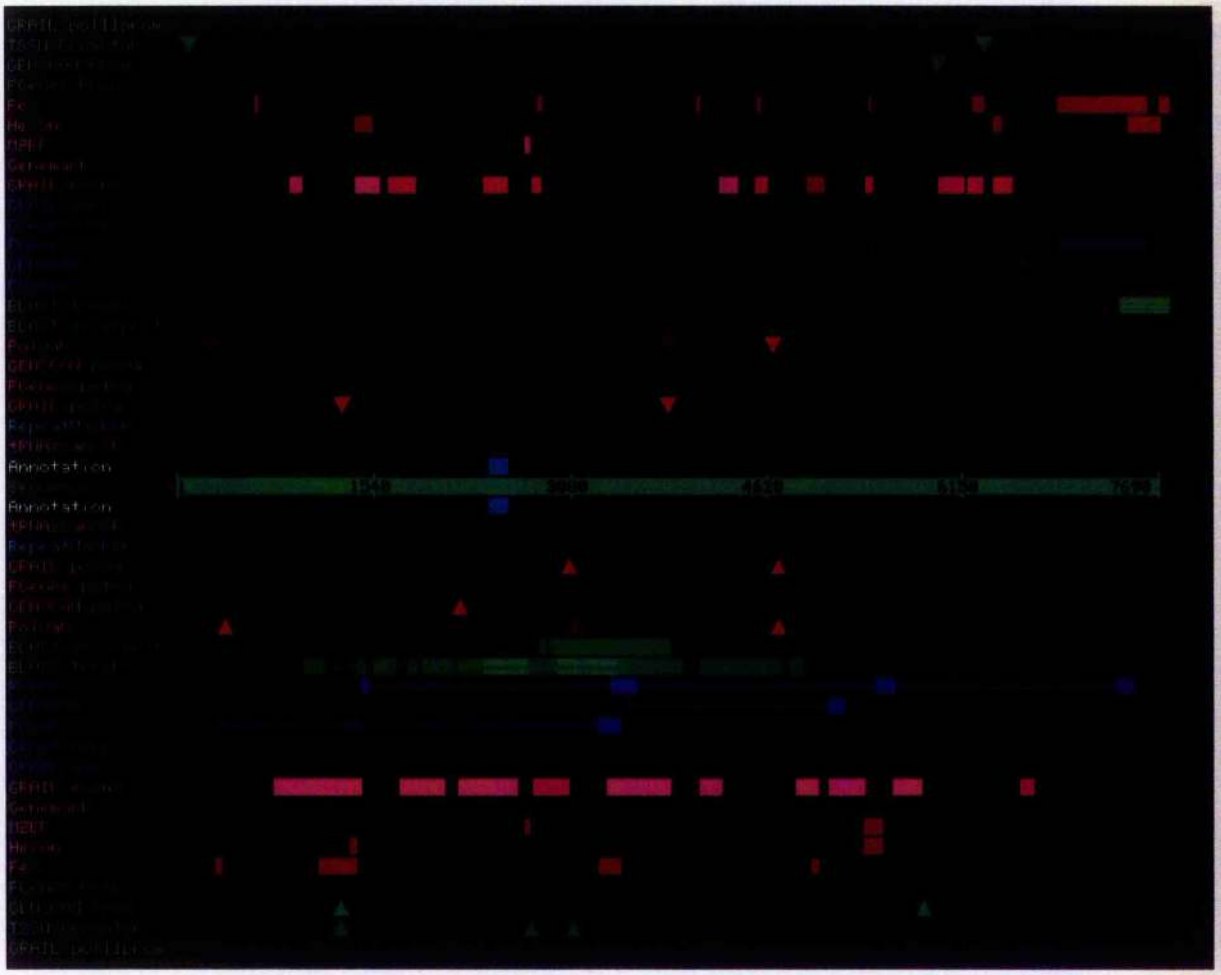


Figure 5.5. Graphical Output of the Computer Analysis of *bvFAS1.7*.
The nucleic acid sequence of the sugar beet genomic fragment *bvFAS1.7* was analysed by a series of programmes (NIX) at the MRC's HGMP site. The graphical output has been simplified to demonstrated only features of interest (see text for full details). Shown across the centre in green is the Sequence line, a representation of *bvFAS1.7* (base 1 to 7690). Features appearing above the Sequence line correspond to the (+) strand, and those below the (-) strand. The region of *bvFAS1.7* that hybridised to the probe *bv160* is shown as a blue box in the Annotation line. The following colour code is used to identify features: putative promoter sites, green triangles: putative poly A sites, red triangles: putative exons, purple/magenta/blue boxes: putative ORFs, green boxes. The more intense the shades of these colours, the stronger the prediction.

one was identified by more than one programme and so, again, confidence is not particularly high.

5.3.3 Analysis of *bvFAS1.7* for Poly A Signal Sites.

Four programmes were used to identify putative poly A sites on the (+) and (-) strand of *bvFAS1.7*. These were GRAIL's 'PolyA', FGENE's 'PolyA', GENESCAN's 'PolyA' and POLYAH. Possible poly A signal sites are shown as red or orange triangles. Four putative sites were identified on the (+) strand but only one site (at 3800) was identified by more than one programme (Fig. 5.5). Four sites were also identified on the (-) strand and two of these (at position 3000 and 4700) were identified by both GRAIL's 'PolyA' and POLYAH (Fig. 5.5).

5.3.4 Analysis of *bvFAS1.7* for Exons.

Nine different programmes were run to establish putative intron / exon boarders. These were FGENE(S), GENESCAN, GENEFINDER, GRAIL's, 'GAP2', GRAIL's 'Exon', GENEMASK, M2EF, HEXON and FEX. Consideration of the analysis of the (+) strand shows a number of small exons (purple or blue bars), some of which are identified by several of the programmes. Consideration of the (-) strand also shows a number of somewhat longer putative ORFs, and in some regions consensus was reached by four of the nine programmes (Fig. 5.5).

5.3.5 Analysis of *bvFAS1.7* by BLAST2.

The entire *bvFAS1.7* sequence was used as a query sequence to search the EMBL, EST, SWISSPROT and TREMBL databases at the European Bioinformatics Institute (EBI) web site in Cambridge (Section 2.2.17). BLAST2 failed to find any strong similarities when either the (+) or (-) strand of *bvFAS1.7* was used to interrogate the EMBL or EST databases (E-value of $<1\ 10^{-50}$, score of < 50). Some good local matches were found

but generally these extended for only a few bases, and most of the 'hits' were to human sequences of unknown function (data not presented).

When the *bvFAS1.7* (+) strand was used by BLAST2 to interrogate the SWISSPROT database, no homologues were identified (Fig. 5.5). However, when the TREMBL database was interrogated, one region of low homology was identified (at position *ca.* 7600). The BLAST2 output from this search identified a retrotransposon GAG (A188) with an E-value of 7 10^{-11} (27% identical over a 120 amino acid region).

In contrast, when the *bvFAS1.7* (-) strand was used by BLAST2 to interrogate the SWISSPROT and TREMBL databases, some good homology was found (Fig. 5.5). Figure 5.6 presents the first few lines of the corresponding output from the BLAST2 *bvFAS1.7* / TREMBL database search. Several plant sequences were identified with comparatively good E-values (>3 10^{-55} to 1 10^{-106}), and these are normally considered to be good matches. However, what is disappointing is that none is a putative chloride channel. Most are matches to putative plant reverse transcriptases although single matches to a protein of unassigned function (F21J9.2, from *Arabidopsis*) and to a putative zinc finger protein was found (O22260, between position 2745 and 3931, also from *Arabidopsis*). Several low score matches to *bvFAS1.7* (-) were found in the SWISSPROT database, which include a mouse retrovirus-related polyprotein (E-value 3 10^{-22}) and a human reverse transcriptase (E-value 5 10^{-17}), but these do not overlap with the expressed *bv160* sequence cloned by RT-PCR.

5.4 Discussion.

The identification of the *bv160* red beet fragment by RT-PCR was quite encouraging (see Chapter 4). The sequence was exactly the size predicted from a voltage gated Cl^- channel, and contained the conserved H and the semi-conserved (I/V/L) dyad motifs at at approximately the correct positions. Further, it is expressed in sugar beet leaves. This chapter describes the experiments that were conducted to isolate a full-length clone containing the *bv160* sequence.

For several reasons, it was decided to try to identify a full-length clone by screening a sugar beet λ-DASH II library that had been prepared in the host laboratory. Initial

A.

BLASTX 2.0.5 [May-5-1998]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query= BV_SAL_, 7767 bases, DCA37A26 checksum.
(7764 letters)

Database: trembl
228,493 sequences; 62,467,864 total letters

Searching.....done

Sequences producing significant alignments:	Score (bits)	E Value	N
1 TR:O22148 O22148 PUTATIVE REVERSE TRANSCRIPTASE.	176	e-106	9
2 TR:O48825 O48825 PUTATIVE TAL-1-LIKE REVERSE TRANSCRIPTASE ...	158	8e-83	10
3 TR:G976278 G976278 REVERSE TRANSCRIPTASE.	155	1e-82	9
4 TR:O04442 O04442 F21J9.2 (FRAGMENT).	149	2e-77	5
5 TR:O22220 O22220 PUTATIVE ZINC-FINGER PROTEIN.	153	1e-69	6
6 TR:G3377824 G3377824 T24H24.17 PROTEIN.	128	3e-55	6
7 TR:O23410 O23410 STRONG HOMOLOGY TO REVERSE TRANSCRIPTASE.	92	2e-53	6
8 TR:O64612 O64612 PUTATIVE REVERSE TRANSCRIPTASE.	92	1e-52	9

B.

BLASTX 2.0.5 [May-5-1998]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query= BV_SAL_, 7767 bases, DCA37A26 checksum.
(7764 letters)

Database: swissprot
77,143 sequences; 27,944,019 total letters

Searching.....done

Sequences producing significant alignments:	Score (bits)	E Value	N
1 SW:POL2_MOUSE P11369 RETROVIRUS-RELATED POL POLYPROTEIN (RE...	49	3e-22	6
2 SW:YTX2_XENLA P14381 TRANSPOSON TX1 HYPOTHETICAL 149 KD PRO...	53	7e-20	5
3 SW:LIN1_HUMAN P08547 LINE-1 REVERSE TRANSCRIPTASE HOMOLOG.	35	5e-17	5
4 SW:LIN1_MYCCO P08548 LINE-1 REVERSE TRANSCRIPTASE HOMOLOG.	40	1e-15	4
5 SW:YMH5_CAEEL P34472 HYPOTHETICAL 136.3 KD PROTEIN F58A4.5 ...	46	7e-08	3
6 SW:PO21_NASVI Q03278 RETROVIRUS-RELATED POL POLYPROTEIN (RE...	33	2e-05	4
7 SW:PO22_FOPJA Q03274 RETROVIRUS-RELATED POL POLYPROTEIN (RE...	32	3e-05	3
8 SW:POL2_HUMAN P12895 RETROVIRUS-RELATED POL POLYPROTEIN (PR...	29	3e-05	3
9 SW:Y2R2_DROME P16425 HYPOTHETICAL 115 KD PROTEIN IN TYPE I ...	28	0.001	4
10 SW:PO23_FOPJA Q05118 RETROVIRUS-RELATED POL POLYPROTEIN (RE...	35	0.003	2

Figure 5.6 BLAST2 Output of TREMBL and Swissprot Database Interrogation with *bvFAS1.7* (-) Strand Sequence.

The (-) sense strand nucleic acid sequence of *bvFAS1.7* was used to interrogate the TREMBL (A) and SWISSPROT (B) databases. Only the first few (most significant) lines from each output are presented.

attempts to clone by 'walking' through the 5' and 3' ends of the gene using a combination of internal *bv160* primers and vector T3 / T7 sites failed, presumably because the *bv160* sequence does not lie within ~ 3kb of either end of any of the cloned 20 kb inserts. This approach was also hampered by the presence of other repetitive / inverted sequences that allowed mispriming of both the forward and reverse internal primers (Fig. 5.2).

Consequently, a conventional λ-library screen was performed on the sugar beet genomic library using *bv160* as probe. This approach failed to identify any positive clones in a 3 . 10^5 clone screen. At this point it was realised that due to the small size of *bv160*, and the amount of purified phage present in a plaque, any true positives were below the limit of detection by this ^{32}P-labelled probe. Asymmetric PCR was then used to try to increase signal strength, but preliminary tests showed only a modest improvement in sensitivity was obtained, the hybridizations were still not sensitive enough to detect true positives. The reasons for this are unclear but may be attributable to the fact that the kinetics of re-association between small (*i.e.* 160 bp) + and - strand probe molecules is much slower than might be predicted, and therefore +/- strand re-annealing during hybridization is not a significant. If this is the case, *bv160* probe generated by asymmetric PCR (or riboprobes) would not be expected to significantly improve the detection limit in hybridizations to dsDNA.

It was decided to try to produce a chimera probe that consisted of the *bv160* sequence for targeting, ligated to a large non-hybridizing ^{32}P-labelled sequence that increases the signal strength. Studies in Chapter 3 suggested that the *Torpedo marmorata clc-0* sequence might be suitable. It is 1.6 kb in length and does not hybridize to sugar beet genomic DNA when washed at high stringency. The chimera probe was found to improve the limit of detection by at least an order of magnitude (~ 1 . 10^{-18} mol of the 160 bp target). A subsequent library screen using the *bv160 / clc-0* chimera as probe identified several putative 'positive' plaques, and after secondary and tertiary screens two clones (*bvFAS1* and *bvFAS2*) were identified. Two 7 kb sub-clones were prepared from these (*bvFAS1.7* and *bvFAS2.7*) which hybridized strongly to *bv160*, and one of these (*bvFAS1.7*) was sequenced using a 'primer-walk' strategy, and this sequence was then analysed for gene structures.

The *bvFAS1.7* fragment was 7751 bp long. Computer analyses of the (+) strand did not identify any strong candidate promoter regions; the only possible site was approximately 2500 bp 5' of the *bv160* sequence. A reasonably good poly A signal site was located at position *ca.* 3800 that might be located at the 3' untranslated end of the *bv160*-containing gene. BLAST2 did not identify any good homologues to the (+) strand when it was used to interrogate the EMBL or SwissProt databases. However, BLAST2 produced a low score match to a mouse retrotransposon sequence (A188) when the TREMBL database was interrogated but this was located at the far 3' end (+ strand) of the *bvFAS1.7* clone and did not overlap the *bv160* sequence (at position 2431 - 2587). A suit of programmes designed to identify exons produced little consensus, and they identified many small putative exon sequences. Presumably, if there are structural genes encoded on the *bvFAS1.7* (+) strand then (apart from the A188 protein) they contain many introns and have no homology to any known sequence.

In contrast, more putative structural features were identified on the *bvFAS1.7* (-) strand. Three possible promoter sites were identified 5' of the *bv160* sequence (at *ca.* 5800, 3100 and 2700, labelled according to the (+) strand), although, as no consensus was achieved by the four promoter identification programmes, little confidence can be attached to any of these sites. Two putative poly A signal sites were identified 3' of the *bv160* sequence on the *bvFAS1.7* (-) strand (at *ca.* 2000 and 3000, (+) strand labelled positions), but again, as no consensus was achieved between the four poly A site identification programmes, confidence in either is low. Several programmes were run to identify exons. The results from these (9) programmes was disappointing as no strong consensus was achieved particularly around the *bv160* sequence region (2431-25870, (+) strand labelled positions). Only one programme, GRAIL's 'Exon', predicted that the *bv160* sequence may form part of a transcript, and yet as *bv160* was isolated by RT-PCR, and hybridizes to transcripts in northern blots (Fig. 4.22), it clearly is expressed. BLAST2 interrogations of the EMBL database failed to identify any sequences with long stretches of homology to *bvFAS1.7* (-) although when the TREMBL and SwissProt databases were searched some good matches were reported. The output from the SwissProt search identified a list of unrelated proteins from a wide range of organisms that include mouse, bacteria and *C. elegans*. However, the region of the *bvFAS1.7* (-) strand that shows homology to SwissProt entries does not overlap with the *bv160* sequence. The BLAST2 output from the TREMBL database contained a long

list of putative plant (*Arabidopsis*) proteins, and the identified homology did span the *bv160* sequence in *bvFAS1.7* (see Fig. 5.5). Some sequences produced what are considered to be very good BLAST scores (>100; e-values of $< 1 . 10^{-50}$), but careful consideration of the alignments with *bvFAS1.7* (-) shows very few regions where exact matches are made over 5-10 amino acids. Instead, there appear to be many runs of 3-5 amino acids where there is good identity or similarity, interspersed with runs of seemingly unrelated sequence, *i.e.* there are good 'global' but poor 'local' sequence alignments. It could be argued proteins that perform identical or similar tasks in different plant families would have the opposite general structure. Regions of close homology reflecting conserved sites (catalytic sites, points of contact with other holoenzyme sub-units, co-factor binding sites, *etc.,*), would be interspersed with long regions where homology is poor (*i.e.* the non-conserved, protein 'scaffolding').

Taken on balance, the *bv160* sequence probably forms part of an expressed sugar beet reverse transcriptase although the uncertainties associated with the location of clear promoter and poly A sites, and with the definition of exon / intron boarders, does give cause for concern. What is certain is that there is no evidence that *bvFAS1.7* contains sequence that codes for a voltage-gated Cl^- channel. The region of *bvFAS1.7* that contains the *bv160* sequence does not appear to contain the conserved ClC GKEGPxxH or GVLFxxEE motifs used to design the degenerate PCR primers that resulted in cloning *bv160*.

CHAPTER SIX. GENERAL DISCUSSION.

Most crop species upon which we rely for food are glycophytes, which lack the mechanisms required to tolerate the water deficit and ion excesses prevailing in saline soil (Greenway and Munns, 1980). Therefore, it is of interest to understand how salt-resistant plants respond to external salinity. What is known is that a number of physiological processes are involved in salt-resistance and each of these processes involves the co-ordinate expression of a number of genes. To engineer crops that can produce high yields when exposed to saline conditions, we need to identify and characterize these genes. This project was focused upon identifying and characterizing genes involved in ion transport that may confer salt-resistance on crop plants.

One way of identifying a putative gene for H^+, Na^+ and Cl^- transporters from salt-resistant plants is to use an approach involving heterologous probes. Therefore, genomic Southern and Northern blot experiments were carried out on barley and sugar beet tissues using a range of probes from different sources. These probes were *adk-1, at57, sod2, nhaA* and *clc-0* (Table 3.1). The genomic Southern blots suggested sugar beet and barley have sequences with good homology to *adk-1,* a *Nicotiana plumbaginifolia* p-type H^+-ATPase; (Boutry *et al.,* 1989), and to *at57,* an *Arabidopsis* v-type H^+-ATPase B subunit (Manolson *et al.,* 1988). A fragment of *clc-0* (*Torpedo marmorata* voltage-gated Cl^- channel; Jentsch, *et al.,* 1990) was also found to have some homology to barley and sugar beet genomic DNA. Northern blot analysis suggested that *clc-0* homologues are expressed in barley; the message does not appear to be poly-adenylated, and has a size of 1.4 kb. These results from these experiments with *clc-0* were encouraging and it was decided to pursue this line of investigation.

Attempts to isolate a voltage-gated chloride channel from a barley genomic λ DASH II library by colony hybridization using *clc-0* as probe were unsuccessful. Approximately $3 .10^5$ out of an intended $1 .10^6$ clones (99.9%) were screened giving an *ca.* 82 % chance of detecting the *clc-0* homolog (Clark and Carbon, 1976). This failure may be due to the detection limit of this heterologous probe (for a single copy 1.4 kb gene,

which corresponds to ~1.5 pg of target per plaque, and this is probably below the limit of detection for a heterologous probe).

At this stage, Maurel and co-workers reported the cloning of the first plant voltage-gated chloride channel (Lurin *et al.,* 1996) by using an RT-PCR approach. However, uncertainties associated with the identity of the *clc-0* target sequence in barley, and in addition, with the success of Maurel and co-workers in cloning by RT-PCR *clc-nt1* from *N. tabaccum*, it was decided to follow a similar approach.

By May 1996, seventeen putative *clc* sequences were deposited in the databases, ten more than were available to Maurel and co-workers when they began their studies in December 1995. The corresponding protein translations of these sequences were subsequently aligned using the PILEUP programme from the GCG 7 computer package. Three regions of consensus were identified (Fig. 1.2) that were suitable for designing PCR primers. Consequently, two forward primers (F1 and F2), and one reverse (R1) primer were synthesized and used in two PCR approaches to identify and clone plant *clc* genes; these were conventional PCR and RT-PCR.

Initially, F1/R1 and F2/R1 primer pairs were used to generate PCR product from high-salt-grown barley and sugar beet cDNA libraries. The F2/R1 primer pair failed to amplify any fragments from either library. In contrast, the F1/R1 primer pair generated two fragments from the sugar beet library, *bv1* and *bv2,* and one fragment from barley library *hv1*. Southern analysis (Fig. 4.3, 4.6, and 4.9) suggested that these sequences were probably primed from the library host cell (*E. coli*) template. BLAST homology searches suggested that none of the three fragments have homology to the *clc* family. Clearly there appears to be a sufficient amount of *E. coli* (host cell) DNA in the barley and sugar beet libraries to allow priming with F1 and R1. These findings provide a strong lesson, wherever possible, use freshly prepared DNA as PCR template, and avoid the use of genomic or cDNA libraries.

The F2/R1 primer pair failed to generate any PCR product when red beet genomic DNA was used as template. However, two fragments, *bv3* and *bv4,* were generated using the F1/R1 primer pair. Although *bv3* and *bv4* were generated from a sterile cell culture of

red beet, BLAST searches using the corresponding *bv3* and *bv4* sequences suggested that bacterial fragments had been generated. Therefore, it was decided not to continue with this approach but to use RT-PCR.

RT-PCR was performed on leaves and root tissue of high-salt-grown sugar beet and barley, and on red beet cells in suspension cultures. Both oligo dT and random primer first strand cDNA synthesis methods were used on the above tissues. No PCR products of the expected size (150-300 bp) were generated from any of the samples when the F1/R1 primer pair was used with oligo dT-synthesized cDNA. In contrast, a single fragment of the expected *bv600* (600 bp) was produced when F2/R1 primer pair was used with this template. Genomic Southern and Northern blot analysis suggested that *bv600* is an authentic *B. vulgaris* sequence that is expressed primarily in the leaf tissues of sugar beet (message size approximately1.4 kb). However, BLAST searches of EMBL database indicated that *bv600* encodes an ATP-dependent protease and has no discernable homology to the *clc* family of voltage-gated chloride channels. At this point, upon consideration of the above results, no further work on *bv600* was undertaken.

The random primer method was used to generate cDNA from barley (roots and leaves), sugar beet (root and leaves) and red beet cell suspension cultures. PCR amplification using the F1/R1 primer pair generated several fragments, but only two (*bv160* and *bv163*) from the red beet cell culture cDNA produced fragments of the expected size. Genomic Southern analysis revealed that *bv160* is an authentic *B. vulgaris* sequence (Fig. 4.21) which is expressed in leaf tissue of several plants, although its expression appears to be higher in low-salt-treated plants. No expression of the *bv160*-like sequences was found in red beet cells; this result was unexpected as red beet cells were the original source of the fragment.

The *bv160* and *bv163* fragments were found to have >95 % sequence identity at the amino acid level. The BLAST searches of the TREMBL and SWISSPROT databases failed to identify any proteins with significant homology to *bv160* or *bv163* (Fig. 4.25 and 4.26). Consideration of the peptide sequence between the two conserved (primer)

motifs GKEGPxxH and GVLFSI, shows little consensus between the 17 *clc* homologues (Fig. 1.2) although *bv160* contains the conserved H and semi-conserved (L/I/V) dyad amino acids motifs at the correct positions (see Fig. 1.2). Northern analysis suggested that *bv160* is expressed mostly in leaf tissue but is suppressed by high external NaCl concentrations (Fig. 4.22A). On the basis of these findings, it was decided to proceed and isolate a full-length clone of the gene by screening a sugar beet genomic library using *bv160* as a probe.

Two methods were used for screening the sugar beet genomic library. The first was a PCR 'primer walking' method. Internal *bv160* primers (forward and reverse) were identified and oligonucleotides synthesized. Combinations of the internal primers and primers complementary to the vector's T3/T7 sites were used in an attempt to amplify the 5' and 3' ends of the *bv160* gene using the PCR. However, this approach failed, presumably because the neither of the internal primer sequences lie within ~3 kb of either end of any of the cloned 20 kb inserts in the sugar beet λ DASH II library. Further, control PCR reactions showed that either of the internal primers was capable of generating products on their own without the T3 or T7 complementary primers. These findings suggest that the full-length *bv160* gene may contain repetitive/invert sequences (Fig. 5.2), and so a conventional library screen was used.

A conventional colony screening method failed to identify any positive clones in the sugar beet λ DASH II genomic library when *bv160* was used as a probe. Control experiments with *bv160* and calculations of target abundance in plaques suggested that with this probe, authentic 'positives' could not be detected as their signal would be below the limit of resolution. Asymmetric PCR was used to try to increase the sensitivity of the *bv160* probe but only modest improvements were found. There is no clear explanation for this observation, but for many short probes, this is not unusual (Dr Edi Cecchini, University of Glasgow, per. comm.). The sensitivity of the hybridization was improved by producing a chimera probe consisting of the target sequence (*bv160*) ligated to a sequence that did not when washed at high stringency (a 1.6 kb fragment of the *T. marmorata clc-0* gene). Using this probe, two positive clones *bvFAS1* And *bvFAS2* were identified. Sub-clones were made from these λ DASH II clones and one 7

kb fragment that hybridized strongly to the *bv160* chimera probe, *bvFAS1.7*, was sequenced.

Extensive sequence analysis proved to be disappointing. BLAST searches of the TREMBL and SWISSPROT data bases suggested that within the region of *bvFAS1.7* that contains the *bv160* sequence, some good global homology exists to several putative plant proteins (reverse transcriptases, zinc fingers, *etc.*,) although, for several reasons, confidence in these predictions is not high. Most worrying were the failure of four programmes designed to identify eukaryote promoter regions to settle on any consensus TATA or CATT box sites 5' of the *bv160* sequence (+ or – strand). Secondly, no concensus poly A initiation sites (AATAAA) were identified 3' of the *bv160* sequence by four programmes routinely used to locate the 3' end of eukaryote genes. These observations can be reconciled if the promoter and poly A sites of the corresponding gene lie outside of the *bvFAS1.7* sequence (*i.e.* the pre-mRNA is greater than 7 kb). However, perhaps more worrying still is the lack of consensus between the 9 programmes that were used to identify intron / exon boundaries, the 5' (C/A)AGGT(A/G)AG and the 3' $(T/C)_nN(C/T)AGG$ motifs. Very little consensus was found here, and therefore, without a clear indication of where the open reading frames are located, the validity of the BLAST searches of the TREMBL database should be treated with caution. There are currently over 27,000 putative reverse transcriptase sequences lodged in the GeneBank database, and yet the number of known retro-viruses falls far short of this number. It is also well established that the databases report some extremely tenuous associations between sequences, and therefore, function is often incorrectly assigned to groups of related proteins. For instance, a sequence has been identified in *Arabidopsis* which has been tagged as a putative Na^+ / H^+ antiporter. However, BLAST 2 and BLITZ interrogations of all of the databases with this sequences failed to identify any Na^+ / H^+ antiporters although many other homologous sequences are identified. Further, structural analysis of the putative protein suggests that it contains no membrane-spanning α-helices, and therefore, it is unlikely to be involved in membrane transport. Unfortunately, since it's putative function has been assigned, other sequences with homology to it (and it alone) have now also been tagged

as putative Na^+ / H^+ antiporters (Dr P Dominy, per. comm). Clearly, with so much sequence information appearing in the databases, any early errors of assigned function to a sequence will result in compounding errors as new homologous sequences are deposited.

What is now required is a confirmation of the *bv160* gene sequence. With hind-site, it would have been more prudent to have attempted to clone the full-length *bv160* gene from mRNA, and then the protein sequence would be known with certainty. It is recommended that such a strategy should be used if the identity of the *bv160* sequence is to be confirmed. However, as the expression of the *bv160* sequence appears to be mainly in the leaf, and suppressed by high NaCl concentrations, what ever it encodes, its is unlikely to play a significant role in Cl^- transport in salt-resistant plant cells.

In September 1998, a new search of the databases for putative chloride channels was undertaken. Over 80 putative chloride channels are now listed. One observation that had been noted was that PILEUP sometimes produced poor alignments for no apparent reason. Often, mis-aligned sequences can be spotted with the eye, and these almost invariably appeared in the sequences near the bottom of the output list and is presumably a reflection on the inability of PILEUP to cope well with more that seven or eight sequences. For this reason, the 17 *clc* sequences used in the original PILEUP alignments were analysed by CLUSTALW which also produces alignments. The output from this CLUSTALW analysis is presented in its entirety Fig. A2 (see Appendix). What is most noticeable from Fig. A2 is that although the three conserved motifs identified by PILEUP are aligned, several other regions of similarity / identity are also marked which PILEUP failed to note. For instance there is a GSGIPExxK motif (between position 200 and 220), and a PGxYAxxGAAA motif (between positions 570 and 585) that would make suitable PCR primers. Further experiments using degenerate oligonucleotides complementary to these sequences could be used in combination with the F1, F2 and R1 primers described in Chapter 4.

There are now six putative plant *clc* gene sequences deposited in the databases (September, 1998). Four of these have been identified from the *Arabidopsis* genome

sequencing programme, another has been identified from potato, and of course the tobacco *clc-nt1* sequence. A CLUSTALW alignment of these six putative plant gene products shows excellent homology between the species and this provides a good basis for carrying forward this project on cloning plant *clc* genes. Figure A3 (see Appendix) presents this alignment. It is recommended that a RT-PCR approach using this information should be used to identify Cl^- channels in salt-resistant plants.

The 17 putative *clc* sequences (encoded for chloride channels) that were used in the original PILEUP alignments, and the six putative plant *clc* gene sequences deposited in the databases, were aligned and analysed by CLUSTALW. The output of the CLUATALW analysis, and the corresponding phylogram, are presented in Fig. A4 and Fig. A5 respectivly (see Appendix). what is most noticeable from Fig. A4 is that the two conserved motifs (GKEGPxxH and GVLFxxE), which were used to design the forward (F1) and reverse (R1) primers, were again identified (between 260 and 320 positions), but these was not the only region of conservation. Unfortunately, the motif that was used for designing the degenerate forward (F2) primer is not found in the CLUSTALW alignment (Fig. A4). Several other regions of similarity with excellent homology are marked, for examples PxAxGSGxP (between position 200 and 220), PGxYAxxGAAA (between position 600 and 620) and PxGxFxP ((between position 540 and 560).

Figure A5 shows a phylogram of the six cloned plant putative *clc* sequences along with those from other phyla. It is clear that the plant sequences are closely related to each other and to the mammalian *clc*-7 class.

It is now likely that conceived regions identified in plant high lighted *clc* sequences by CLUSTAW, can be used to design PCR primers that will allow *clc* homologous to be cloned from salt-resistant plants.

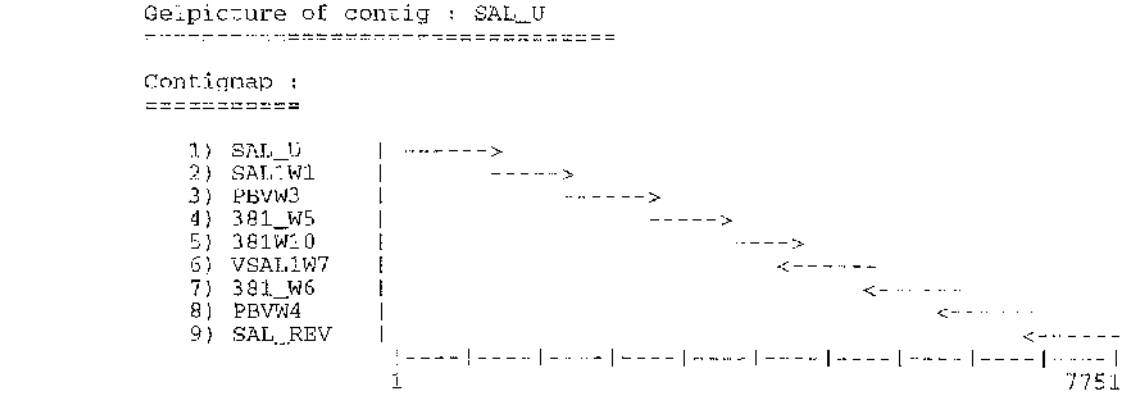
It is recommended that this information should now be used to move this project forward.

Figure A1. Sequence of Sugar Beet Genomic Clone *bvFAS1.7*

The sub-clone *bvFAS1.7* isolated from a sugar beet λ-DASH II genomic library was ligated into pBluescript SK (+/-) and sequenced using the 'primer walking' method (MWG Biotech). Nine contig. fragments were required to cover the full clone (7751bp). The region of homology to *bv160*, the probe used to isolate *bvFAS1.7* is shown (position 2430 to 2589); bases shown here in bold are identical to *bv160*. The underlined sequences correspond to the primer-binding regions.

Gelpicture of contig : SAL_U

Contigmap :



Contig statistics :

Contiglength	=	7751	
Total number of bases sequenced	=	9006	
Average coverage	=	1.2	
Number of Fragments	=	9	
Average reading length	=	1001 bases	
Number of ambiguities in consensus	=	1	(rate = 0.0%)
Double stranded sequence	=	231 bases	(rate = 3.0%)

Contig :

```
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   Consens        1 ----------|---------|---------|---------|---------|   50

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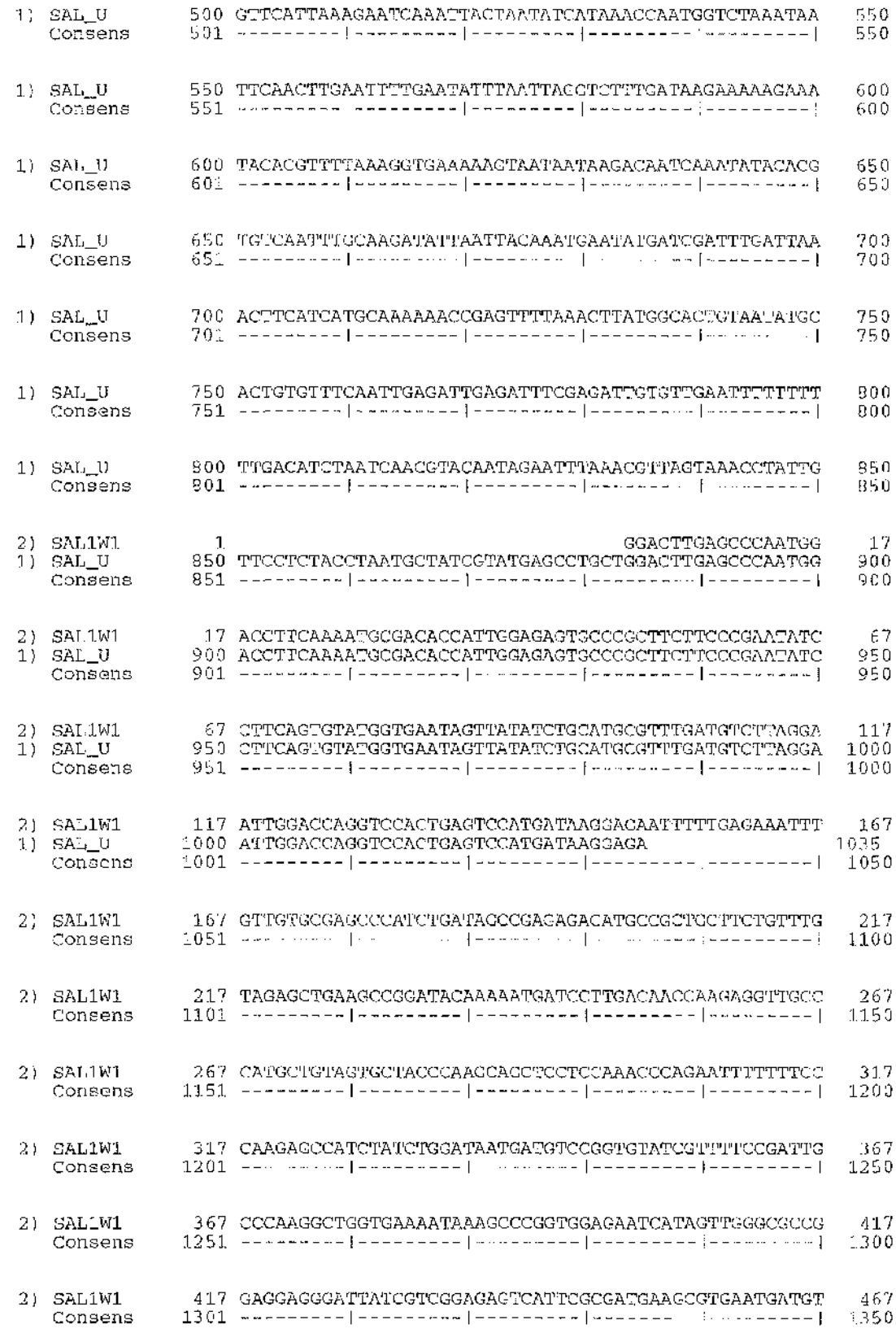

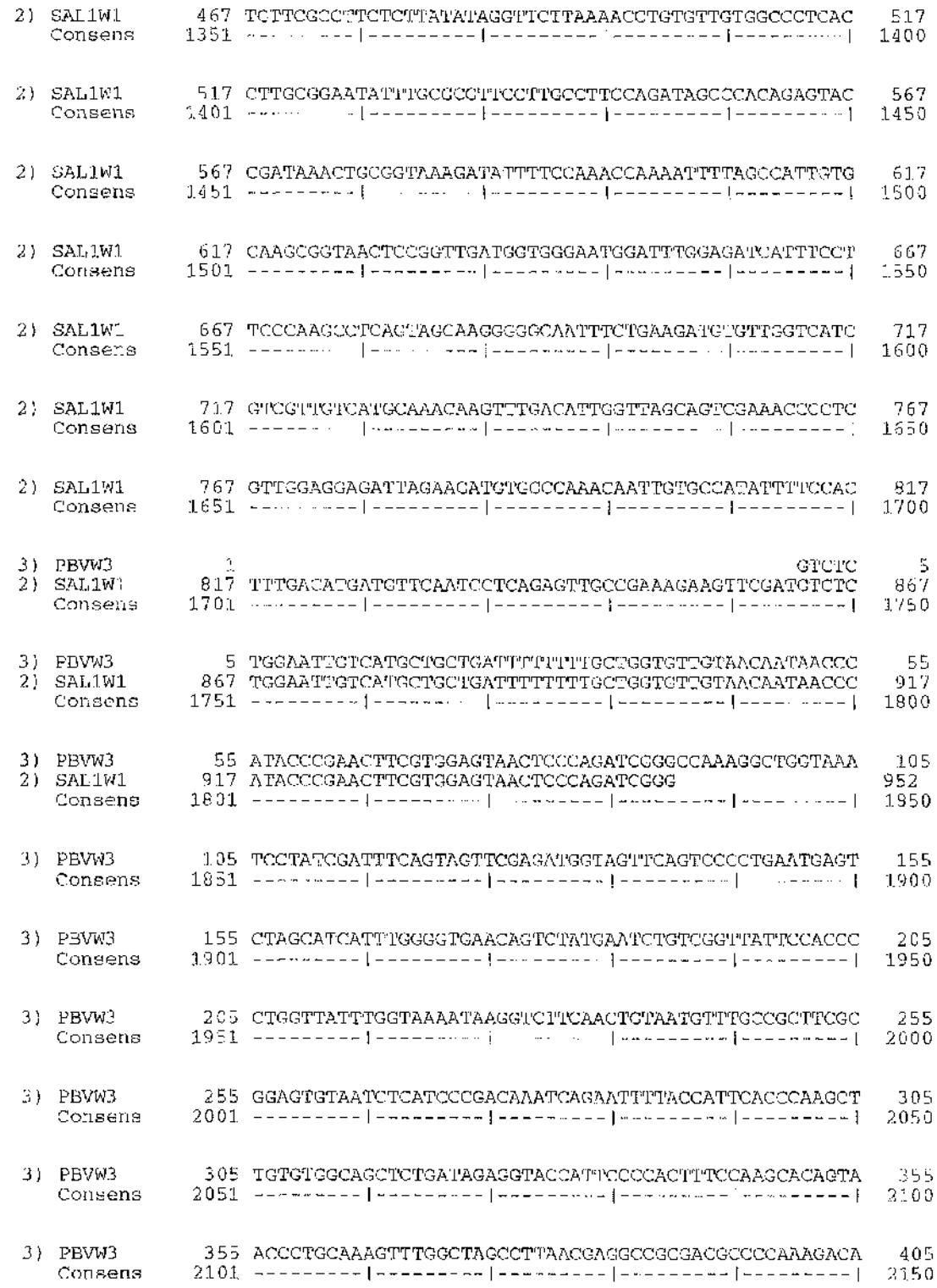
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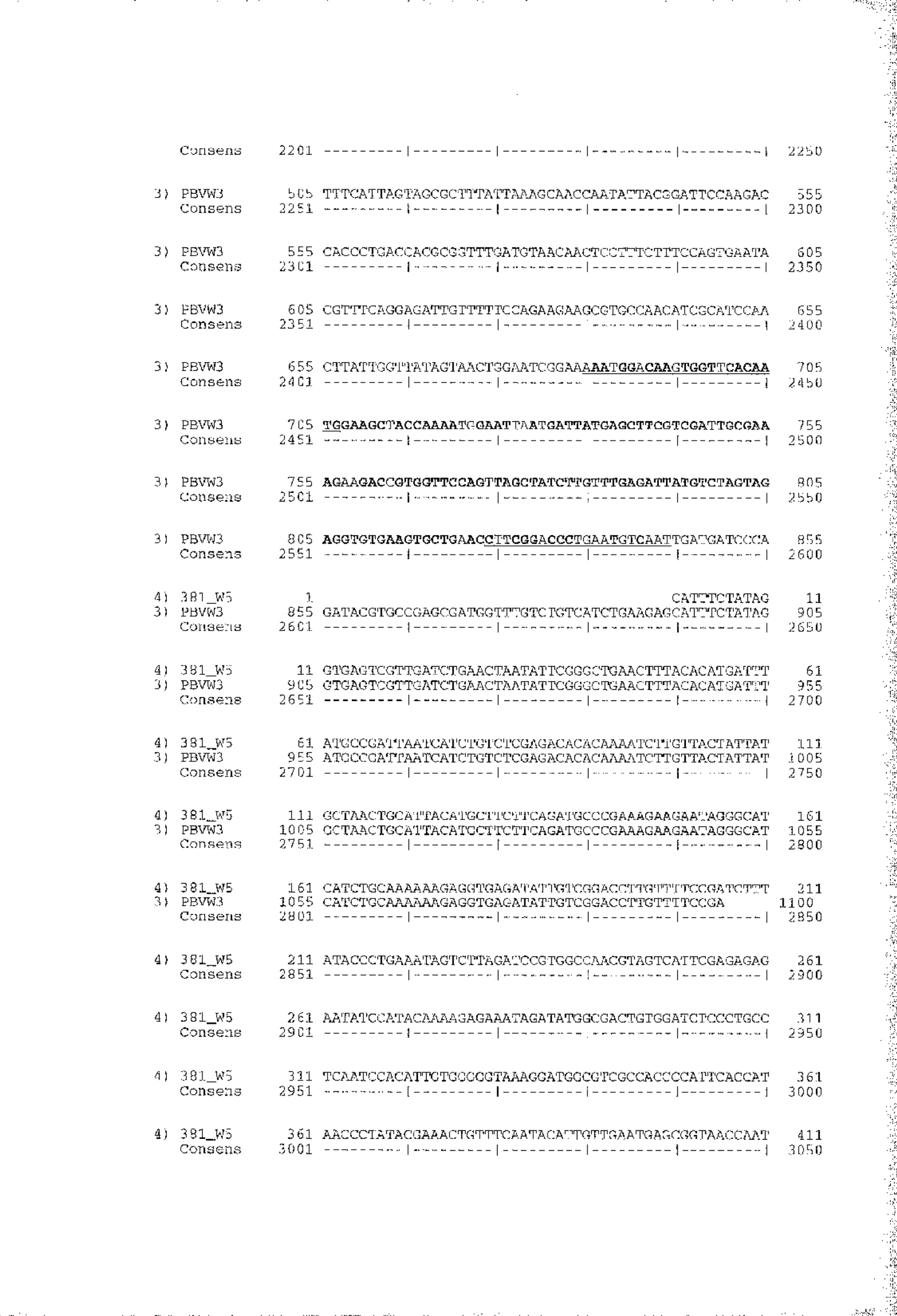
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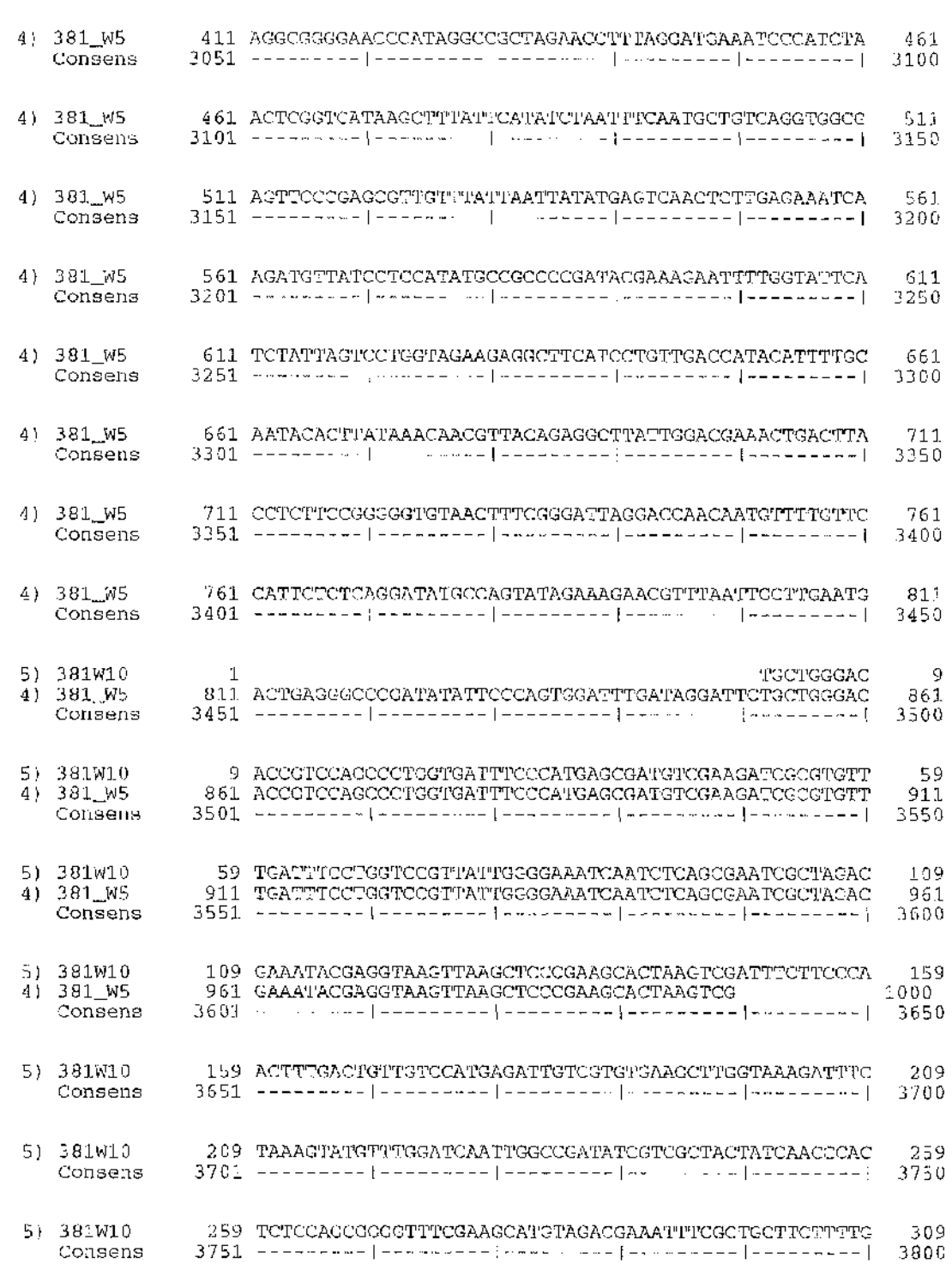
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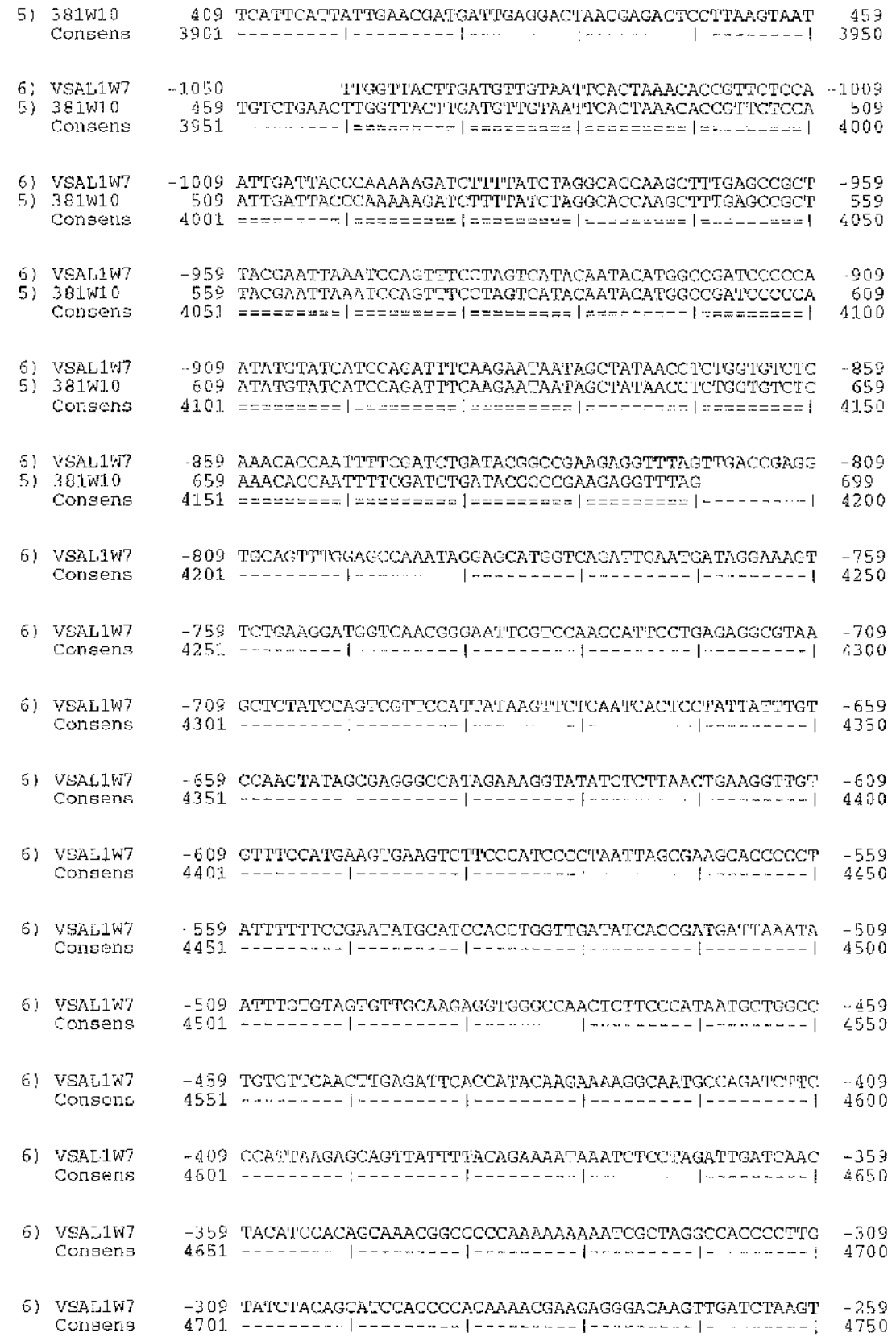
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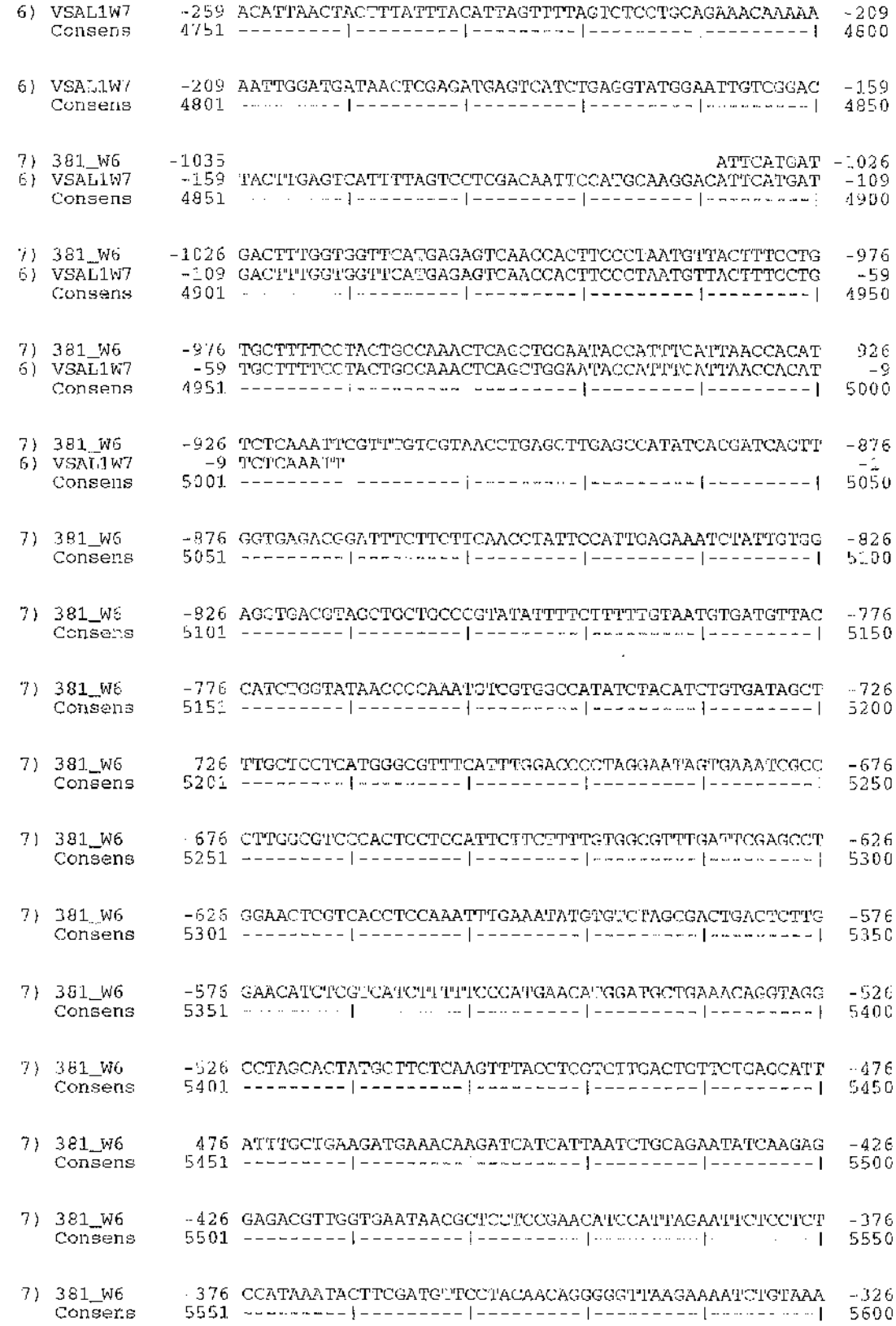
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Consens 5201 5250
7) 381_W6 -676 CTTGGCGTCCCACTCCTCCATTCTTCTTTTGTGGCGTTTGATTCGAGCCT -626
Consens 5251 5300
7) 381_W6 -626 GGAACTCGTCACCTCCAAATTTGAAATATGTGTCTAGCGACTGACTCTTG -576
Consens 5301 5350
7) 381_W6 -576 GAACATCTCGTCATCTTTTTCCCATGAACATGGATGCTGAAACAGGTAGG -526
Consens 5351 5400
7) 381_W6 -526 CCTAGCACTATGCTTCTCAAGTTTACCTCGTCTTGACTGTTCTCAGCATT -476
Consens 5401 5450
7) 381_W6 476 ATTTGCTGAAGATGAAACAAGATCATCATTAATCTGCAGAATATCAAGAG -426
Consens 5451 5500
7) 381_W6 -426 GAGACGTTGGTGAATAACGCTCCTCCGAACATCCATTAGAATTCTCCTCT -376
Consens 5501 5550
7) 381_W6 -376 CCATAAATACTTCGATGTTCCTACAACAGGGGGTTAAGAAAATCTGTAAA -326
Consens 5551 5600

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7)  381_W6      -326 GGGTGGAGGAGATATGCGAAATCGACTATTAATAATTTGCGAAATGTTAC  -276
    Consens     5601 ---------|---------|---------|---------|---------|  5650

7)  381_W6      -276 GAAGCTCATCTGGTTCGTTGCTGGAGTTAGAGAGAGGATGATTTTGTGGA  -226
    Consens     5651 ---------|---------|---------|---------|---------|  5700

7)  381_W6      -226 TGCAGTCTAATATCTACGTAACCATGCAAATCATTGAGGGCCTGCTCTTG  -176
    Consens     5701 ---------|---------|---------|---------|---------   5750

8)  PBVW4      -1060                                    TCTTGTGGCCCAGGAGGTA -1041
7)  381_W6      -176 CGCCCTATCAACCCAGTTACTCGGCCCAGCTTCTTGTGGCCCAGGAGGTA  -126
    Consens     5751 ---------|---------|---------|---------|---------|  5800

8)  PBVW4      -1041 CAATTGGCCGTCCTTGAGCTACCAGATTTTCAAGAGAGTTTCTTTGATCA  -991
7)  381_W6      -126 CAATTGGCCGTCCTTGAGCTACCAGATTTTCAAGAGAGTTTCTTTGATCA   -76
    Consens     5801 ---------|---------|---------|---------|---------|  5850

8)  PBVW4       -991 TAAGTTTGTTCTATGAATGGAGGGTTTGGGCTAGCTGGCCTAGTATGACC  -941
7)  381_W6       -76 TAAGTTTGTTCTATGAATGGAGGGTTTGGGCTAGCTGGCCTAGTATGACC   -26
    Consens     5851 ---------|---------|---------|---------|---------|  5900

8)  PBVW4       -941 GAAAGATCCACACAAGTGTCACGCTATCTAAGCTTGTTGTAGCCTAAAAA  -891
7)  381_W6       -26 GAAAGATCCACACAAGTGTCACGCTA                          -1
    Consens     5901 ---------|---------|---------|---------|---------|  5950

8)  PBVW4       -891 TCAACGGCTACCCTTATGGCACATGGCACTATCTCAGCCCTACATGTGCA  -841
    Consens     5951 ---------|---------|---------|---------|---------|  6000

8)  PBVW4       -841 TTCGCCTATGCAAAGAAAAGTACAAAGGCCTGCAGGTTCTTTGGGCTAAA  -791
    Consens     6001 ---------|---------|---------|---------|---------|  6050

8)  PBVW4       -791 GTACATTTTTCTGAGAAAAAGGTGTACTGAAAAGCTCCCTTATGGCCCTG  -741
    Consens     6051 ---------|---------|---------|---------|---------|  6100

8)  PBVW4       -741 AAATTGTACTTAGGCCCAATGAAGTAAAGCCTGATTAAGCTAAAGGGTAA  -691
    Consens     6101 ---------|---------|---------|---------|---------|  6150

8)  PBVW4       -691 AATGTACTGACCAATCAGCGTTTGCCACGTGTCTTGATATTATTGCTCAG  -641
    Consens     6151 ---------|---------|---------|---------|---------|  6200

8)  PBVW4       -641 CCAATCAGATGCTCCCAAATTAGGGTGACATCAAGGTACATCAGTCATTT  -591
    Consens     6201 ---------|---------|---------|---------|---------|  6250

8)  PBVW4       -591 CAAACAATATAAATACTCCTTGAAATCAGGAGAAAGGTACGTTTTTCAGA  -541
    Consens     6251 ---------|---------|---------|---------|---------|  6300

8)  PBVW4       -541 GCATAGTATTGTTATTCTGCCATAAATTCTCTCTCTCTCTCTACTTTCTC  -491
    Consens     6301 ---------|---------|---------|---------|---------|  6350

8)  PBVW4        491 TCTCTAAAAGTTCCTTCATAGCCCTGATATCTGACTTAAGCATCGGAGGG  -441
    Consens     6351 ---------|---------|---------|---------|---------|  6400

8)  PBVW4       -441 GGAAATACCACCCAAACCTCCACGGTGGGTCCCTTTTTGCAGGTTGCTGG  -391
    Consens     6401 ---------|---------|---------|---------|---------|  6450
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8) PBVW4      -391 TCAGAGGTTTGCACCGAGATCCGAGCCAATCATTGTTGCACAAGTCTCCA -341
   Consens    6451 ----------|----------|----------|----------|----------| 6500

8) PBVW4      -341 AACTAAGAATCTAAGGTTGGGAGTTATTGTATTAGTTTACTAGTTTCGTA -291
   Consens    6501 ----------|----------|----------|----------|----------| 6550

8) PBVW4      -291 CTACGTAACATTTGGCGCCGTCTGTGGGGAGACGCAACAATGACGGATTG -241
   Consens    6551 ----------|----------|----------|----------|----------| 6600

8) PBVW4      -241 TCATTCATTGAATGTCGCAAGGAACATAAGTTTTAGAGTTCAAGTGGAAG -191
   Consens    6601 ----------|----------|----------|----------|----------| 6650

9) SAL_REV   -1075                                 CAGTGGACGGGACAGAGGCCATGCT -1050
8) PBVW4      -191 ACACTGTGAATGACGAGAGGCCACCCAGTGGACGGGACAGAGGCCATGCT -141
   Consens    6651 ----------|----------|----------|----------|----------| 6700

9) SAL_REV   -1050 GCCGTCCATCTGGAGGGTAGGACTGTGACCCCAGTCATTCAGGAAAACCT -1000
8) PBVW4      -141 GCCGTCCATCTGGAGGGTAGGACTGTGACCCCAGTCATTCAGGAAAACCT  -91
   Consens    6701 ----------|----------|----------|----------|----------| 6750

9) SAL_REV   -1000 TGATCATCCAATGACAGCTGGTCACTTGGAGAATGTCTTAAATGATTTCC  -950
8) PBVW4       -91 TGATCATCCAATGACAGCTGGTCACTTGGAGAATGTCTTAAATGATTTCC   -41
   Consens    6751 ----------|----------|----------|----------|----------| 6800

9) SAL_REV    -950 AGGCCAGAATGGCCGCTGTCATGGAAGAGCAGATTAAGAACAATATGTTG  -900
8) PBVW4       -41 AGGCCAGAATGGCCGCTGTCATGGAAGAGCAGATTAAGAAC            -1
   Consens    6801 ----------|----------|----------|----------|----------| 6850

9) SAL_REV    -900 TTCTTTCAGCTAAGTCCAGGAGGAGAGGCAGCAGAGCCACTCAAAGGGAG  -850
   Consens    6851 ----------|----------|----------|----------|----------| 6900

9) SAL_REV    -850 ATCCAAAGAGGGTCGTCCAGGGACTAGCCGGCCACCTCGGCCAGAGTTGA  -800
   Consens    6901 ----------|----------|----------|----------|----------| 6950

9) SAL_REV    -800 CCAGGCCGCTTTCCAAGGGGAAGGGGCCAGCTGTTAGTGACTCTAGGAGG  -750
   Consens    6951 ----------|----------|----------|----------|----------| 7000

9) SAL_REV    -750 AAGATGGACTGGAGGTTGGTAGTCCGTCCAGGAGACAATATTCCCCCTAC  -700
   Consens    7001 ----------|----------|---------- ---------|----------| 7050

9) SAL_REV    -700 CGGCCACAAAATGGAAACTGATGCTAGGGAATATCTGGAAGCCAAAAGAG  -650
   Consens    7051 ----------|----------|----------|----------|----------| 7100

9) SAL_REV    -650 CGGCAGCCCAACAGCGTTCGGCCTCTGGTTCTACTCCTTATATGAAGGAT  -600
   Consens    7101 ----------|----------|----------|----------|----------| 7150

9) SAL_REV    -600 CTGTTAGTAAAGCTCCCGTCCAGAACAGTAGGGAGAAGTCATGGACCTTC  -550
   Consens    7151 ----------|----------|----------|----------|----------| 7200

9) SAL_REV    -550 GGCTTTTCCAGCCAAGGCAAGAAAAAACAGTTCTGCAGCCAATGGTGTACA  -500
   Consens    7201 ----------|----------|----------|----------|----------| 7250

9) SAL_REV    -500 GGCGTCAAGCCTTAGCTAACACCTCTTCTAATCCGGTACTAAAGGAGTAT  -450
   Consens    7251 ----------|----------|----------|----------|----------| 7300
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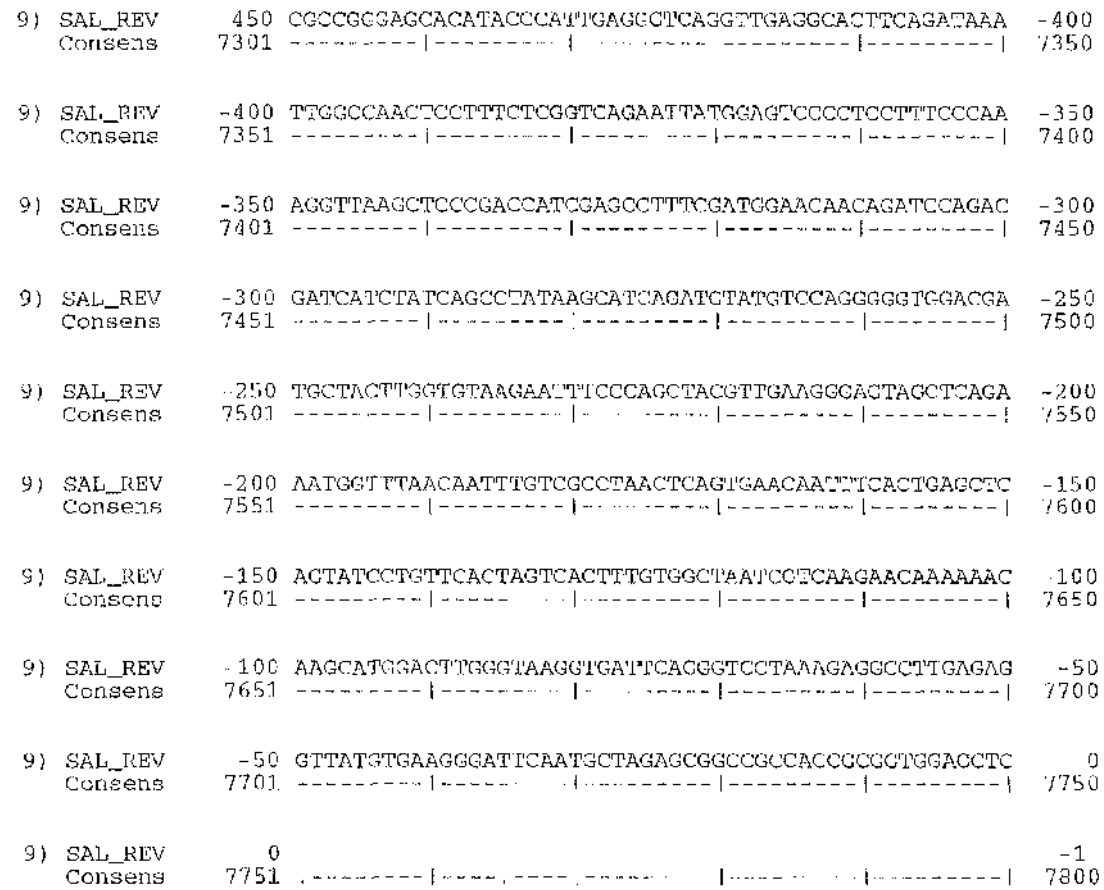
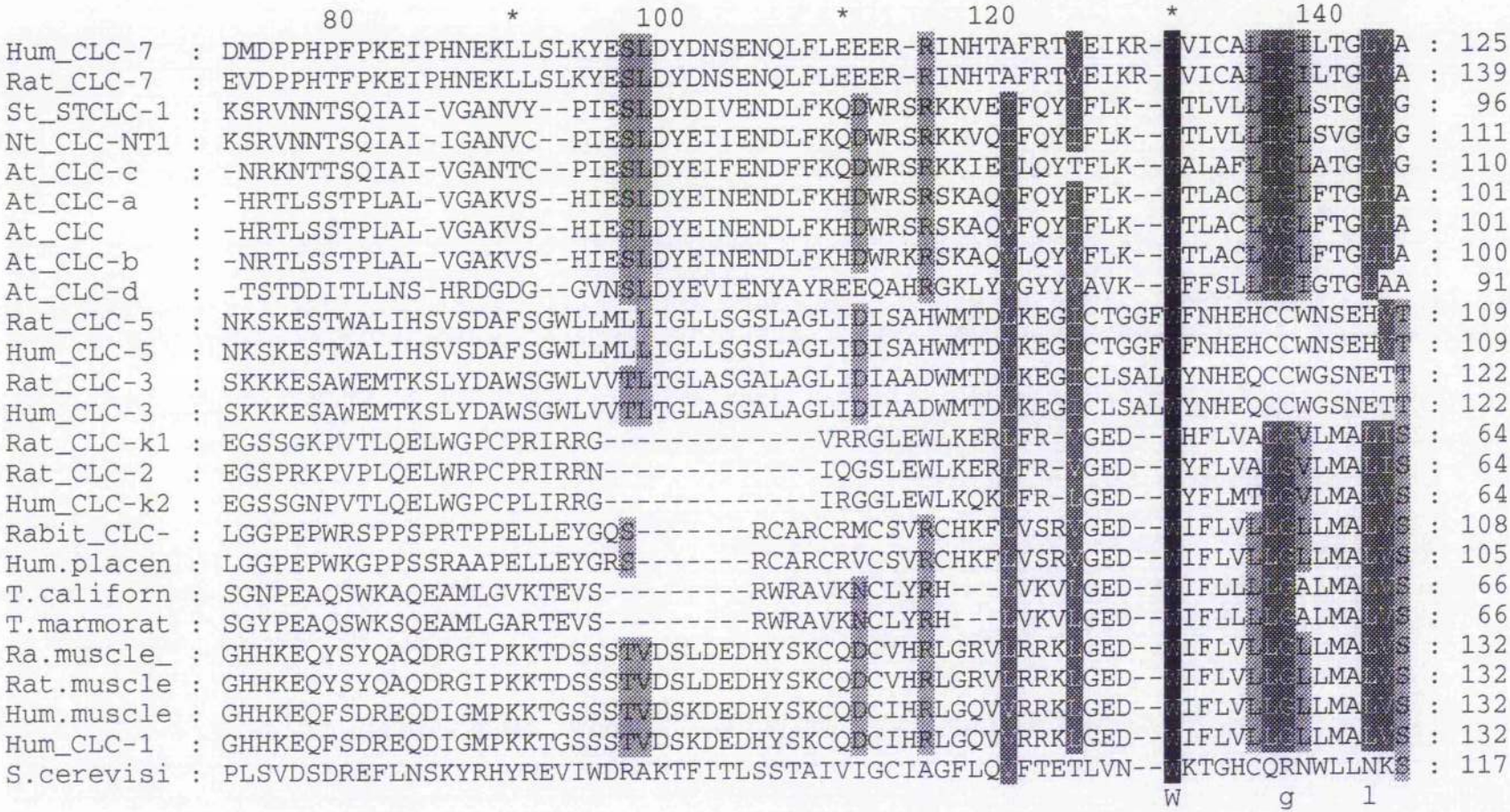

9) SAL_REV 450 CGCCGCGAGCACATACCCATTGAGGCTCAGGTTGAGGCACTTCAGATAAA -400
Consens 7301
9) SAL_REV -400 TTGGCCAACTCCTTTCTCGGTCAGAATTATGGAGTCCCCTCCTTTCCCAA -350
Consens 7351
9) SAL_REV -350 AGGTTAAGCTCCCGACCATCGAGCCTTTCGATGGAACAACAGATCCAGAC -300
Consens 7401
9) SAL_REV -300 GATCATCTATCAGCCTATAAGCATCAGATGTATGTCCAGGGGGTGGACGA -250
Consens 7451
9) SAL_REV -250 TGCTACTTGGTGTAAGAACTTCCCAGCTACGTTGAAGGCACTAGCTCAGA -200
Consens 7501
9) SAL_REV -200 AATGGTTTAACAATTTGTCGCCTAACTCAGTGAACAATTTCACTGAGCTC -150
Consens 7551
9) SAL_REV -150 ACTATCCTGTTCACTAGTCACTTTGTGGCTAATCCTCAAGAACAAAAAAC -100
Consens 7601
9) SAL_REV -100 AAGCATGGACTTGGGTAAGGTGATTCAGGGTCCTAAAGAGGCCTTGAGAG -50
Consens 7651
9) SAL_REV -50 GTTATGTGAAGGGATTCAATGCTAGAGCGGCCGCCACCGCGGTGGAGCTC 0
Consens 7701
9) SAL_REV 0 -1
Consens 7751 7800

Figure A.2. CLUSTALW Alignment of 17 Putative *CLC*-type Chloride Channels. In September 1998, the CLUSTALW programme at the EMBL European Bioinformatics Institute, Cambridge was used to align the same 17 *CLC* protein sequences aligned by GCG's PILEUP programme (see Fig. 1.2). Several more regions of conserved sequence were identified, over and above those identified by PILEUP. The sequence accession numbers are as follows: rnmrna, x64139: human placenta CLC-2, s77770: oc15652, chinese hampster CLC-2: *Torpedo californica* CLC-0, tcvgcc: *Torpedo marmorata* CLC-0, tmclchan: human muscle CLC-1, hsclc1mcc: human muscle CLC-1, hsclc1mr: rat skeletal muscle, rrsmcc: rat protein kinase A activated Cl channel, rrclck2a: human kidney CLC-2, s80315: human CLC-5, hsclcn5gn: rat CLC-5; rnclc5: human chloride channel protein, hsclcpx: rat CLC-3, rnclc3: human CLC-7, hsclc7mr: rat CLC-7, rnclc7mr: *S. cerevisiae* GEF1, scclcy.


* 20 * 40 * 60 *
Hum_CLC-7 : ----------DEEAAPLLRRTARPGGGTPLLNGAGPGAARQSPRSALFRVGHMSSVELDDELLDP : 55
Rat_CLC-7 : -MANVSKKVSWSGRDRDDEEGAPLLRRTGQPDEETPLLNGAGPG-ARQS-HSALFRIGQMNNVELDDELLDP : 69
St_STCLC-1 : ----------MIEEEKFD-LERNFSTISES----------GSI-----RVPLLKS : 29
Nt_CLC-NT1 : --------MEDQGDIENEGGGIGVMIMENGKD-LERNISAVSES----------G-V-----RQPLLSS : 44
At_CLC-c : ----------MDDRHEGDHHDIEVEGGALHGFERKISGILDD----------GSVG---FRQPLLAR : 44
At_CLC-a : ----------MDEDGNLQISNSNYNGEEEG----------EDPENNTLNQPLLKR : 35
At_CLC : ----------MDEDGNLQISNSNYNGEEEG----------EDPENNTLNQPLLKR : 35
At_CLC-b : ----------MVEEDLNQIGGNSNYNG--EG----------GDPESNTLNQPLVKA : 34
At_CLC-d : ----------MLSNHLQNGIESD----------NLLWS---RVPESDD : 25
Rat_CLC-5 : ----------MDFLEEPIPGVGTYDDFNTIDWVR----------EKSRDR---DRHREIT : 37
Hum_CLC-5 : ----------MDFLEEPIPGVGTYDDFNTIDWVR----------EKSRDR---DRHREIT : 37
Rat_CLC-3 : --------MTNGGSINSSTHLLDLLDEPIPGVGTYDDFHTIDWVR----------EKCKDR---ERHRRIN : 50
Hum_CLC-3 : --------MTNGGSINSSTHLLDLLDEPIPGVGTYDDFHTIDWVR----------EKCKDR---ERHRRIN : 50
Rat_CLC-k1 : ----------MEELVGLR : 8
Rat_CLC-2 : ----------MEEIVGLR : 8
Hum_CLC-k2 : ----------MEEFVGLR : 8
Rabit_CLC- : ----------MAAPAAAAVEEGMEPRALQYEQTLMYG----------RYTQDLGAFAKEEAARIR : 45
Hum.placen : ----------MAAAAAEEGMEPRALQHEQTLMYG----------RYTQDLGAFAKEEAARIR : 42
T.californ : ----------MSHEKNEA : 8
T.marmorat : ----------MSHEKNEA : 8
Ra.muscle_ : MERSQSQQHGGEQSWWGTAPQYQYMPFEHCTSYGLPSENGGLQH----------RPRKDLGPRHNAHPTQIY : 62
Rat.muscle : MERSQSQQHGGEQSWWGTAPQYQYMPFEHCTSYGLPSENGGLQH----------RPRKDLGPRHNAHPTQIY : 62
Hum.muscle : MEQSRSQQRGGEQSWWGSDPQYQYMPFEHCTSYGLPSENGGLQH----------RLRKDAGPRHNVHPTQIY : 62
Hum_CLC-1 : MEQSRSQQRGGEQSWWGSDPQYQYMPFEHCTSYGLPSENGGLQH----------RLRKDAGPRHNVHPTQIY : 62
S.cerevisi : ----------MPTTYVPINQPIRDGEDVIDTNRFTNIPETQN----------FDQFVTIDKIAEENR : 47


80 * 100 * 120 * 140
Hum_CLC-7 : DMDPPHPFPKEIPHNEKLLSLKYESLDYDNSENQLFLEEER-RINHTAFRTWEIKR-WVICALLCILTGLVA : 125
Rat_CLC-7 : EVDPPHTFPKEIPHNEKLLSLKYESLDYDNSENQLFLEEER-RINHTAFRTWEIKR-WVICALLCILTGLVA : 139
St_STCLC-1 : KSRVNNTSQIAI-VGANVY--PIESLDYDIVENDLFKQDWRSRKKVEWFQYWFLK--WTLVLLLCLSTGLVG : 96
Nt_CLC-NT1 : KSRVNNTSQIAI-IGANVC--PIESLDYEIIENDLFKQDWRSRKKVQWFQYWFLK--WTLVLLLCLSVGLVG : 111
At_CLC-c : -NRKNTTSQIAI-VGANTC--PIESLDYEIFENDFFKQDWRSRKKIEWLQYTFLK--WALAFLLCLATGLVG : 110
At_CLC-a : -HRTLSSTPLAL-VGAKVS--HIESLDYEINENDLFKHDWRSRSKAQWFQYWFLK--WTLACLVCLFTGLIA : 101
At_CLC : -HRTLSSTPLAL-VGAKVS--HIESLDYEINENDLFKHDWRSRSKAQWFQYWFLK--WTLACLVCLFTGLIA : 101
At_CLC-b : -NRTLSSTPLAL-VGAKVS--HIESLDYEINENDLFKHDWRKRSKAQWLQYWFLK--WTLACLVCLFTGLIA : 100
At_CLC-d : -TSTDDITLLNS-HRDGDG--GVNSLDYEVIENYAYREEQAHRGKLYWGYYWAVK--WFFSLLLCIGTGLAA : 91
Rat_CLC-5 : NKSKESTWALIHSVSDAFSGWLLMLLIGLLSGSLAGLIDISAHWMTDLKEGWCTGGFWFNHEHCCWNSEHWT : 109
Hum_CLC-5 : NKSKESTWALIHSVSDAFSGWLLMLLIGLLSGSLAGLIDISAHWMTDLKEGWCTGGFWFNHEHCCWNSEHWT : 109
Rat_CLC-3 : SKKKESAWEMTKSLYDAWSGWLVVTLTGLASGALAGLIDIAADWMTDLKEGWCLSALWYNHEQCCWGSNETT : 122
Hum_CLC-3 : SKKKESAWEMTKSLYDAWSGWLVVTLTGLASGALAGLIDIAADWMTDLKEGWCLSALWYNHEQCCWGSNETT : 122
Rat_CLC-k1 : EGSSGKPVTLQELWGPCPRIRRG----------VRRGLEWLKERLFR-WGED--WHFLVALCVLMALLS : 64
Rat_CLC-2 : EGSPRKPVPLQELWRPCPRIRRN----------IQGSLEWLKERLFR-WGED--WYFLVALCVLMALLS : 64
Hum_CLC-k2 : EGSSGNPVTLQELWGPCPLIRRG----------IRGGLEWLKQKLFR-WGED--WYFLMTLCVLMALVS : 64
Rabit_CLC- : LGGPEPWRSPPSPRTPPELLEYGQS-------RCARCRMCSVRCHKFLVSRWGED--WIFLVLLCLLMALVS : 108
Hum.placen : LGGPEPWKGPPSSRAAPELLEYGRS-------RCARCRVCSVRCHKFLVSRWGED--WIFLVLLCLLMALVS : 105
T.californ : SGNPEAQSWKAQEAMLGVKTEVS---------RWRAVKNCLYRH---LVKVWGED--WIFLLLLCALMALVS : 66
T.marmorat : SGYPEAQSWKSQEAMLGARTEVS---------RWRAVKNCLYRH---LVKVWGED--WIFLLLLCALMALVS : 66
Ra.muscle_ : GHHKEQYSYQAQDRGIPKKTDSSSTVDSLDEDHYSKCQDCVHRLGRVLRRKWGED--WIFLVLLCLLMALVS : 132
Rat.muscle : GHHKEQYSYQAQDRGIPKKTDSSSTVDSLDEDHYSKCQDCVHRLGRVLRRKWGED--WIFLVLLCLLMALVS : 132
Hum.muscle : GHHKEQFSDREQDIGMPKKTGSSSTVDSKDEDHYSKCQDCIHRLGQVWRRKWGED--WIFLVLLCLLMALVS : 132
Hum_CLC-1 : GHHKEQFSDREQDIGMPKKTGSSSTVDSKDEDHYSKCQDCIHRLGQVWRRKWGED--WIFLVLLCLLMALVS : 132
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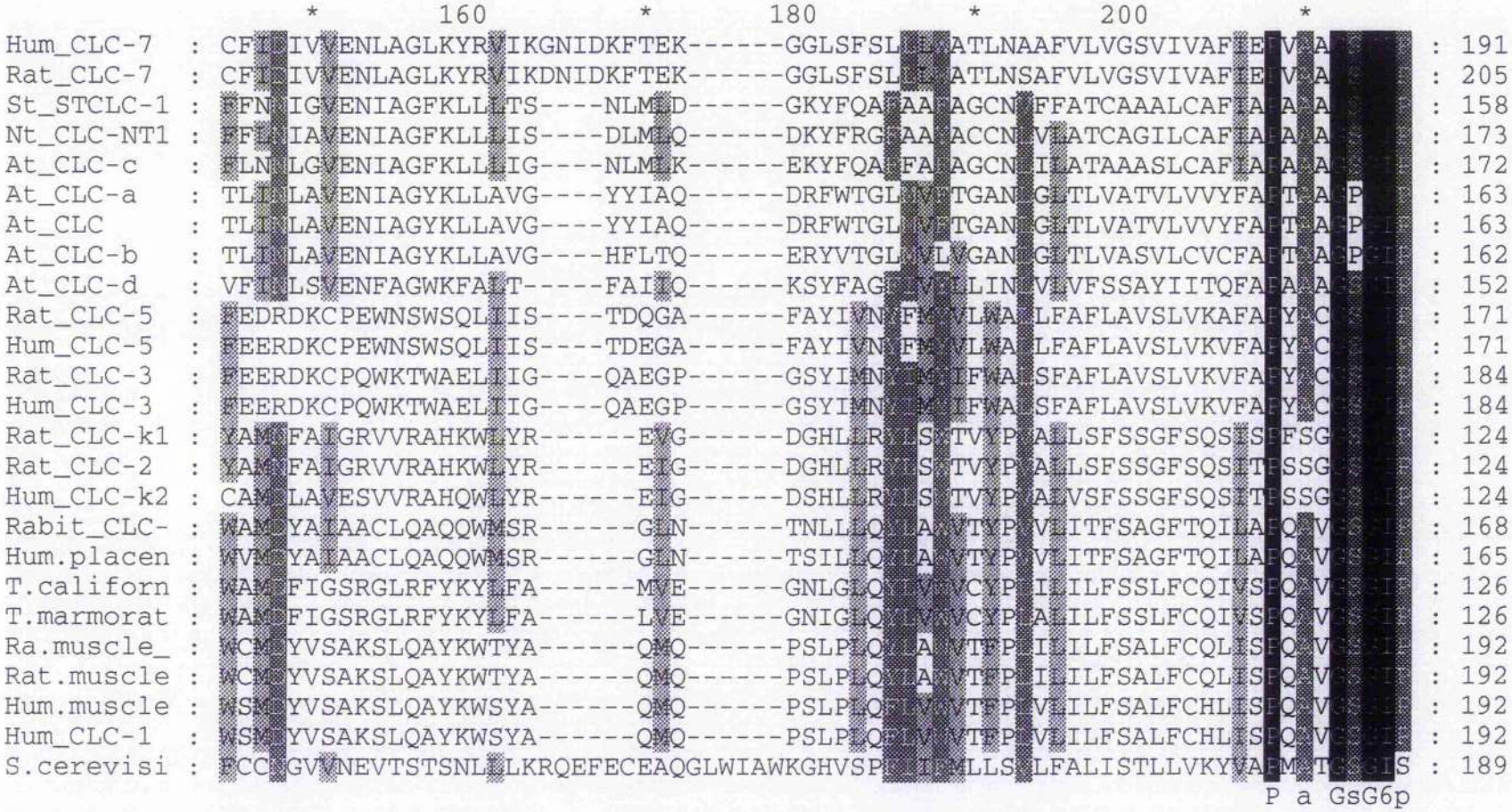
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180
200
Hum_CLC-7 : 191
Rat_CLC-7 : 205
St_STCLC-1 : 158
Nt_CLC-NT1 : 173
At_CLC-c : 172
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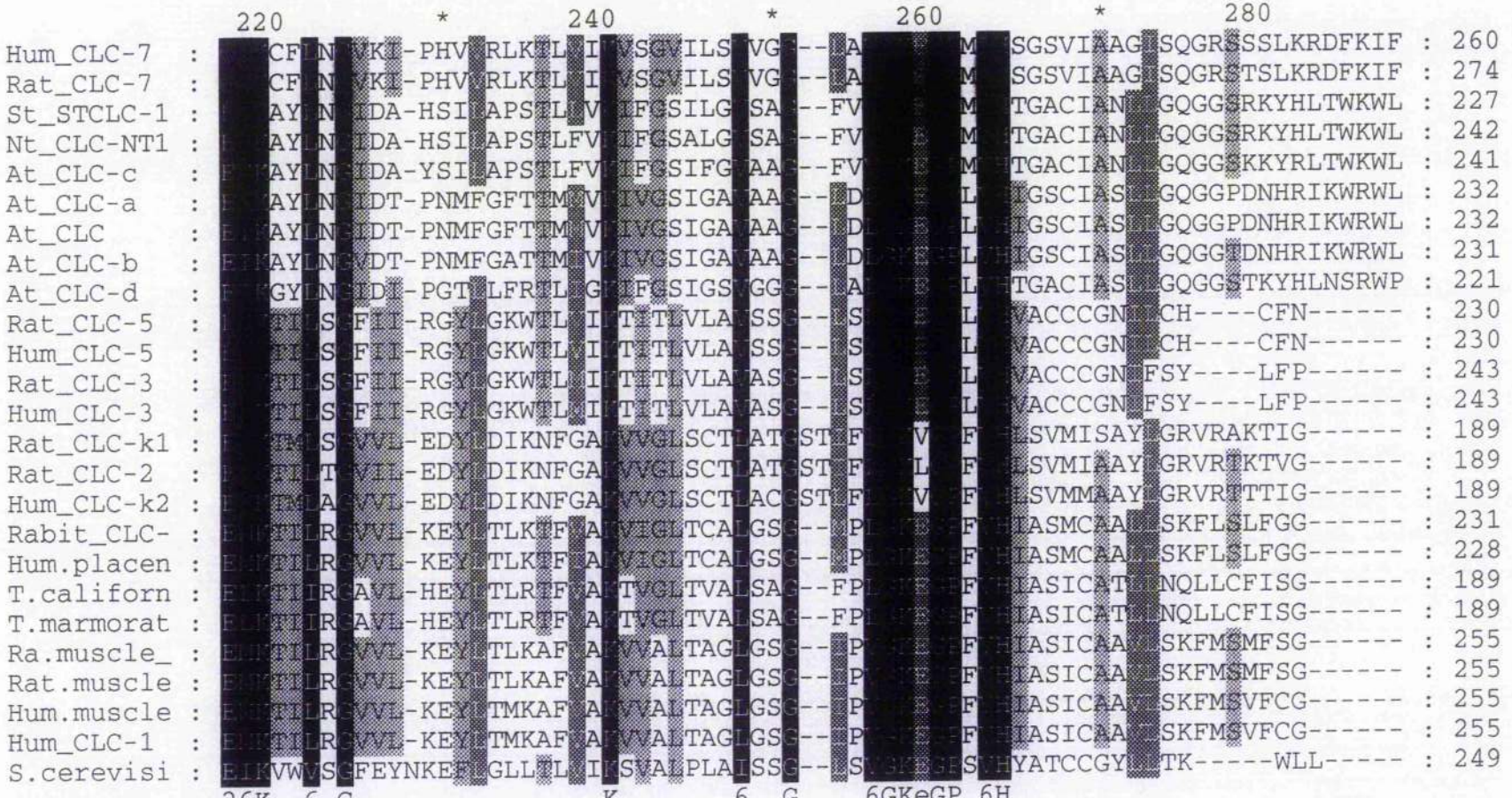
At_CLC : 163
At_CLC-b : 162
At_CLC-d : 152
Rat_CLC-5 : 171
Hum_CLC-5 : 171
Rat_CLC-3 : 184
Hum_CLC-3 : 184
Rat_CLC-k1 : 124
Rat_CLC-2 : 124
Hum_CLC-k2 : 124
Rabit_CLC- : 168
Hum.placen : 165
T.californ : 126
T.marmorat : 126
Ra.muscle_ : 192
Rat.muscle : 192
Hum.muscle : 192
Hum_CLC-1 : 192
S.cerevisi : 189
P a GsG6p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Hum_CLC-7 : CFLNWKI-PHVWRLKTLWIVSGVILSVG--LA M SGSVIAAGISQGRSSLKRDFKIF : 260
Rat_CLC-7 : CFLNWKI-PHVWRLKTLWIVSGVILSVG--LA M SGSVIAAGISQGRSTSLKRDFKIF : 274
St_STCLC-1 : AYLNWDA-HSILAPSTLVVIFGSILGSA--FV M TGACIANLGQGGSRKYHLTWKWL : 227
Nt_CLC-NT1 : AYLNWDA-HSILAPSTLFVIFGSALGSA--FV M TGACIANLGQGGSRKYHLTWKWL : 242
At_CLC-c : AYLNWDA-YSILAPSTLFVIFGSIFGAA--FV M TGACIANLGQGGSKKYRLTWKWL : 241
At_CLC-a : AYLNWDT-PNMFGFTTMVVIVGSIGAAA--LD L IGSCIASLGQGGPDNHRIKWRWL : 232
At_CLC : AYLNWDT-PNMFGFTTMVVIVGSIGAAA--LD L IGSCIASLGQGGPDNHRIKWRWL : 232
At_CLC-b : AYLNWDT-PNMFGATTMVVIVGSIGAAA--LD L IGSCIASLGQGGTDNHRIKWRWL : 231
At_CLC-d : GYLNWDI-PGTLLFRTLIGVIFGSIGSVGG--LA L TGACIASLGQGGSTKYHLNSRWP : 221
Rat_CLC-5 : TILSWFII-RGYLGKWTLVIITTLVLAVSS--LS L VACCCGNLCH----CFN------ : 230
Hum_CLC-5 : TILSWFII-RGYLGKWTLVIITTLVLAVSS--LS L VACCCGNLCH----CFN------ : 230
Rat_CLC-3 : TILSWFII-RGYLGKWTLVIITTLVLAVAS--LS L VACCCGNLFSY----LFP------ : 243
Hum_CLC-3 : TILSWFII-RGYLGKWTLVIITTLVLAVAS--LS L VACCCGNLFSY----LFP------ : 243
Rat_CLC-k1 : TMLSWVL-EDYLDIKNFGAVVGLSCTLATGSTLF V F LSVMISAYLGRVRAKTIG------ : 189
Rat_CLC-2 : TILTWVL-EDYLDIKNFGAVVGLSCTLATGSTLF L F LSVMIAAYLGRVRTKTVG------ : 189
Hum_CLC-k2 : TMLAWVL-EDYLDIKNFGAVVGLSCTLACGSTLF V F LSVMMAAYLGRVRTTTIG------ : 189
Rabit_CLC- : TILRWVL-KEYLTLKTFWAVVIGLTCALGSG--MP F LASMCAALLSKFLSLFGG------ : 231
Hum.placen : TILRWVL-KEYLTLKTFWAVVIGLTCALGSG--MP F LASMCAALLSKFLSLFGG------ : 228
T.californ : TILRAVL-HEYLTLRTFWAVTVGLTVALSAG--FP F LASICATLLNQLLCFISG------ : 189
T.marmorat : TILRAVL-HEYLTLRTFWAVTVGLTVALSAG--FP F LASICATLLNQLLCFISG------ : 189
Ra.muscle_ : TILRWVL-KEYLTLKAFWAVVALTAGLGSG--LP F LASICAAMLSKFMSMFSG------ : 255
Rat.muscle : TILRWVL-KEYLTLKAFWAVVALTAGLGSG--LP F LASICAAMLSKFMSMFSG------ : 255
Hum.muscle : TILRWVL-KEYLTMKAFWAVVALTAGLGSG--LP F LASICAAMLSKFMSVFCG------ : 255
Hum_CLC-1 : TILRWVL-KEYLTMKAFWAVVALTAGLGSG--LP F LASICAAMLSKFMSVFCG------ : 255
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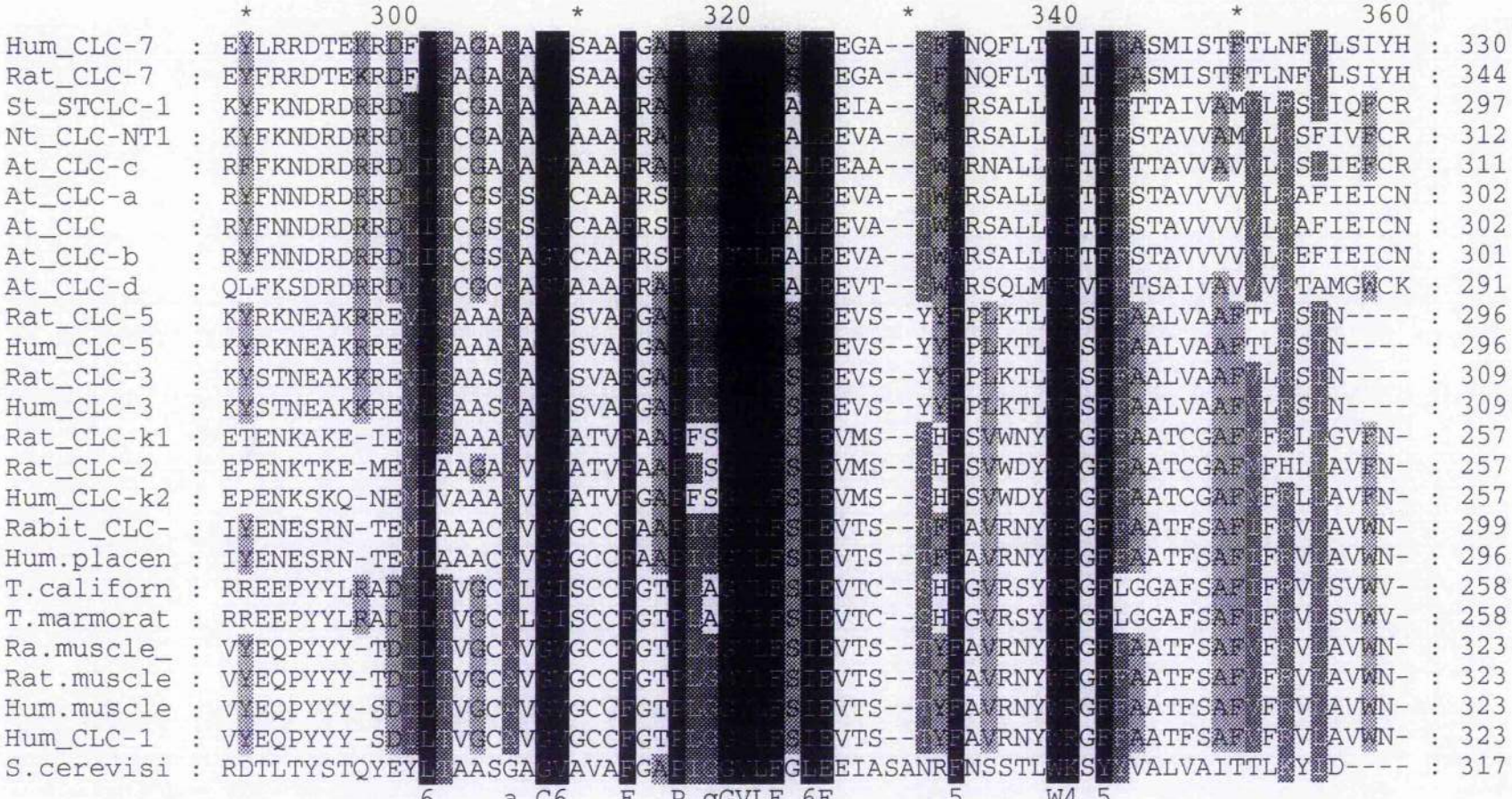
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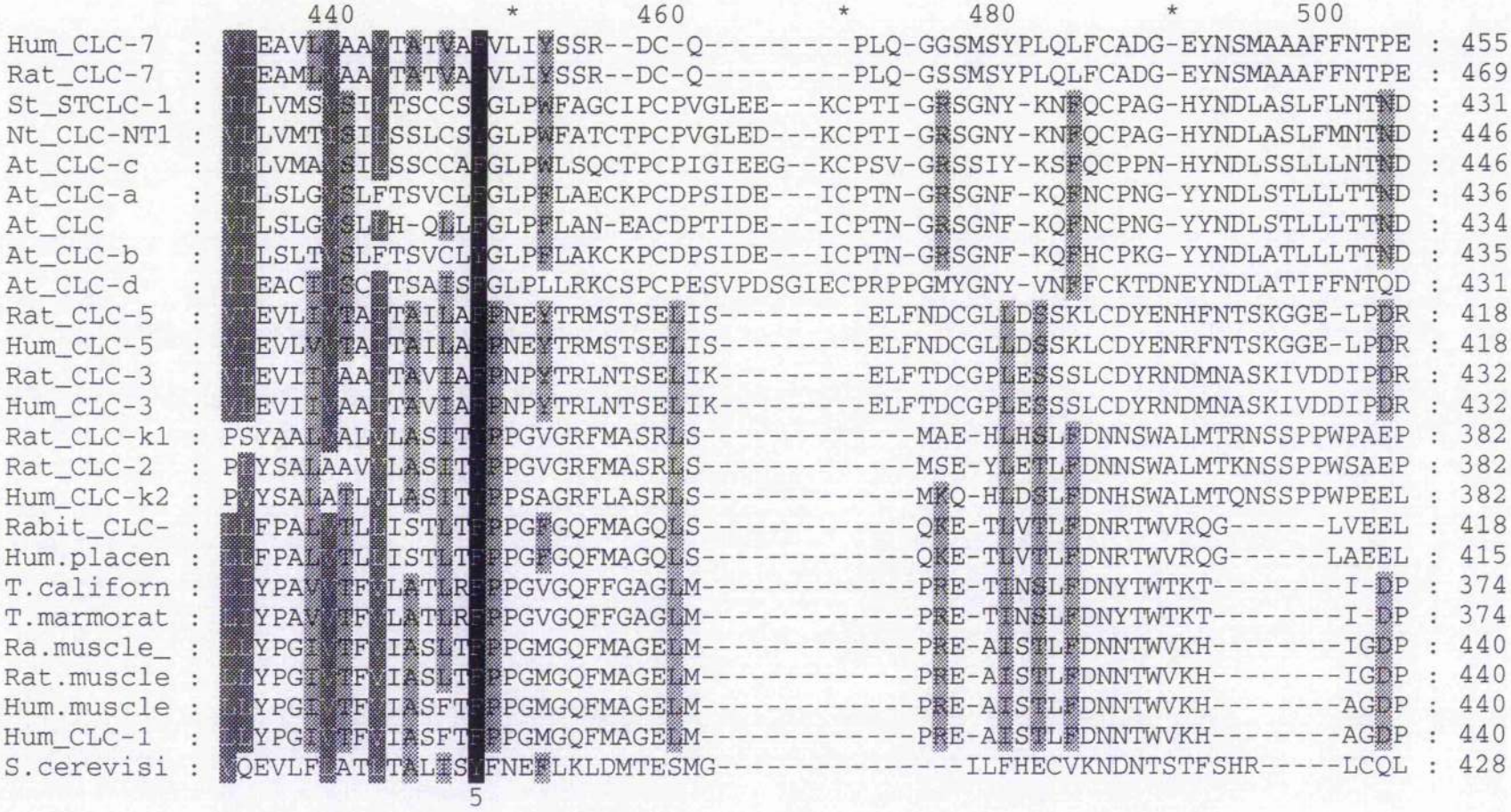
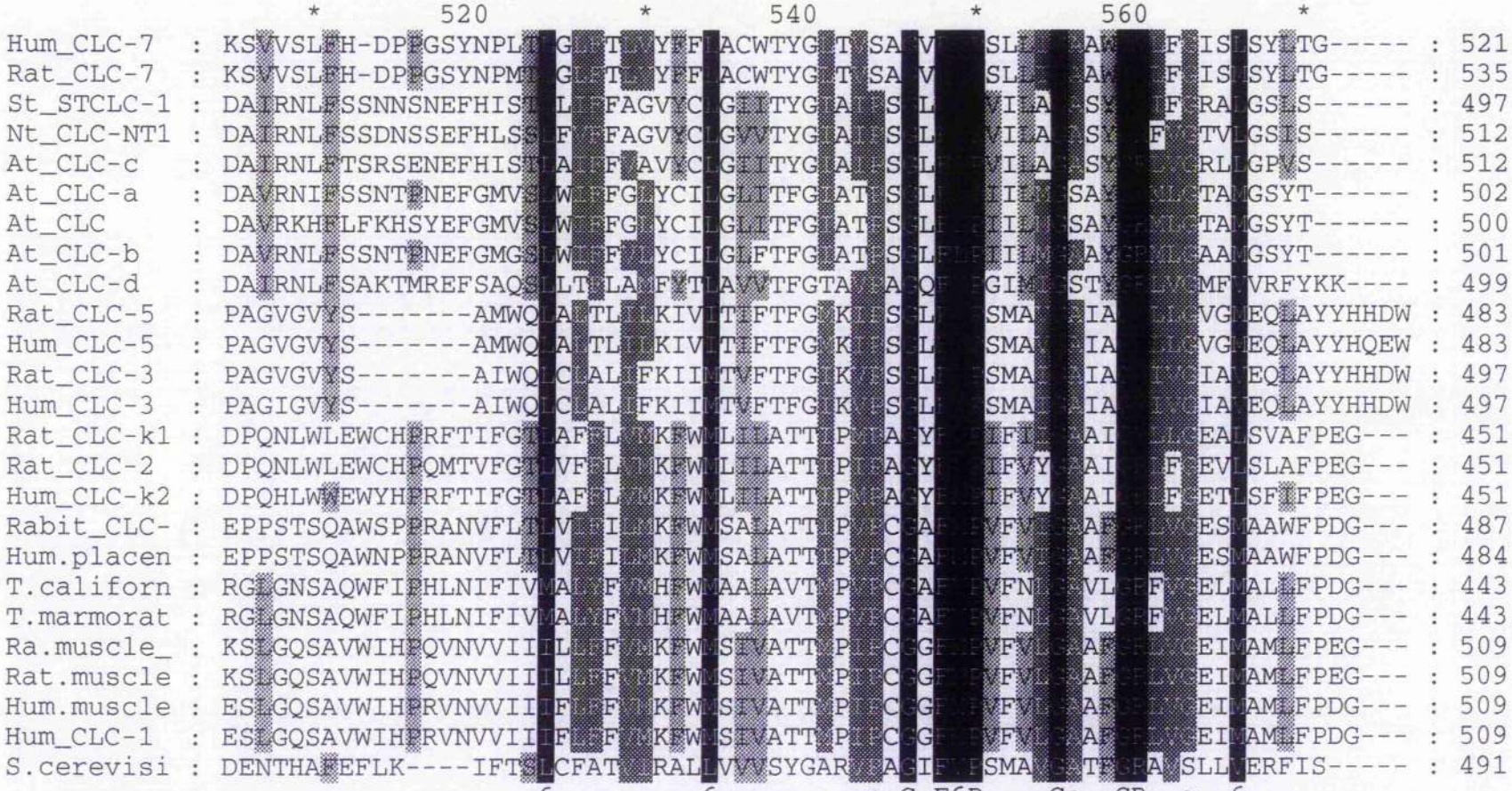
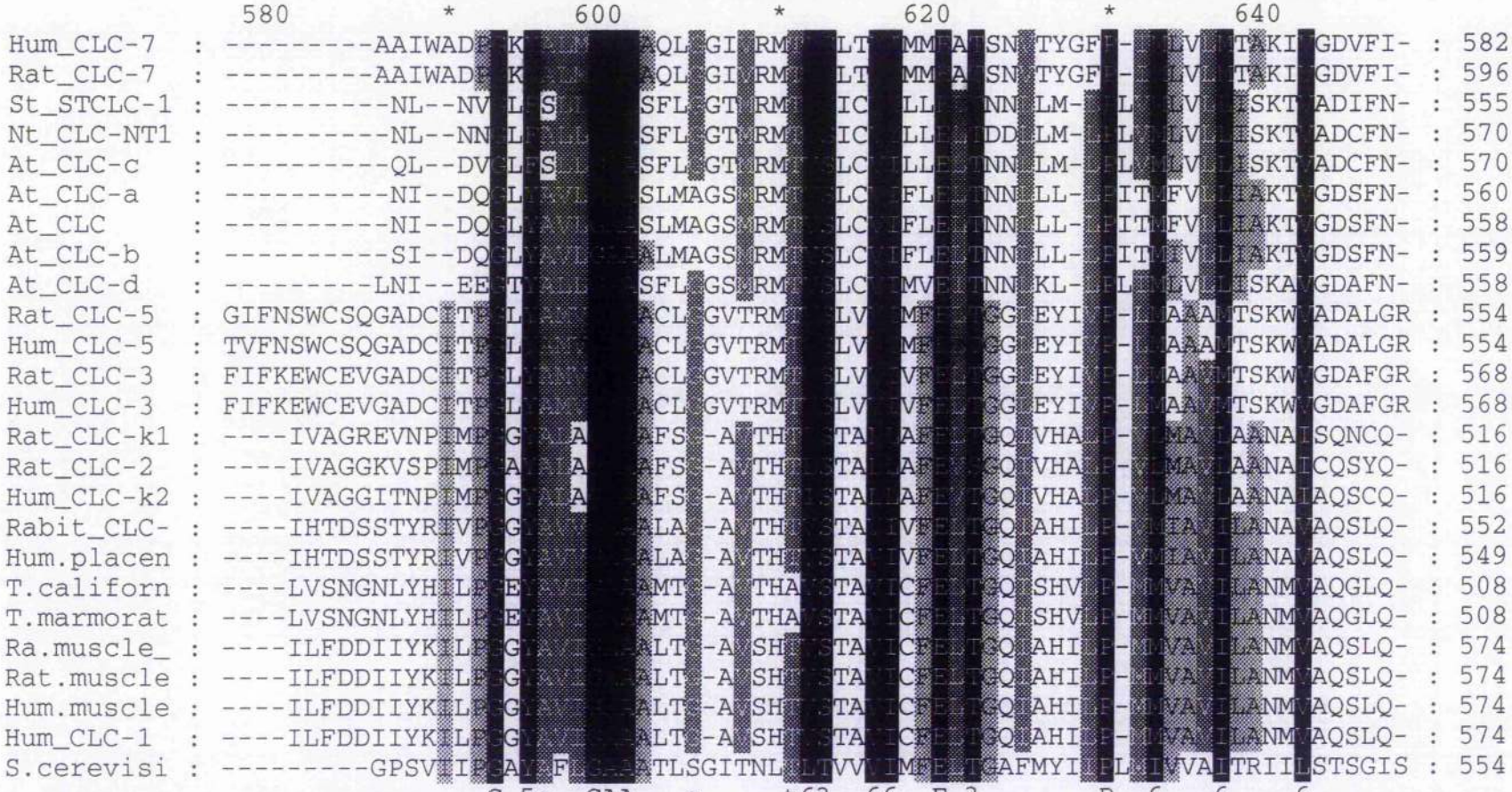
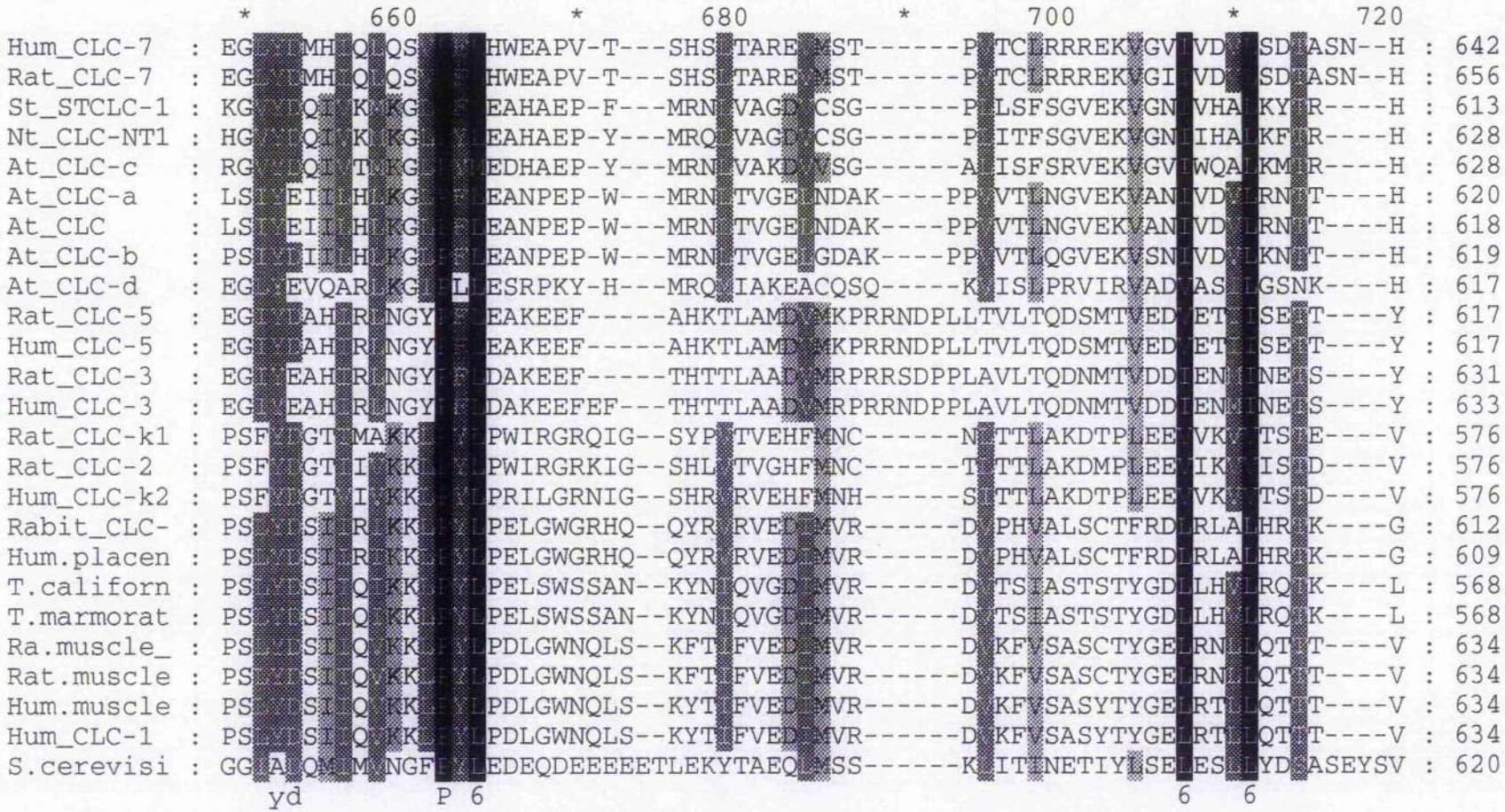
St_STCLC-1 : 297
Nt_CLC-NT1 : 312
At_CLC-c : 311
At_CLC-a : 302
At_CLC : 302
At_CLC-b : 301
At_CLC-d : 291
Rat_CLC-5 : 296
Hum_CLC-5 : 296
Rat_CLC-3 : 309
Hum_CLC-3 : 309
Rat_CLC-k1 : 257
Rat_CLC-2 : 257
Hum_CLC-k2 : 257
Rabit_CLC- : 299
Hum.placen : 296
T.californ : 258
T.marmorat : 258
Ra.muscle_ : 323
Rat.muscle : 323
Hum.muscle : 323
Hum_CLC-1 : 323
S.cerevisi : 317

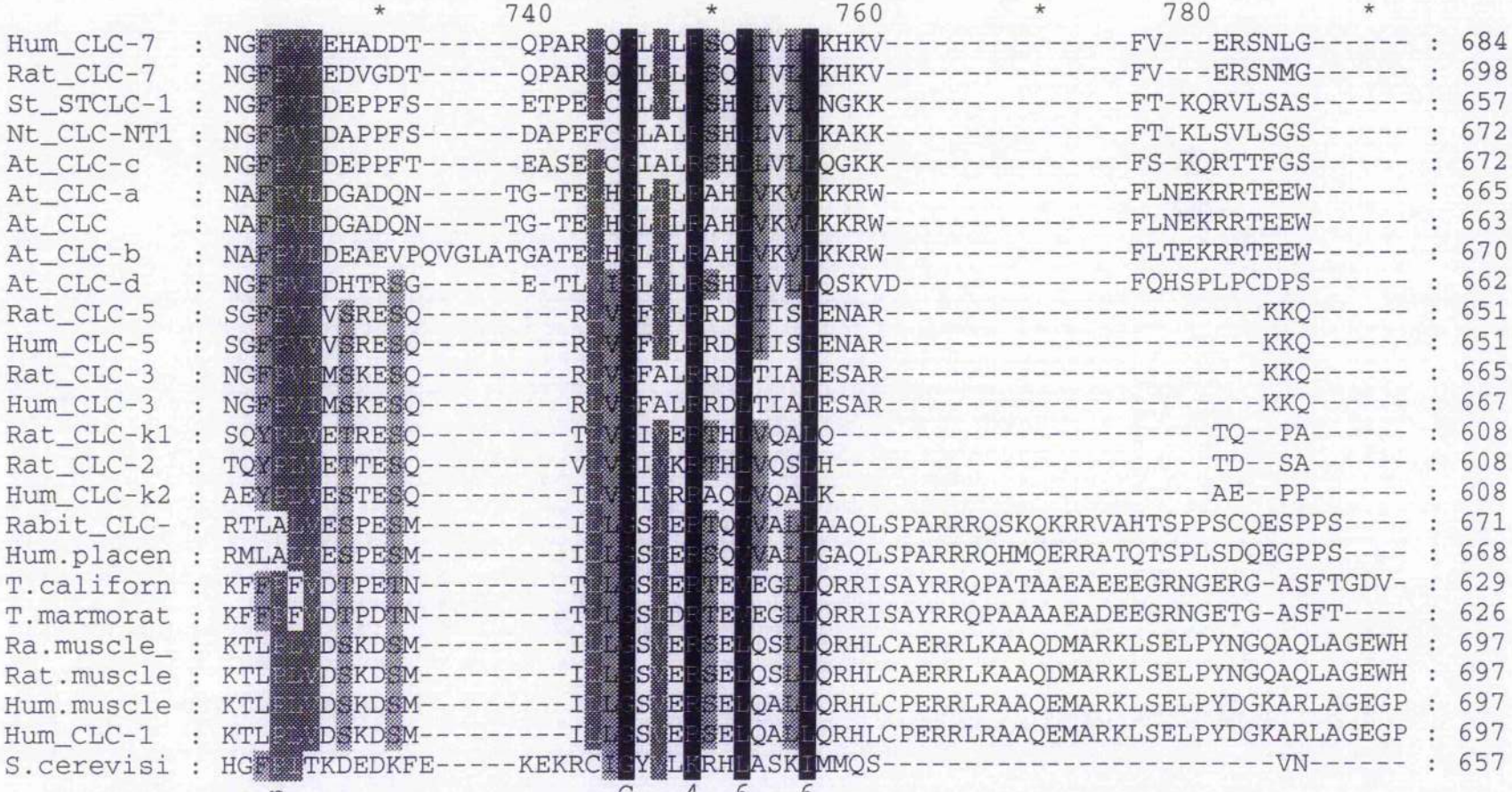

* 380 * 400 * 420 *
Hum_CLC-7 : -GNMWDLSSPGLIN GRFDSEKMAYTIHE PVFIA VG VLCAV NALNYWLTMFRI--RYIHR--PCLQ : 397
Rat_CLC-7 : -GNMWDLSSPGLIN GRFDSEKMAYTIHE PVFIA VG VLCAV NALNYWLTMFRI--RYIHR--PCLQ : 411
St_STCLC-1 : GGNCGLFGQGGLI DVNSGVSNYNTIDV ALIF- LG LLSL NYLVDKVLRTY---AVINERGPAFK : 365
Nt_CLC-NT1 : SGKCGLFGQGGLI DVNSGAPNYNTIDV AVLL- LG LLSL NYLVDKVLRTY---SIINERGPAFK : 380
At_CLC-c : SGRCGLFGKGGLI DVNSGPVLYSTPDL AIVF- IG VLSL NYLVDKVLRTY---SIINEKGPRFK : 379
At_CLC-a : SGKCGLFGSGGLI DVSHVEVRYHAAHI PVTL- FG LLSL NHLLHKVLRLY---NLINQKGKIHK : 370
At_CLC : SGKCGLFGSGGLI DVSHVEVRYHAADI PVTL- FG LLSL NHLLHKVLRLY---NLINQKGKIHK : 370
At_CLC-b : SGKCGLFGKGGLI DVSHVTYTYHVTDI PVML- IG LLSL NHLLHKVLRLY---NLINEKGKIHK : 369
At_CLC-d : SGICGHFGGGGFI DVSDGQDDYYFKEL PMAV- IG LLCAL NQLTLYMTSWRR--NSLHKKGNRVK : 360
Rat_CLC-5 : -----PFGNSRLVL YVEFHTP-WHLFEL PFIV- FG LWCAL IRTNIAWCRKR-----KTTQLGKYP : 356
Hum_CLC-5 : -----PFGNSRLVL YVEFHTP-WHLFEL PFIL- FG LWCAL IRTNIAWCRKR-----KTTQLGKYP : 356
Rat_CLC-3 : -----PFGNSRLVL YVEYHTP-WYLFELF PFIL- FG LWCAF IRANIAWCRRR-----KSTKFGKYP : 369
Hum_CLC-3 : -----PFGNSRLVL YVEYHTP-WYLFELF PFIL- FG LWCAF IRANIAWCRRR-----KSTKFGKYP : 369
Rat_CLC-k1 : -----SEQETITSL KTRFRVDVPFDLPE FFFVA FIC VLSCA LFCQRTFLRFIKTNRYTSRLLATSK : 324
Rat_CLC-2 : -----SEQETITSL KTSFPVDIPFDLPE FFFVA AIC LLSCG NYCQRTSLFFLKSNGFTSKLLATSK : 324
Hum_CLC-k2 : -----SEQETITSL KTSFRVDVPFDLPE FFFVV GLC LLGSA LFCQRIFFGFIRNNRFSSKLLATSK : 324
Rabit_CLC- : -----RDEETITAL KTRFRLDFPFDLQE PAFAV AS FG ALF VYLNRKIVQVMRKQKTINRFLMRKR : 366
Hum.placen : -----RDEETITAL KTRFRLDFPFDLQE PAFAV AS FG ALF VYLNRKIVQVMRKQKTINRFLMRKR : 363
T.californ : -----KDTVTLTAL KTNFRGDIPFDLQE PAFAI AS FF ALF VYLNRQIIVFMRKKNFVTKILKKQR : 325
T.marmorat : -----KDTVTLTAL KTNFRGDIPFDLQE PAFAI AS FF ALF VYLNRQIIVFMRKKNFVTKILKKQR : 325
Ra.muscle_ : -----KDAVTITAL RTNFRMDFPFDLKE PAFAV CC FL AVF VYLHRQVMLGVRKHKALSQFLAKHR : 390
Rat.muscle : -----KDAVTITAL RTNFRMDFPFDLKE PAFAV CC FL AVF VYLHRQVMLGVRKHKALSQFLAKHR : 390
Hum.muscle : -----KDAVTITAL RTNFRMDFPFDLKE PAFAA CC LL AVF VYLHRQVMLGVRKHKALSQFLAKHR : 390
Hum_CLC-1 : -----KDAVTITAL RTNFRMDFPFDLKE PAFAA CC LL AVF VYLHRQVMLGVRKHKALSQFLAKHR : 390
S.cerevisi : -----PFRNGRVI NVTYDRD--WKVQE PIFIA FG LY KYISKWNINFIHFR------KMYLSSWP : 376
5 6G G g


440
*
460
*
480
*
500
Hum_CLC-7 : 455
Rat_CLC-7 : 469
St_STCLC-1 : 431
Nt_CLC-NT1 : 446
At_CLC-c : 446
At_CLC-a : 436
At_CLC : 434
At_CLC-b : 435
At_CLC-d : 431
Rat_CLC-5 : 418
Hum_CLC-5 : 418
Rat_CLC-3 : 432
Hum_CLC-3 : 432
Rat_CLC-k1 : 382
Rat_CLC-2 : 382
Hum_CLC-k2 : 382
Rabit_CLC- : 418
Hum.placen : 415
T.californ : 374
T.marmorat : 374
Ra.muscle_ : 440
Rat.muscle : 440
Hum.muscle : 440
Hum_CLC-1 : 440
S.cerevisi : 428

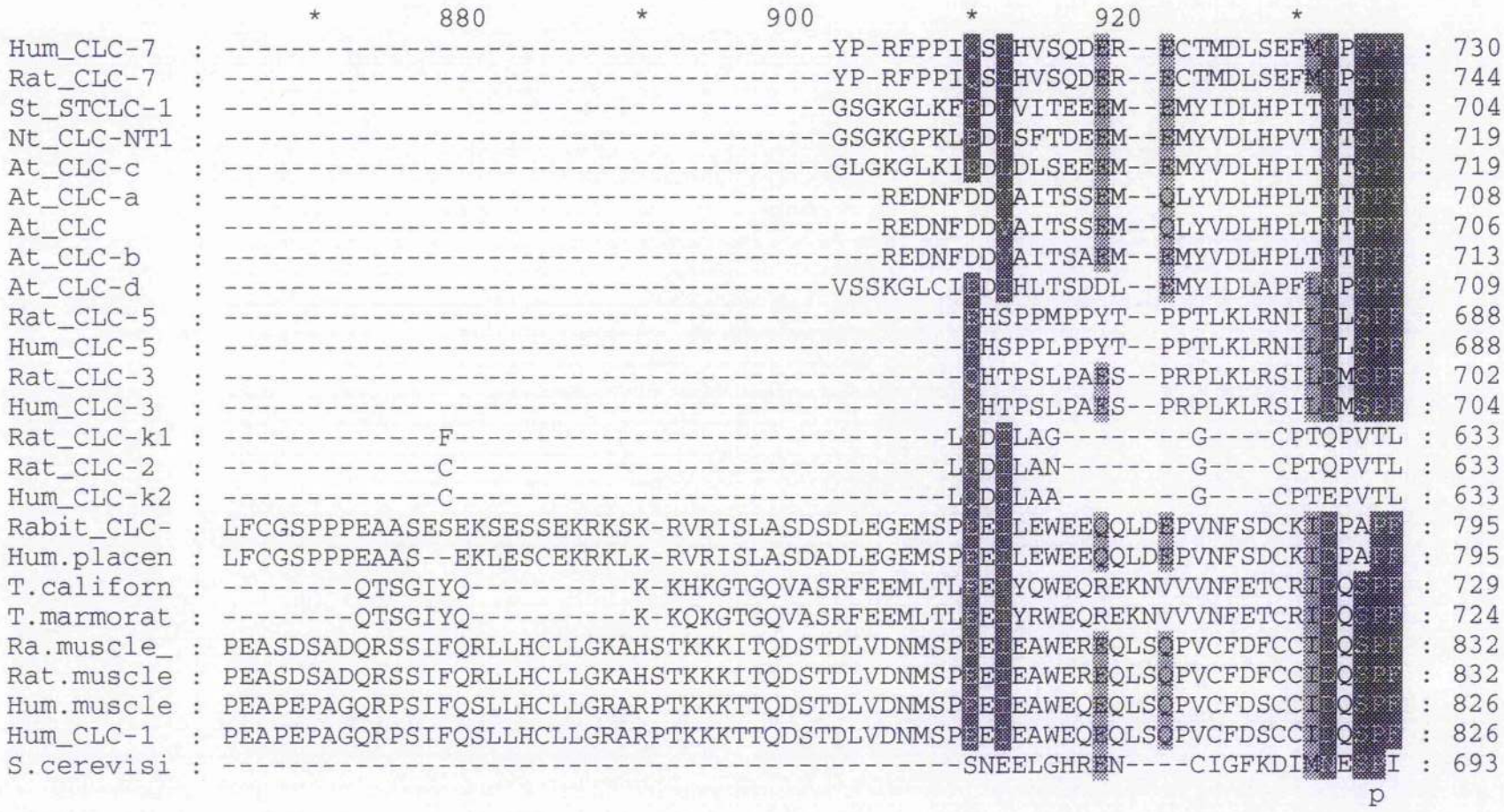

520
540
560
Hum_CLC-7 : KSVVSLFH-DPPGSYNPL : 521
Rat_CLC-7 : KSVVSLFH-DPPGSYNPM : 535
St_STCLC-1 : DAIRNLFSSNNSNEFHIS : 497
Nt_CLC-NT1 : DAIRNLFSSDNSSEFHLS : 512
At_CLC-c : DAIRNLFTSRSENEFHIS : 512
At_CLC-a : DAVRNIFSSNTPNEFGMV : 502
At_CLC : DAVRKHFLFKHSYEFGMV : 500
At_CLC-b : DAVRNLFSSNTPNEFGMG : 501
At_CLC-d : DAIRNLFSAKTMREFSAQ : 499
Rat_CLC-5 : PAGVGVYS-------AMWQ : 483
Hum_CLC-5 : PAGVGVYS-------AMWQ : 483
Rat_CLC-3 : PAGVGVYS-------AIWQ : 497
Hum_CLC-3 : PAGIGVYS-------AIWQ : 497
Rat_CLC-k1 : DPQNLWLEWCHPRFTIFG : 451
Rat_CLC-2 : DPQNLWLEWCHPQMTVFG : 451
Hum_CLC-k2 : DPQHLWWEWYHPRFTIFG : 451
Rabit_CLC- : EPPSTSQAWSPPRANVFL : 487
Hum.placen : EPPSTSQAWNPPRANVFL : 484
T.californ : RGLGNSAQWFIPHLNIFI : 443
T.marmorat : RGLGNSAQWFIPHLNIFI : 443
Ra.muscle_ : KSLGQSAVWIHPQVNVVI : 509
Rat.muscle : KSLGQSAVWIHPQVNVVI : 509
Hum.muscle : ESLGQSAVWIHPRVNVVI : 509
Hum_CLC-1 : ESLGQSAVWIHPRVNVVI : 509
S.cerevisi : DENTHAFEFLK----IFT : 491

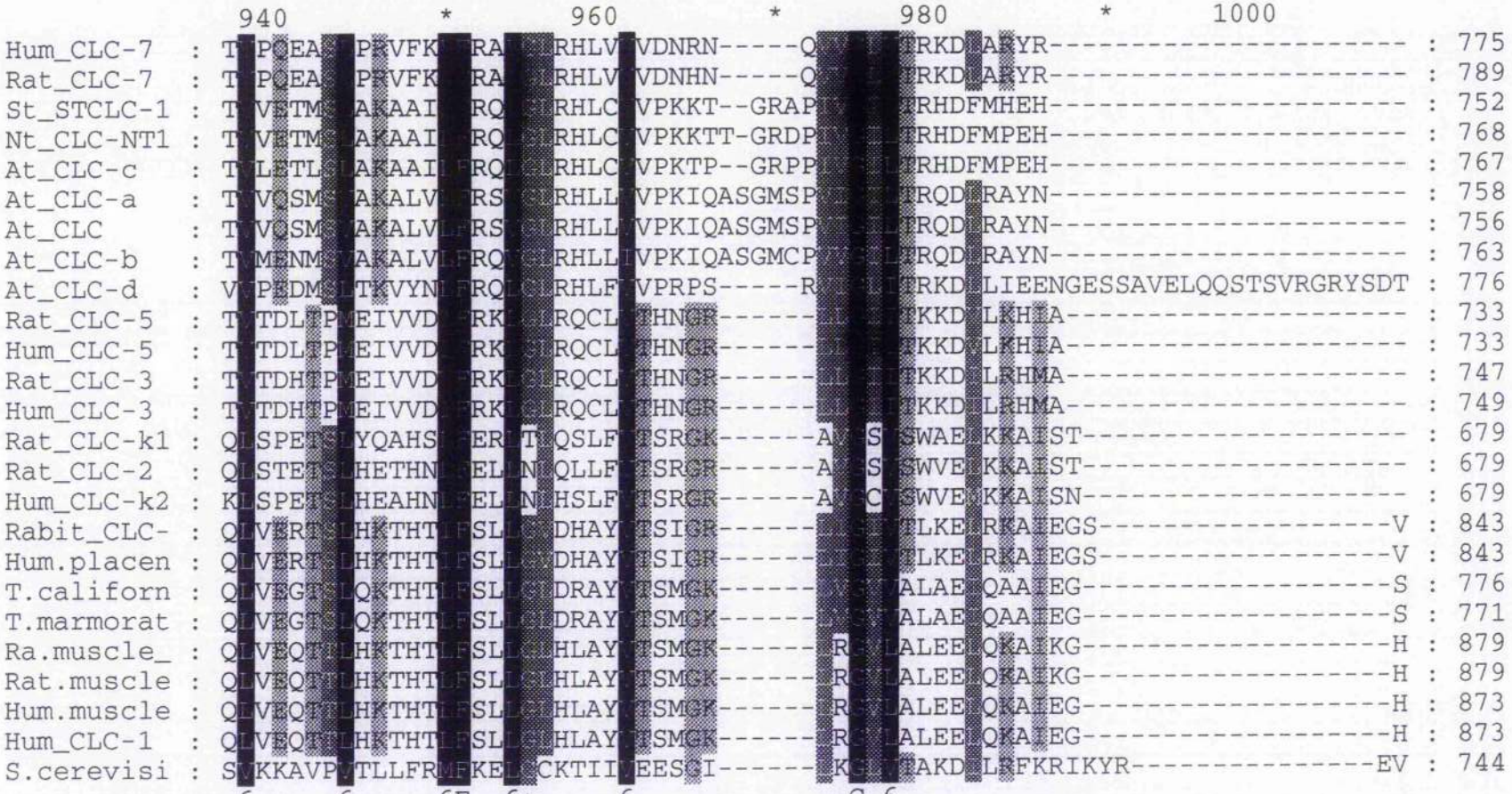

580
600
620
640
Hum_CLC-7 : ---------AAIWADP-KQLMGQLGIRMGLTMMPATSNITYGFP-GLLVLMTAKIIGDVFI- : 582
Rat_CLC-7 : ---------AAIWADP-KQLMGQLGIRMGLTMMPATSNITYGFP-GLLVLMTAKIIGDVFI- : 596
St_STCLC-1 : ----------NL--NVLLSLSFLGTRMGICLLNNLM-GLLLVLESKTVADIFN- : 555
Nt_CLC-NT1 : ----------NL--NNLLLLSFLGTRMGICLLDDLM-GLLLVLESKTVADCFN- : 570
At_CLC-c : ----------QL--DVLLSLSFLGTRMGLCLLETNNLM-GLLMLVLESKTVADCFN- : 570
At_CLC-a : ----------NI--DQGLTVLSLMAGSRMGLCFLETNNLL-GITMFVLLAKTVGDSFN- : 560
At_CLC : ----------NI--DQGLAVLSLMAGSRMGLCFLETNNLL-GITMFVLLAKTVGDSFN- : 558
At_CLC-b : ----------SI--DQGLAVLSLMAGSRMGLCFLETNNLL-GITMFVLLAKTVGDSFN- : 559
At_CLC-d : ---------LNI--EEGTVLLSFLGSRMGLCMVETNNLKL-GLLMLVLESKAVGDAFN- : 558
Rat_CLC-5 : GIFNSWCSQGADCGTPGLAMTACLGVTRMGLVMMFLTGGLEYIVP-LLAAAMTSKWVADALGR : 554
Hum_CLC-5 : TVFNSWCSQGADCGTPGLAMTACLGVTRMGLVMMFLTGGLEYIVP-LLAAAMTSKWVADALGR : 554
Rat_CLC-3 : FIFKEWCEVGADCGTPGLAMTACLGVTRMGLVVFLTGGLEYIVP-LMAAVMTSKWVGDAFGR : 568
Hum_CLC-3 : FIFKEWCEVGADCGTPGLAMTACLGVTRMGLVVFLTGGLEYIVP-LMAAVMTSKWVGDAFGR : 568
Rat_CLC-k1 : ----IVAGREVNPMPGGLAAAFS-AGTHGSTALAAFLTGQLVHAVP-VLMAVLAANAISQNCQ- : 516
Rat_CLC-2 : ----IVAGGKVSPMPGALAAAFS-AGTHGSTALAAFLTGQLVHAVP-VLMAVLAANAICQSYQ- : 516
Hum_CLC-k2 : ----IVAGGITNPMPGGLAAAFS-AGTHGSTALAAFLTGQLVHAVP-VLMAVLAANAIAQSCQ- : 516
Rabit_CLC- : ----IHTDSSTYRVPGGLAVAALA-AGTHGSTAIVFFLTGQLAHILP-VMIAVILANAVAQSLQ- : 552
Hum.placen : ----IHTDSSTYRVTGGLAVAALA-AGTHGSTAIVFFLTGQLAHILP-VMIAVILANAVAQSLQ- : 549
T.californ : ----LVSNGNLYHVLPEVAVAAMT-AGTHASTAICFFLTGQLSHVLP-MLVAILANMVAQGLQ- : 508
T.marmorat : ----LVSNGNLYHVLPEVAVAAMT-AGTHASTAICFFLTGQLSHVLP-MLVAILANMVAQGLQ- : 508
Ra.muscle_ : ----ILFDDIIYKVLPGGLAVAALT-AGSHGSTAICFFLTGQLAHILP-MLVAILANMVAQSLQ- : 574
Rat.muscle : ----ILFDDIIYKVLPGGLAVAALT-AGSHGSTAICFFLTGQLAHILP-MLVAILANMVAQSLQ- : 574
Hum.muscle : ----ILFDDIIYKVLPGGLAVAALT-AGSHGSTAICFFLTGQLAHILP-MLVAILANMVAQSLQ- : 574
Hum_CLC-1 : ----ILFDDIIYKVLPGGLAVAALT-AGSHGSTAICFFLTGQLAHILP-MLVAILANMVAQSLQ- : 574
S.cerevisi : ---------GPSVTIPGAYFFLGAATLSGITNLGLTVVLIMFELTGAFMYILPLMIVVAITRIILSTSGIS : 554

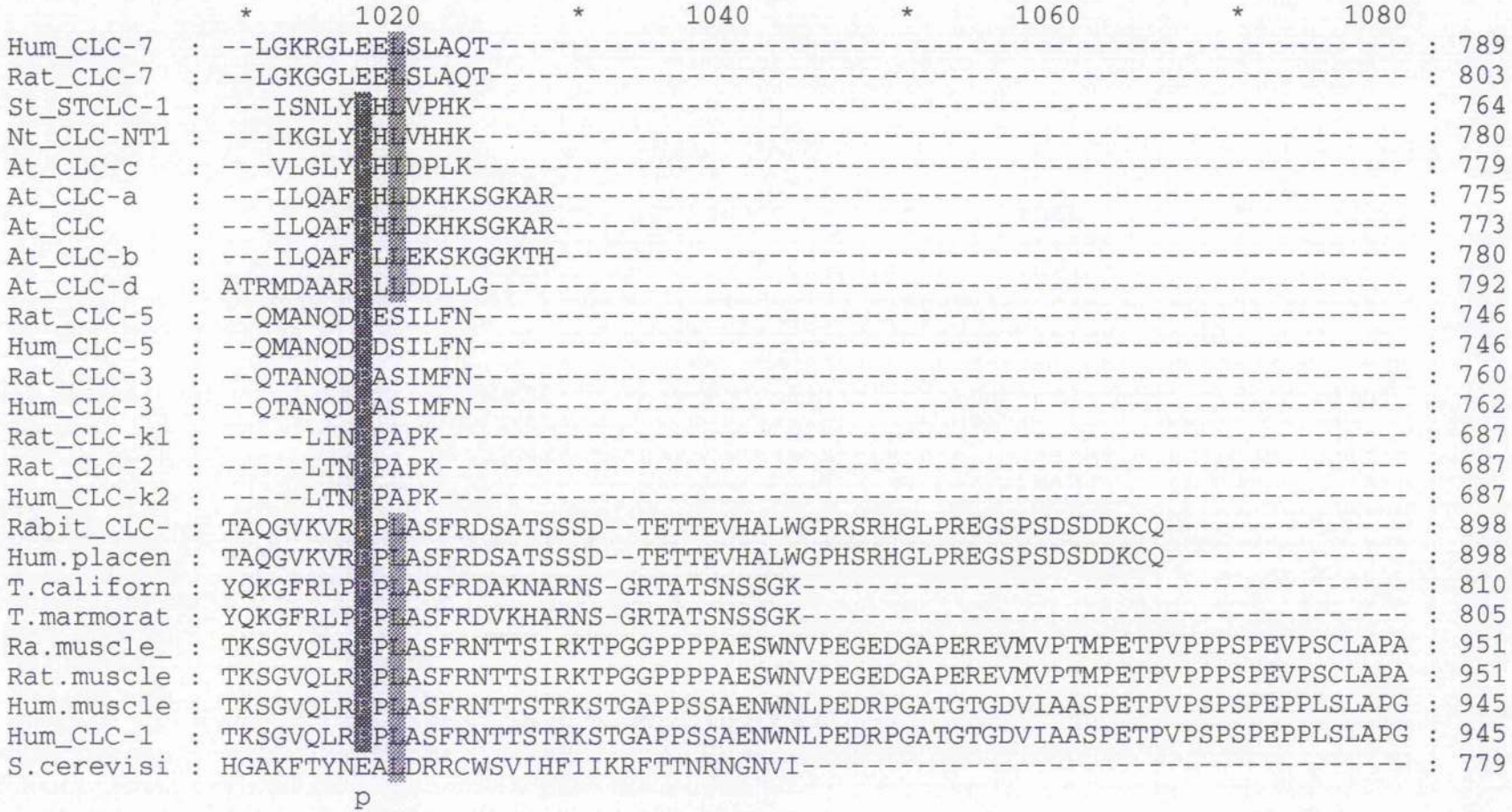

660
680
700
720
Hum_CLC-7 : 642
Rat_CLC-7 : 656
St_STCLC-1 : 613
Nt_CLC-NT1 : 628
At_CLC-c : 628
At_CLC-a : 620
At_CLC : 618
At_CLC-b : 619
At_CLC-d : 617
Rat_CLC-5 : 617
Hum_CLC-5 : 617
Rat_CLC-3 : 631
Hum_CLC-3 : 633
Rat_CLC-k1 : 576
Rat_CLC-2 : 576
Hum_CLC-k2 : 576
Rabit_CLC- : 612
Hum.placen : 609
T.californ : 568
T.marmorat : 568
Ra.muscle_ : 634
Rat.muscle : 634
Hum.muscle : 634
Hum_CLC-1 : 634
S.cerevisi : 620


740
760
780
Hum_CLC-7 : 684
Rat_CLC-7 : 698
St_STCLC-1 : 657
Nt_CLC-NT1 : 672
At_CLC-c : 672
At_CLC-a : 665
At_CLC : 663
At_CLC-b : 670
At_CLC-d : 662
Rat_CLC-5 : 651
Hum_CLC-5 : 651
Rat_CLC-3 : 665
Hum_CLC-3 : 667
Rat_CLC-k1 : 608
Rat_CLC-2 : 608
Hum_CLC-k2 : 608
Rabit_CLC- : 671
Hum.placen : 668
T.californ : 629
T.marmorat : 626
Ra.muscle_ : 697
Rat.muscle : 697
Hum.muscle : 697
Hum_CLC-1 : 697
S.cerevisi : 657

```
                  800         *       820         *       840         *       860
Hum_CLC-7   : -----LVQRRLRLKDFRDA-------------------------------------------------- : 698
Rat_CLC-7   : -----LVQRRLRLKDFRDA-------------------------------------------------- : 712
St_STCLC-1  : -----NILSRFHAFDFAKP-------------------------------------------------- : 671
Nt_CLC-NT1  : -----SILRSFHAFDFAKP-------------------------------------------------- : 686
At_CLC-c    : -----QILRSCKARDFGKA-------------------------------------------------- : 686
At_CLC-a    : -----EVREKFTPVELAE--------------------------------------------------- : 678
At_CLC      : -----EVREKFTPVELAE--------------------------------------------------- : 676
At_CLC-b    : -----EVREKFPWDELAE--------------------------------------------------- : 683
At_CLC-d    : -----ARNIRHSFSEFAKP-------------------------------------------------- : 676
Rat_CLC-5   : -----DGVVSTSIIYFT---------------------------------------------------- : 663
Hum_CLC-5   : -----DGVVSTSIIYFT---------------------------------------------------- : 663
Rat_CLC-3   : -----EGVVGSSRVCFA---------------------------------------------------- : 677
Hum_CLC-3   : -----EGIVGSSRVCFA---------------------------------------------------- : 679
Rat_CLC-k1  : ---------SWAPGQER---------------------------------------------------- : 616
Rat_CLC-2   : ---------SWAPGQQP---------------------------------------------------- : 616
Hum_CLC-k2  : ---------SWAPGHQQ---------------------------------------------------- : 616
Rabit_CLC-  : ----PETSVCFQVKAED-----AQGEPHKPLKPALKRGCSNSVNLG----------ESPTGHVESAGIALRS : 724
Hum.placen  : ----PEASVCFQVNTEDSAFPAARGETHKPLKPALKRGPSVTRNLG----------ESPTGSAESAGIALRS : 726
T.californ  : PG---EAETSFAYIDQEEA-EGQQQREGLEAVKVQTEDPRPPSPVP---------AEEP-----------T- : 676
T.marmorat  : -G---EAESSFAYIDQEDA-EGQQ-REGLEAVKVQTEDPRPPSPVP---------AEEP-----------T- : 671
Ra.muscle_  : PG---GRPESFAFVDEDED-EDVSRKT--ELPQTPTPPPPPPPPLPPQFPIAPSYPEEPNGPLPSH---KQP : 760
Rat.muscle  : PG---GRPESFAFVDEDED-EDVSRKT--ELPQTPTPPPPPPPPLPPQFPIAPSYPEEPNGPLPSH---KQP : 760
Hum.muscle  : PGAPPGRPESFAFVDEDED-EDLSGKS--ELPPSLALHPSTTAPLS---------PEEPNGPLPGH---KQQ : 754
Hum_CLC-1   : PGAPPGRPESFAFVDEDED-EDLSGKS--ELPPSLALHPSTTAPLS---------PEEPNGPLPGH---KQQ : 754
S.cerevisi  : -----STKAQTTLVYFNK-------------------------------------------------- : 670
```

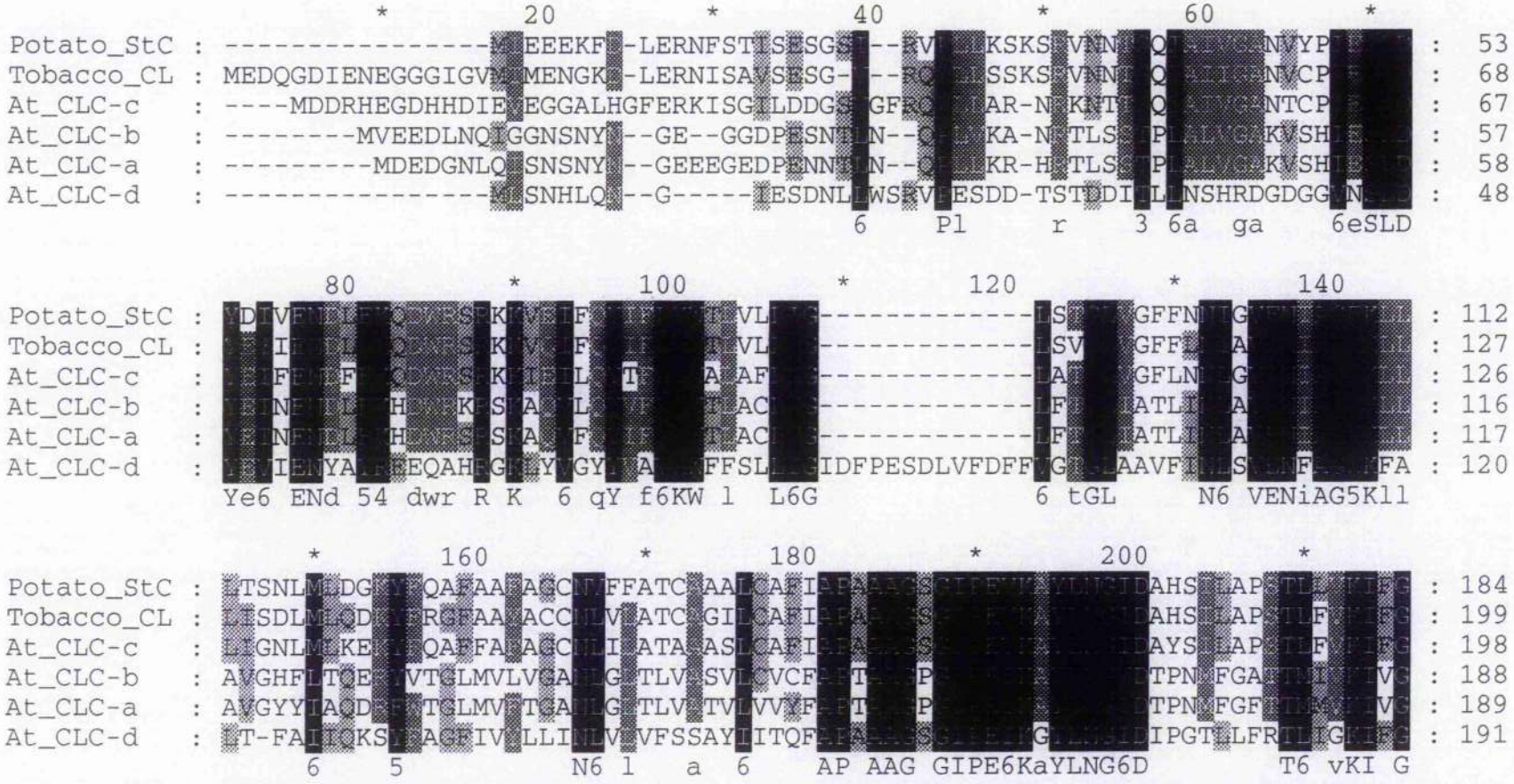

* 880 * 900 * 920 *
Hum_CLC-7 : 730
Rat_CLC-7 : 744
St_STCLC-1 : 704
Nt_CLC-NT1 : 719
At_CLC-c : 719
At_CLC-a : 708
At_CLC : 706
At_CLC-b : 713
At_CLC-d : 709
Rat_CLC-5 : 688
Hum_CLC-5 : 688
Rat_CLC-3 : 702
Hum_CLC-3 : 704
Rat_CLC-k1 : 633
Rat_CLC-2 : 633
Hum_CLC-k2 : 633
Rabit_CLC- : 795
Hum.placen : 795
T.californ : 729
T.marmorat : 724
Ra.muscle_ : 832
Rat.muscle : 832
Hum.muscle : 826
Hum_CLC-1 : 826
S.cerevisi : 693
p

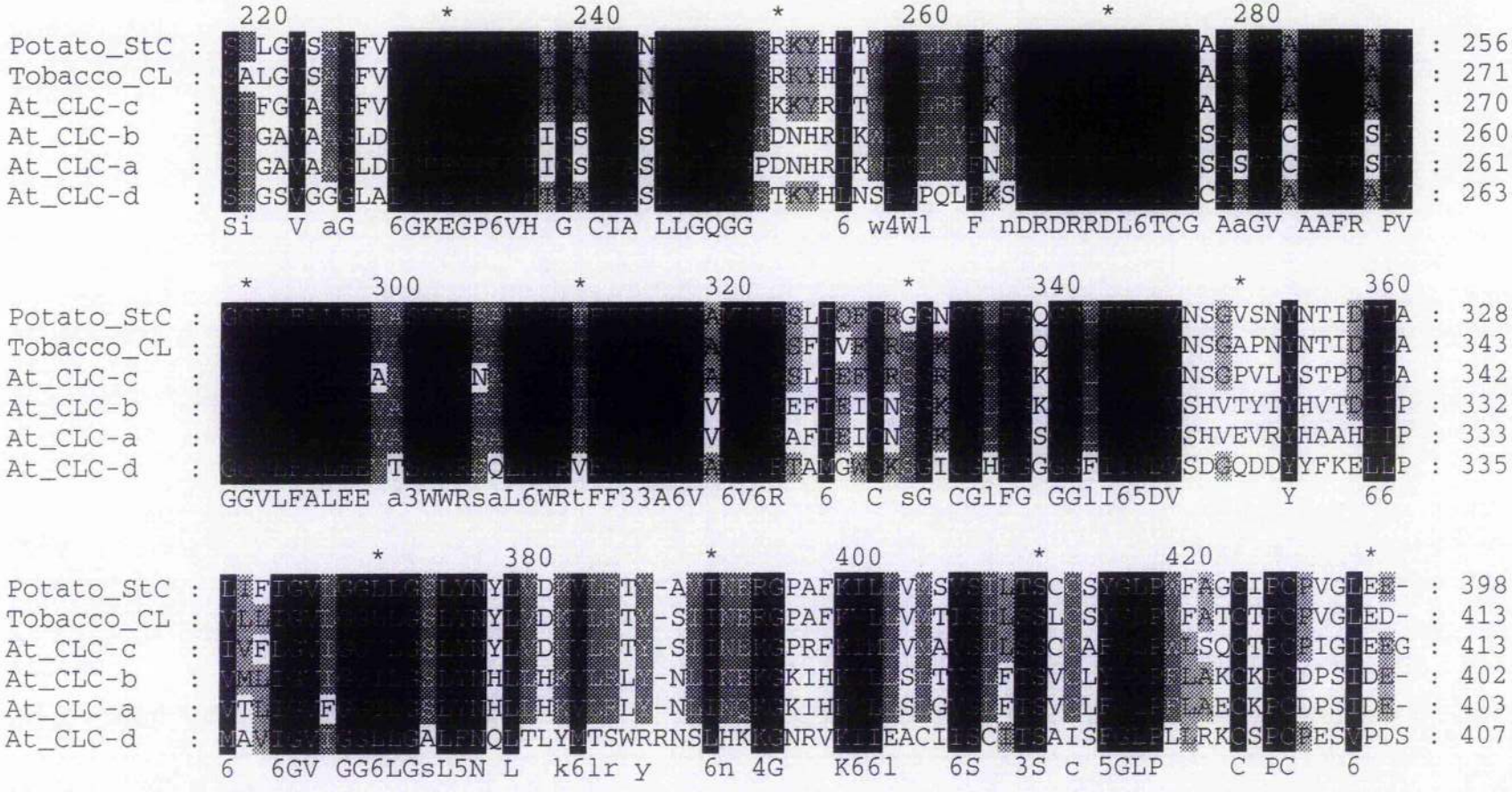

940
960
980
1000
Hum_CLC-7 : 775
Rat_CLC-7 : 789
St_STCLC-1 : 752
Nt_CLC-NT1 : 768
At_CLC-c : 767
At_CLC-a : 758
At_CLC : 756
At_CLC-b : 763
At_CLC-d : 776
Rat_CLC-5 : 733
Hum_CLC-5 : 733
Rat_CLC-3 : 747
Hum_CLC-3 : 749
Rat_CLC-k1 : 679
Rat_CLC-2 : 679
Hum_CLC-k2 : 679
Rabit_CLC- : 843
Hum.placen : 843
T.californ : 776
T.marmorat : 771
Ra.muscle_ : 879
Rat.muscle : 879
Hum.muscle : 873
Hum_CLC-1 : 873
S.cerevisi : 744

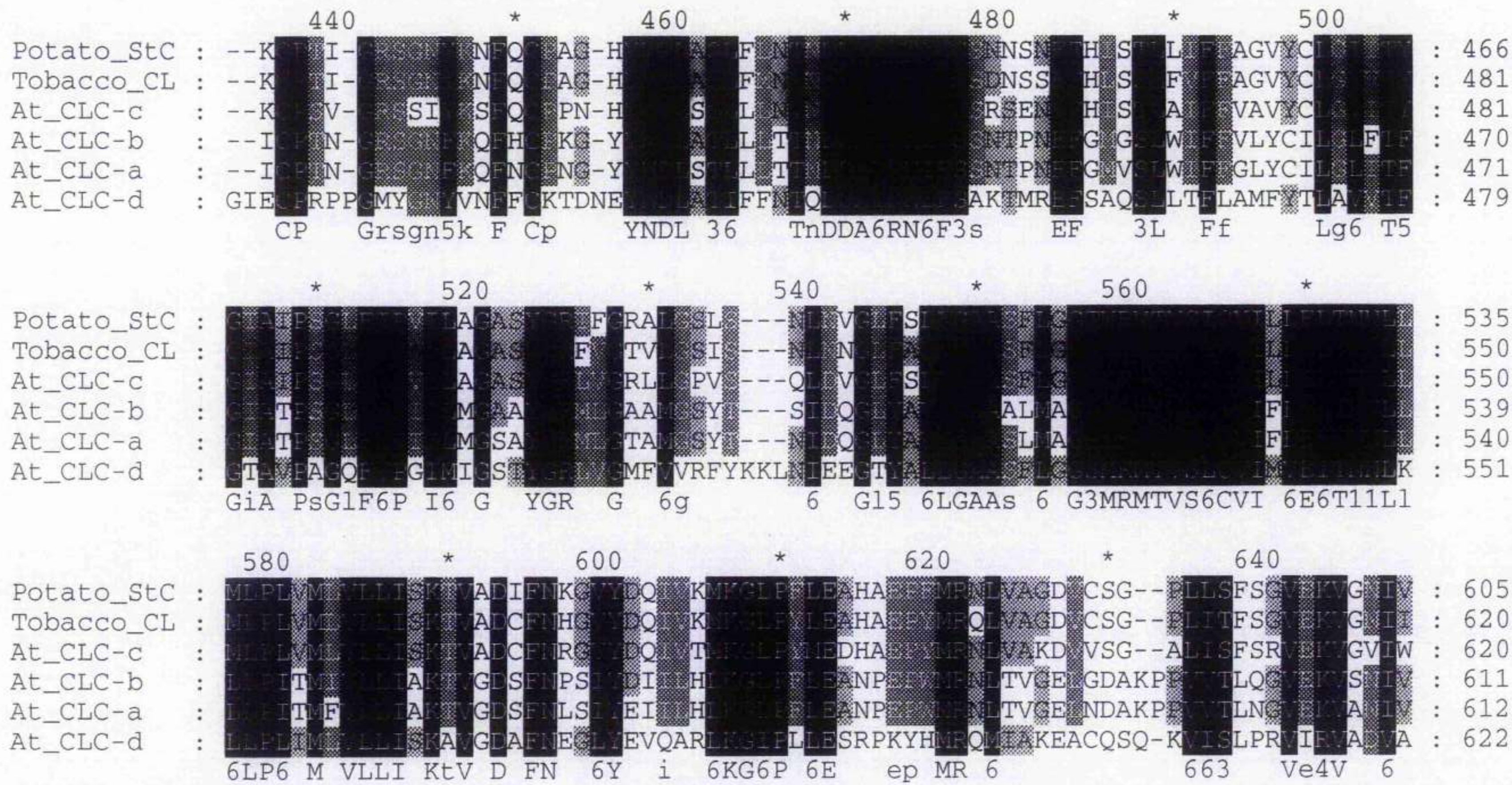

* 1020 * 1040 * 1060 * 1080
Hum_CLC-7 : --LGKRGLEELSLAQT---------- : 789
Rat_CLC-7 : --LGKGGLEELSLAQT---------- : 803
St_STCLC-1 : ---ISNLYTHLVPHK---------- : 764
Nt_CLC-NT1 : ---IKGLYTHLVHHK---------- : 780
At_CLC-c : ---VLGLYTHLDPLK---------- : 779
At_CLC-a : ---ILQAFTHLDKHKSGKAR---------- : 775
At_CLC : ---ILQAFTHLDKHKSGKAR---------- : 773
At_CLC-b : ---ILQAFTLLEKSKGGKTH---------- : 780
At_CLC-d : ATRMDAARTLLDDLLG---------- : 792
Rat_CLC-5 : --QMANQDTESILFN---------- : 746
Hum_CLC-5 : --QMANQDTDSILFN---------- : 746
Rat_CLC-3 : --QTANQDTASIMFN---------- : 760
Hum_CLC-3 : --QTANQDTASIMFN---------- : 762
Rat_CLC-k1 : -----LINTPAPK---------- : 687
Rat_CLC-2 : -----LTNTPAPK---------- : 687
Hum_CLC-k2 : -----LTNTPAPK---------- : 687
Rabit_CLC- : TAQGVKVRTPLASFRDSATSSSD--TETTEVHALWGPRSRHGLPREGSPSDSDDKCQ---------- : 898
Hum.placen : TAQGVKVRTPLASFRDSATSSSD--TETTEVHALWGPHSRHGLPREGSPSDSDDKCQ---------- : 898
T.californ : YQKGFRLPTPLASFRDAKNARNS-GRTATSNSSGK---------- : 810
T.marmorat : YQKGFRLPTPLASFRDVKHARNS-GRTATSNSSGK---------- : 805
Ra.muscle_ : TKSGVQLRTPLASFRNTTSIRKTPGGPPPPAESWNVPEGEDGAPEREVMVPTMPETPVPPPSPEVPSCLAPA : 951
Rat.muscle : TKSGVQLRTPLASFRNTTSIRKTPGGPPPPAESWNVPEGEDGAPEREVMVPTMPETPVPPPSPEVPSCLAPA : 951
Hum.muscle : TKSGVQLRTPLASFRNTTSTRKSTGAPPSSAENWNLPEDRPGATGTGDVIAASPETPVPSPSPEPPLSLAPG : 945
Hum_CLC-1 : TKSGVQLRTPLASFRNTTSTRKSTGAPPSSAENWNLPEDRPGATGTGDVIAASPETPVPSPSPEPPLSLAPG : 945
S.cerevisi : HGAKFTYNEALDRRCWSVIHFIIKRFTTNRNGNVI---------- : 779
p

```
                        *      1100          *      1120
Hum_CLC-7   : ---------------------------------------------- :    -
Rat_CLC-7   : ---------------------------------------------- :    -
St_STCLC-1  : ---------------------------------------------- :    -
Nt_CLC-NT1  : ---------------------------------------------- :    -
At_CLC-c    : ---------------------------------------------- :    -
At_CLC-a    : ---------------------------------------------- :    -
At_CLC      : ---------------------------------------------- :    -
At_CLC-b    : ---------------------------------------------- :    -
At_CLC-d    : ---------------------------------------------- :    -
Rat_CLC-5   : ---------------------------------------------- :    -
Hum_CLC-5   : ---------------------------------------------- :    -
Rat_CLC-3   : ---------------------------------------------- :    -
Hum_CLC-3   : ---------------------------------------------- :    -
Rat_CLC-k1  : ---------------------------------------------- :    -
Rat_CLC-2   : ---------------------------------------------- :    -
Hum_CLC-k2  : ---------------------------------------------- :    -
Rabit_CLC-  : ---------------------------------------------- :    -
Hum.placen  : ---------------------------------------------- :    -
T.californ  : ---------------------------------------------- :    -
T.marmorat  : ---------------------------------------------- :    -
Ra.muscle_  : RVEGELEELEMVGNLGPEEDLADILHGPSLRSTDEEDEDELIL : 994
Rat.muscle  : RVEGELEELEMVGNLGPEEDLADILHGPSLRSTDEEDEDELIL : 994
Hum.muscle  : KVEGELEELELVESPGLEEELADILQGPSLRSTDEEDEDELIL : 988
Hum_CLC-1   : KVEGELEELELVESPGLEEELADILQGPSLRSTDEEDEDELIL : 988
S.cerevisi  : ---------------------------------------------- :    -
```

Figure A.3. CLUSTALW Alignment of 6 Putative Plant *CLC*-type Cl^- Channels.
The sequences of six putative plant voltage-gated Cl^- channels are deposited in the database. The translations of these six sequences were aligned using the CLUSTALW programe at the EMBL European Bioinformatics Institute, Cambridge. The sequence abbreviations are as follows: Nt_CLC-NT1, *Nicotiana tabaccum clc-Nt1*: St_STCLC1, *Solanum tuberosum StCLC1*:At_CLC a-d, *Arabidopsis thaliana CLC-a, CLC-b, CLC-c* and *CLC-d.*


Potato_StC
Tobacco_CL
At_CLC-c
At_CLC-b
At_CLC-a
At_CLC-d


220 * 240 * 260 * 280
Potato_StC : 256
Tobacco_CL : 271
At_CLC-c : 270
At_CLC-b : 260
At_CLC-a : 261
At_CLC-d : 263
Si V aG 6GKEGP6VH G CIA LLGQGG 6 w4Wl F nDRDRRDL6TCG AaGV AAFR PV
* 300 * 320 * 340 * 360
Potato_StC : 328
Tobacco_CL : 343
At_CLC-c : 342
At_CLC-b : 332
At_CLC-a : 333
At_CLC-d : 335
GGVLFALEE a3WWRsaL6WRtFF33A6V 6V6R 6 C sG CGlFG GGlI65DV Y 66
* 380 * 400 * 420 *
Potato_StC : 398
Tobacco_CL : 413
At_CLC-c : 413
At_CLC-b : 402
At_CLC-a : 403
At_CLC-d : 407
6 6GV GG6LGsL5N L k6lr y 6n 4G K66l 6S 3S c 5GLP C PC 6


440 460 480 500
Potato_StC : 466
Tobacco_CL : 481
At_CLC-c : 481
At_CLC-b : 470
At_CLC-a : 471
At_CLC-d : 479
520 540 560
Potato_StC : 535
Tobacco_CL : 550
At_CLC-c : 550
At_CLC-b : 539
At_CLC-a : 540
At_CLC-d : 551
580 600 620 640
Potato_StC : 605
Tobacco_CL : 620
At_CLC-c : 620
At_CLC-b : 611
At_CLC-a : 612
At_CLC-d : 622

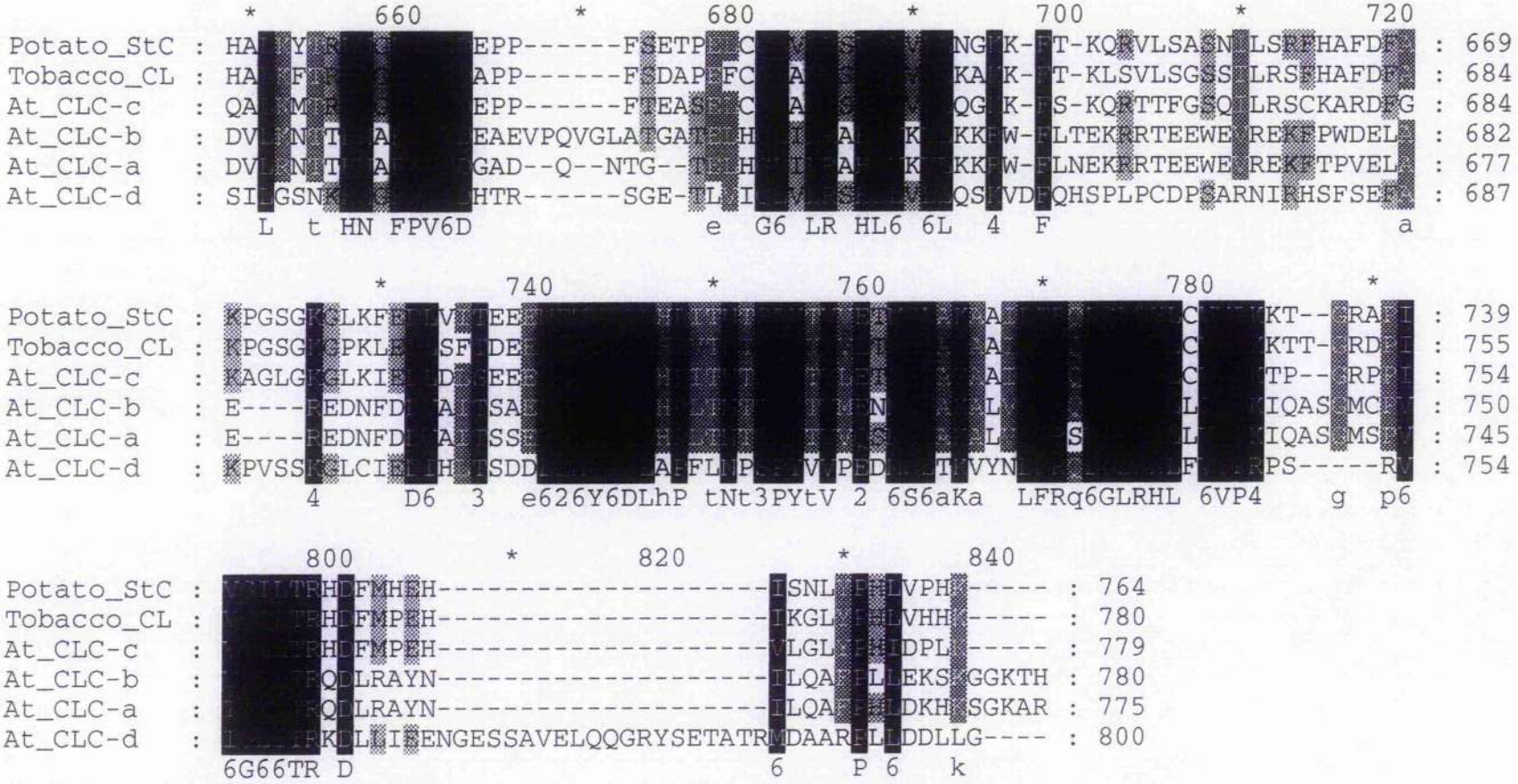
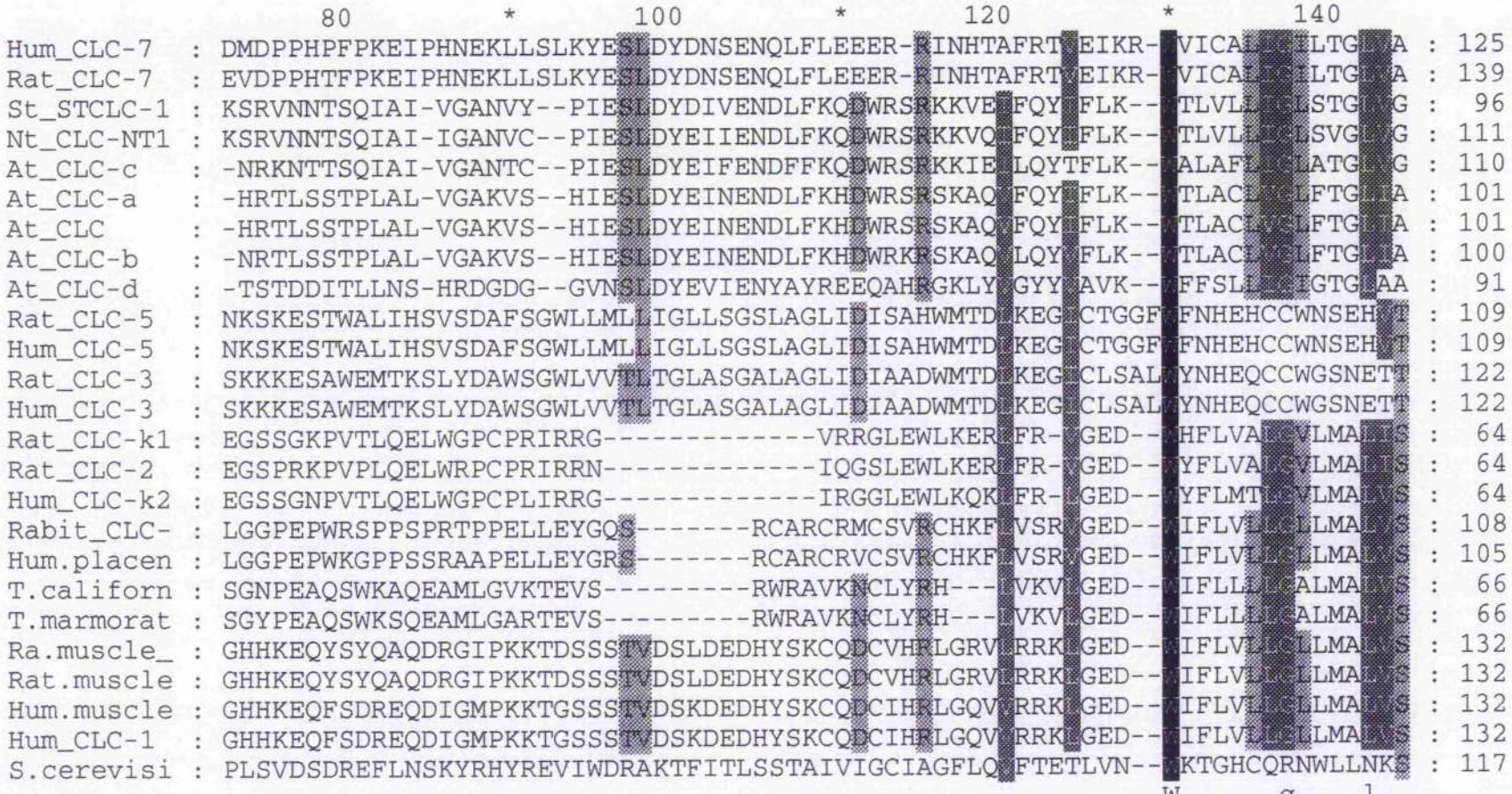

660
680
700
720
Potato_StC : 669
Tobacco_CL : 684
At_CLC-c : 684
At_CLC-b : 682
At_CLC-a : 677
At_CLC-d : 687
740
760
780
Potato_StC : 739
Tobacco_CL : 755
At_CLC-c : 754
At_CLC-b : 750
At_CLC-a : 745
At_CLC-d : 754
800
820
840
Potato_StC : 764
Tobacco_CL : 780
At_CLC-c : 779
At_CLC-b : 780
At_CLC-a : 775
At_CLC-d : 800

Figure A.4. CLUSTALW Alignment of Putative *CLC*-type Cl^- Channels.

The sequences of animals and plants voltage-gated Cl^- channels are deposited in the databases. The translations of these sequences were aligned using the CLUSTALW programe at the EMBL European Bioinformatics Institute, Cambridge. The sequence names are as follows: Human CLC-7, Rat CLC-7, Human CLC-5, Ratm CLC-5, Human CLC-3, Rat CLC-3, Human CLC-k2, Rat CLC-k2, Rat CLC-k1, Rabit CLC, Human placenta CLC, *T. califoria*, *T. marmorata*, Rat. Muscle CLC, Human muscle CLC, Human CLC-1, *S. cerevisiae* GEF1, NtCLC NT1 (*Nicotiana tabaccum*) St STCLC1, (*Solanum tuberosum*), At CLC a-d (*Arabidopsis thaliana CLC-a, CLC-b, CLC-c* and *CLC-d*).


* 20 * 40 * 60 *
Hum_CLC-7 : ----------DEEAAPLRRTARPGGGTPLLNGAGPGAARQSPRSALFRVGHMSSVELDDELLDP : 55
Rat_CLC-7 : -MANVSKKVSWSGRDRDDEEGAPLRRTGQPDEETPLLNGAGPG-ARQS-HSALFRIGQMNNVELDDELLDP : 69
St_STCLC-1 : ----------MIEEEKFD-LERNFSTISES----------GSI-----RVPLLKS : 29
Nt_CLC-NT1 : --------MEDQGDIENEGGGIGVMIMENGKD-LERNISAVSES----------G-V-----RQPLLSS : 44
At_CLC-c : ----------MDDRHEGDHHDIEVEGGALHGFERKISGILDD----------GSVG---FRQPLLAR : 44
At_CLC-a : ----------MDEDGNLQISNSNYNGEEEG----------EDPENNTLNQPLLKR : 35
At_CLC : ----------MDEDGNLQISNSNYNGEEEG----------EDPENNTLNQPLLKR : 35
At_CLC-b : ----------MEEDLNQIGGNSNYNG--EG----------GDPESNTLNQPLVKA : 34
At_CLC-d : ----------MLSNHLQNGIESD----------NLLWS---RVPESDD : 25
Rat_CLC-5 : ----------MDFEEPIPGVGTYDDFNTIDWVR----------EKSRDR---DRHREIT : 37
Hum_CLC-5 : ----------MDFEEPIPGVGTYDDFNTIDWVR----------EKSRDR---DRHREIT : 37
Rat_CLC-3 : --------MTNGGSINSSTHLLDLDEPIPGVGTYDDFHTIDWVR----------EKCKDR---ERHRRIN : 50
Hum_CLC-3 : --------MTNGGSINSSTHLLDLDEPIPGVGTYDDFHTIDWVR----------EKCKDR---ERHRRIN : 50
Rat_CLC-k1 : ----------MEELVGLR : 8
Rat_CLC-2 : ----------MEEIVGLR : 8
Hum_CLC-k2 : ----------MEEFVGLR : 8
Rabit_CLC- : ----------MAAPAAAAVEEGMEPRALQYEQTLMYG----------RYTQDLGAFAKEEAARIR : 45
Hum.placen : ----------MAAAAAEEGMEPRALQHEQTLMYG----------RYTQDLGAFAKEEAARIR : 42
T.californ : ----------MSHEKNEA : 8
T.marmorat : ----------MSHEKNEA : 8
Ra.muscle_ : MERSQSQQHGGEQSWWGTAPQYQYMPFEHCTSYGLPSENGGLQH----------RPRKDLGPRHNAHPTQIY : 62
Rat.muscle : MERSQSQQHGGEQSWWGTAPQYQYMPFEHCTSYGLPSENGGLQH----------RPRKDLGPRHNAHPTQIY : 62
Hum.muscle : MEQSRSQQRGGEQSWWGSDPQYQYMPFEHCTSYGLPSENGGLQH----------RLRKDAGPRHNVHPTQIY : 62
Hum_CLC-1 : MEQSRSQQRGGEQSWWGSDPQYQYMPFEHCTSYGLPSENGGLQH----------RLRKDAGPRHNVHPTQIY : 62
S.cerevisi : ----------MPTTYVPINQPIRDGEDVIDTNRFTNIPETQN----------FDQFVTIDKIAEENR : 47


80 * 100 * 120 * 140
Hum_CLC-7 : DMDPPHPFPKEIPHNEKLLSLKYESLDYDNSENQLFLEEER-RINHTAFRTWEIKR- VICALLGLLTGLVA : 125
Rat_CLC-7 : EVDPPHTFPKEIPHNEKLLSLKYESLDYDNSENQLFLEEER-RINHTAFRTWEIKR- VICALLGLLTGLVA : 139
St_STCLC-1 : KSRVNNTSQIAI-VGANVY--PIESLDYDIVENDLFKQDWRSRKKVEIFQYTFLK- TLVLLLGLSTGLVG : 96
Nt_CLC-NT1 : KSRVNNTSQIAI-IGANVC--PIESLDYEIIENDLFKQDWRSRKKVQIFQYTFLK- TLVLLLGLSVGLVG : 111
At_CLC-c : -NRKNTTSQIAI-VGANTC--PIESLDYEIFENDFFKQDWRSRKKIELQYTFLK- ALAFLLGLATGLVG : 110
At_CLC-a : -HRTLSSTPLAL-VGAKVS--HIESLDYEINENDLFKHDWRSRSKAQVFQYTFLK- TLACLVGLFTGLIA : 101
At_CLC : -HRTLSSTPLAL-VGAKVS--HIESLDYEINENDLFKHDWRSRSKAQVFQYTFLK- TLACLVGLFTGLIA : 101
At_CLC-b : -NRTLSSTPLAL-VGAKVS--HIESLDYEINENDLFKHDWRKRSKAQVLQYTFLK- TLACLVGLFTGLIA : 100
At_CLC-d : -TSTDDITLLNS-HRDGDG--GVNSLDYEVIENYAYREEQAHRGKLYIGYYIAVK- FFSLLIGIGTGLAA : 91
Rat_CLC-5 : NKSKESTWALIHSVSDAFSGWLLMLLIGLLSGSLAGLIDISAHWMTDLKEGICTGGFVFNHEHCCWNSEHYT : 109
Hum_CLC-5 : NKSKESTWALIHSVSDAFSGWLLMLLIGLLSGSLAGLIDISAHWMTDLKEGICTGGFVFNHEHCCWNSEHYT : 109
Rat_CLC-3 : SKKKESAWEMTKSLYDAWSGWLVVTLTGLASGALAGLIDIAADWMTDLKEGICLSALVYNHEQCCWGSNETT : 122
Hum_CLC-3 : SKKKESAWEMTKSLYDAWSGWLVVTLTGLASGALAGLIDIAADWMTDLKEGICLSALVYNHEQCCWGSNETT : 122
Rat_CLC-k1 : EGSSGKPVTLQELWGPCPRIRRG----------VRRGLEWLKERLFR-VGED--VHFLVALGVLMALLS : 64
Rat_CLC-2 : EGSPRKPVPLQELWRPCPRIRRN----------IQGSLEWLKERLFR-VGED--VYFLVALGVLMALLS : 64
Hum_CLC-k2 : EGSSGNPVTLQELWGPCPLIRRG----------IRGGLEWLKQKLFR-LGED--VYFLMTLGVLMALVS : 64
Rabit_CLC- : LGGPEPWRSPPSPRTPPELLEYGQS-------RCARCRMCSVRCHKFLVSRVGED--VIFLVLLGLLMALVS : 108
Hum.placen : LGGPEPWKGPPSSRAAPELLEYGRS-------RCARCRVCSVRCHKFLVSRVGED--VIFLVLLGLLMALVS : 105
T.californ : SGNPEAQSWKAQEAMLGVKTEVS---------RWRAVKNCLYRH---LVKVLGED--VIFLLLLGALMALVS : 66
T.marmorat : SGYPEAQSWKSQEAMLGARTEVS---------RWRAVKNCLYRH---LVKVLGED--VIFLLLLGALMALVS : 66
Ra.muscle_ : GHHKEQYSYQAQDRGIPKKTDSSSTVDSLDEDHYSKCQDCVHRLGRVLRRKLGED--VIFLVLLGLLMALVS : 132
Rat.muscle : GHHKEQYSYQAQDRGIPKKTDSSSTVDSLDEDHYSKCQDCVHRLGRVLRRKLGED--VIFLVLLGLLMALVS : 132
Hum.muscle : GHHKEQFSDREQDIGMPKKTGSSSTVDSKDEDHYSKCQDCIHRLGQVLRRKLGED--VIFLVLLGLLMALVS : 132
Hum_CLC-1 : GHHKEQFSDREQDIGMPKKTGSSSTVDSKDEDHYSKCQDCIHRLGQVLRRKLGED--VIFLVLLGLLMALVS : 132
S.cerevisi : PLSVDSDREFLNSKYRHYREVIWDRAKTFITLSSTAIVIGCIAGFLQVFTETLVN--VKTGHCQRNWLLNKS : 117
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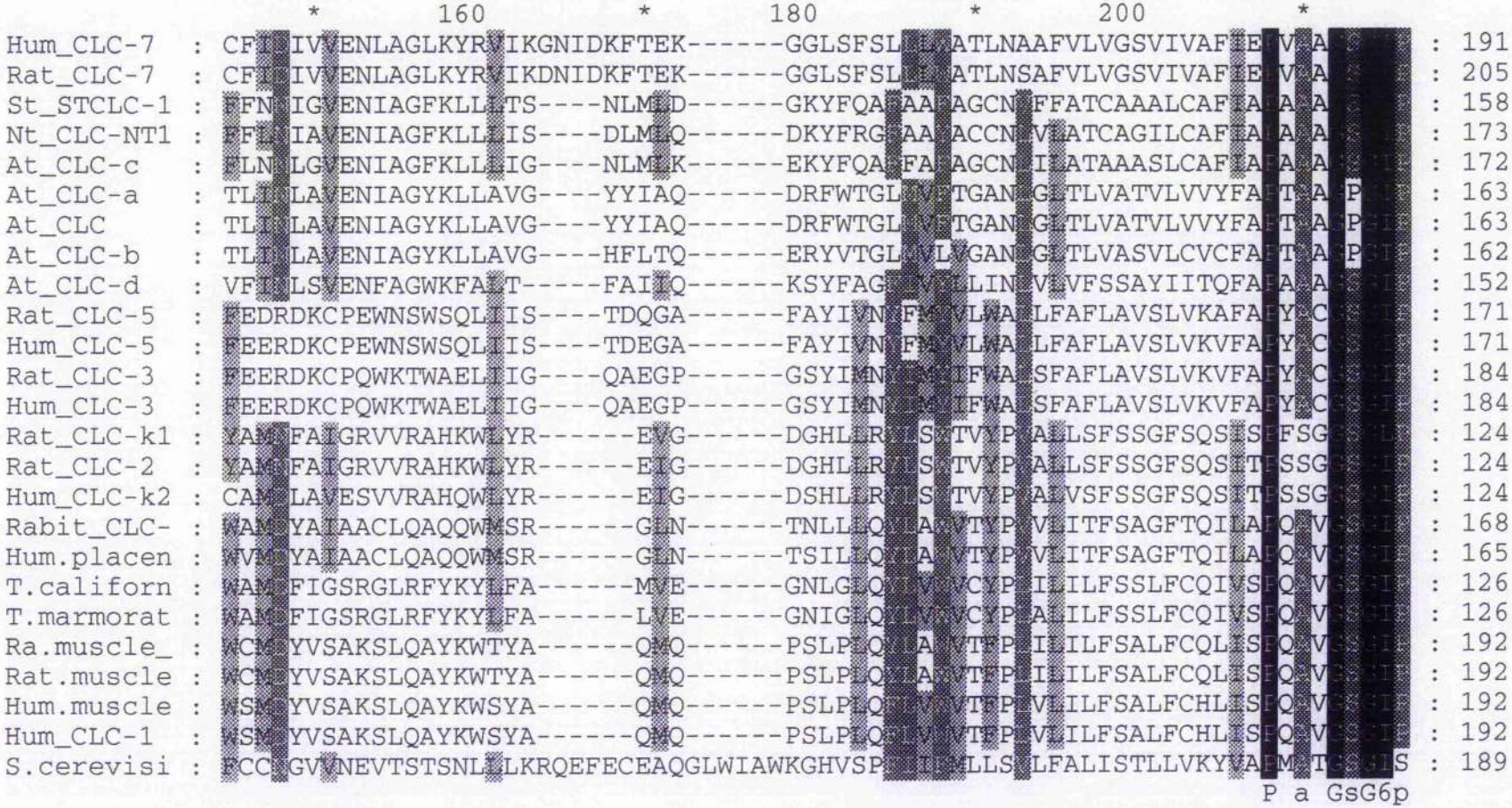
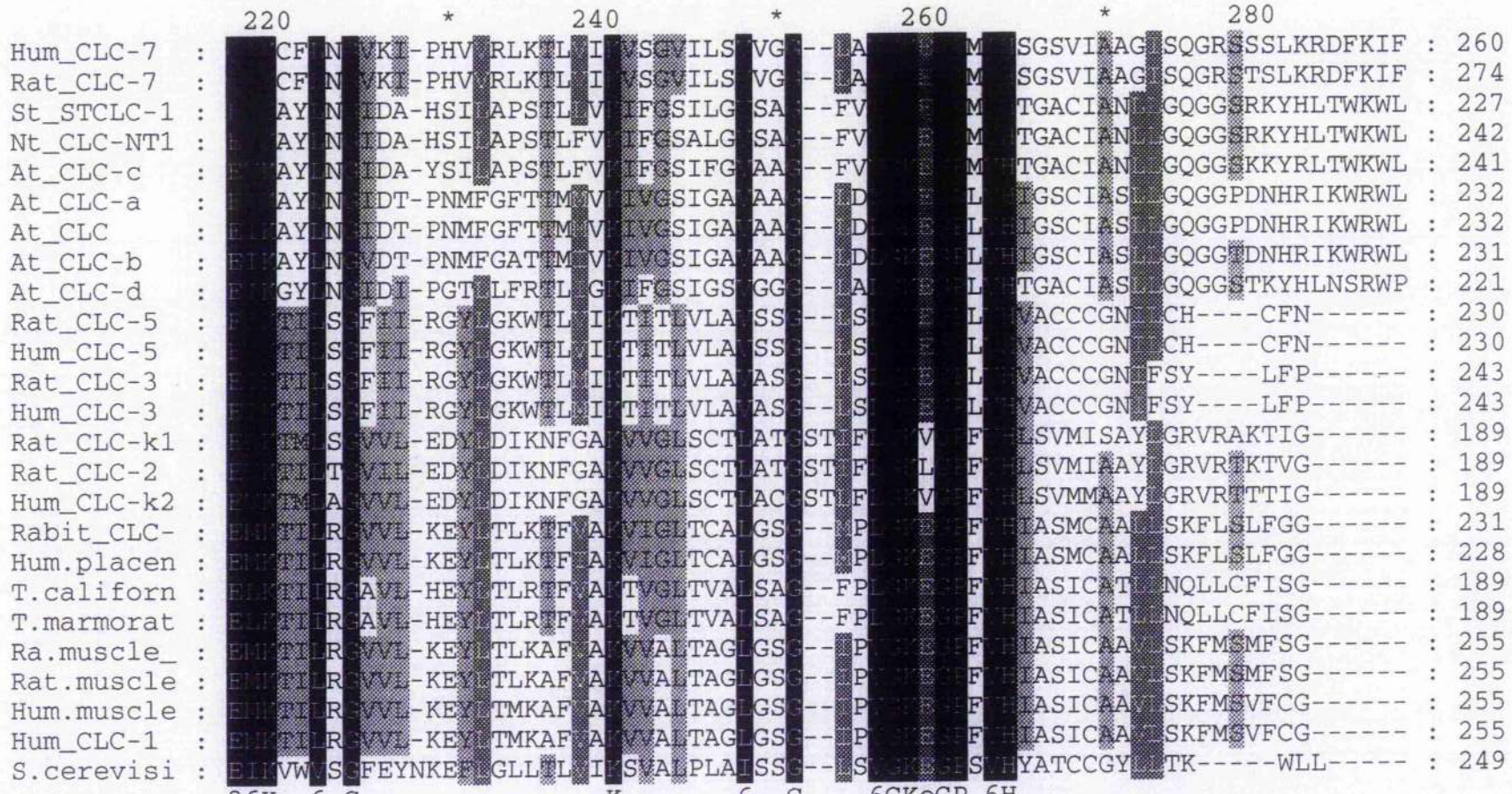
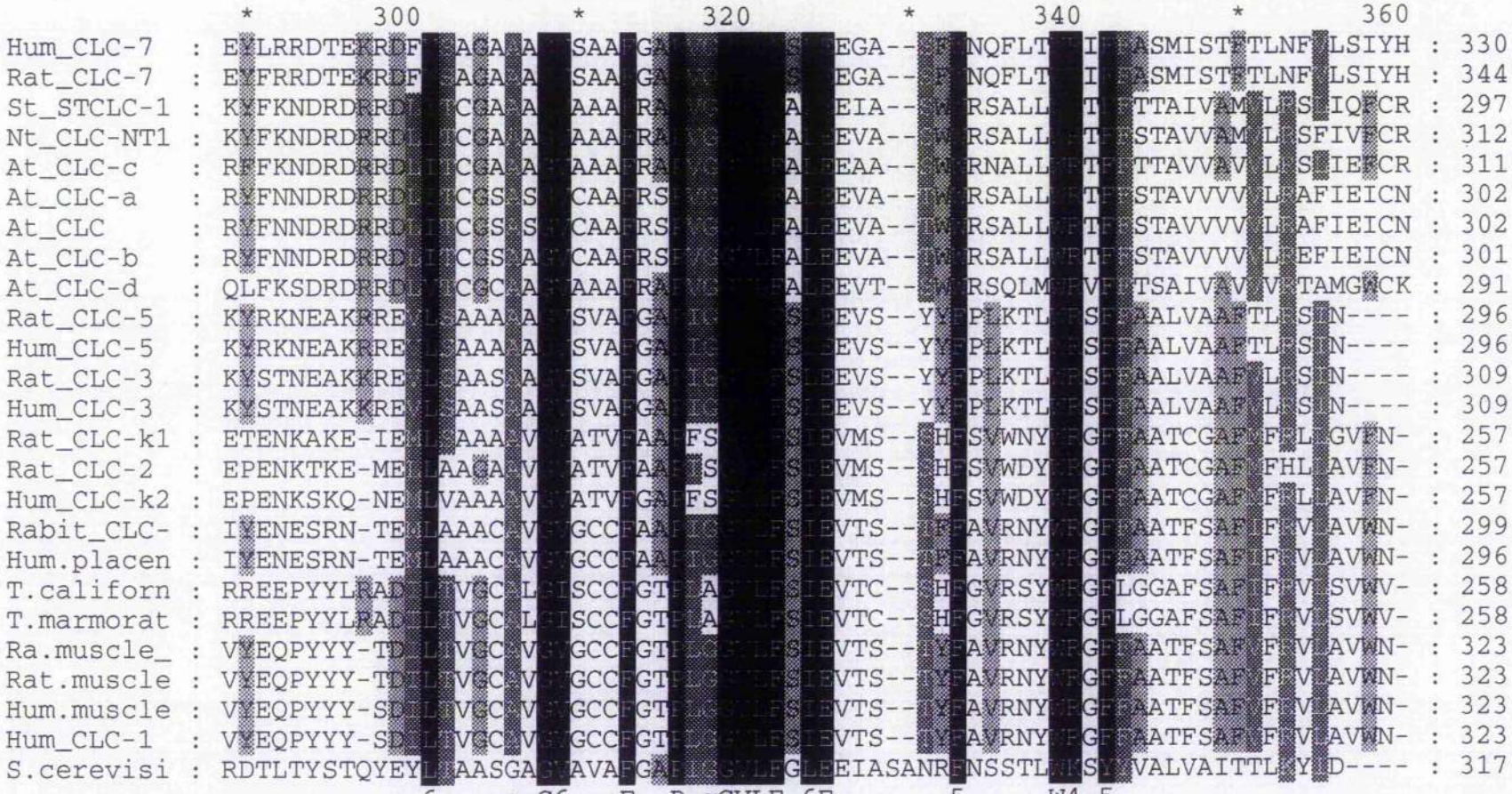
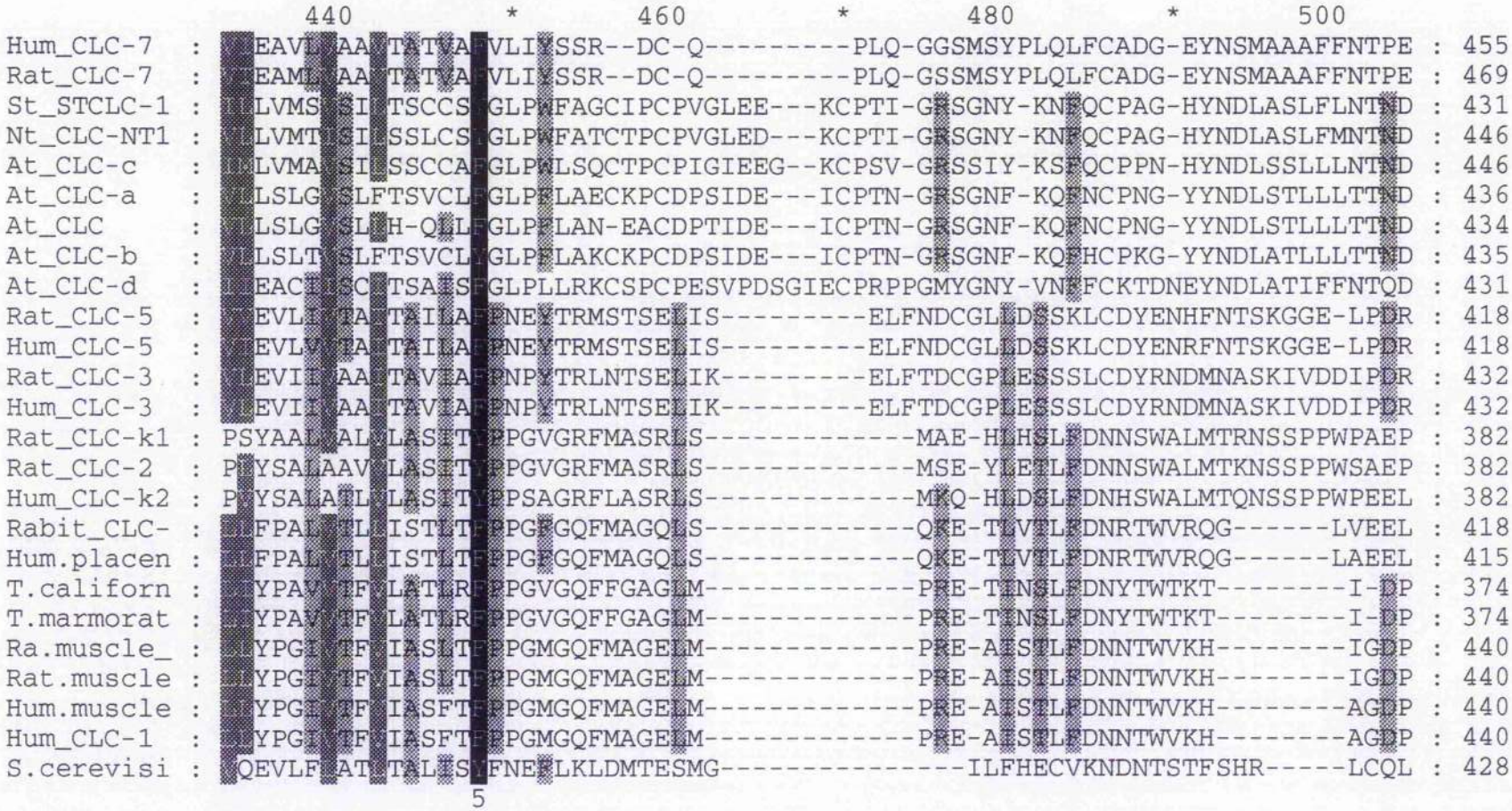
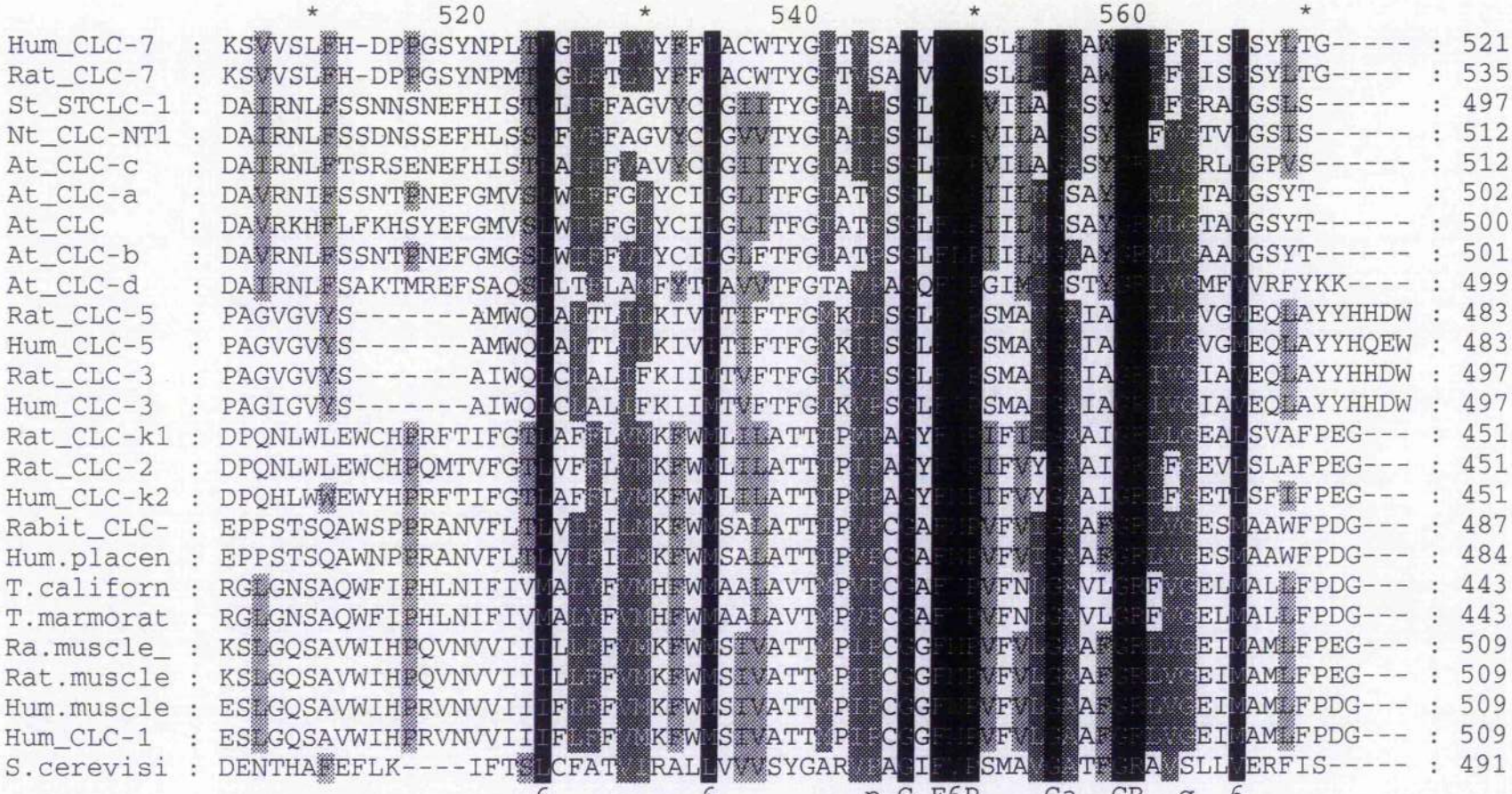

160
180
200
Hum_CLC-7 : 191
Rat_CLC-7 : 205
St_STCLC-1 : 158
Nt_CLC-NT1 : 173
At_CLC-c : 172
At_CLC-a : 163
At_CLC : 163
At_CLC-b : 162
At_CLC-d : 152
Rat_CLC-5 : 171
Hum_CLC-5 : 171
Rat_CLC-3 : 184
Hum_CLC-3 : 184
Rat_CLC-k1 : 124
Rat_CLC-2 : 124
Hum_CLC-k2 : 124
Rabit_CLC- : 168
Hum.placen : 165
T.californ : 126
T.marmorat : 126
Ra.muscle_ : 192
Rat.muscle : 192
Hum.muscle : 192
Hum_CLC-1 : 192
S.cerevisi : 189
P a GsG6p

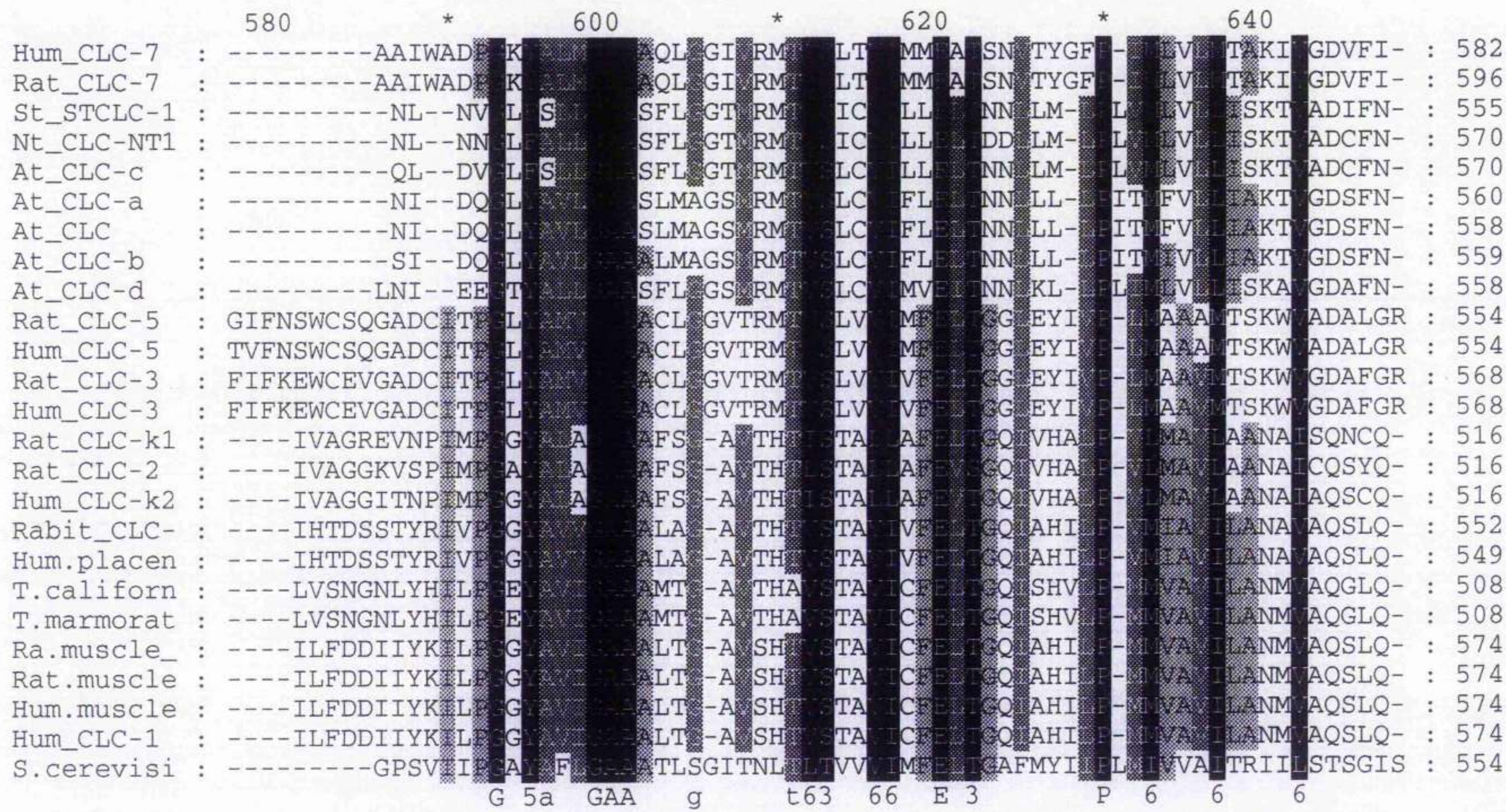

220 * 240 * 260 * 280
Hum_CLC-7 : CFNVKI-PHVRLKTLIVSGVILSVG--LA M SGSVIAAGSQGRSSSLKRDFKIF : 260
Rat_CLC-7 : CFNVKI-PHVRLKTLIVSGVILSVG--LA M SGSVIAAGSQGRSTSLKRDFKIF : 274
St_STCLC-1 : AYNIDA-HSILAPSTLVIFGSILGSA--FV M TGACIANLGQGGSRKYHLTWKWL : 227
Nt_CLC-NT1 : AYNIDA-HSILAPSTLFVIFGSALGSA--FV M TGACIANLGQGGSRKYHLTWKWL : 242
At_CLC-c : AYNIDA-YSILAPSTLFVIFGSIFGAAG--FV M TGACIANLGQGGSKKYRLTWKWL : 241
At_CLC-a : AYNIDT-PNMFGFTTMVIVGSIGALAAG--LD L IGSCIASLGQGGPDNHRIKWRWL : 232
At_CLC : AYNIDT-PNMFGFTTMVIVGSIGALAAG--LD L IGSCIASLGQGGPDNHRIKWRWL : 232
At_CLC-b : AYNVDT-PNMFGATTMVIVGSIGALAAG--LD L IGSCIASLGQGGTDNHRIKWRWL : 231
At_CLC-d : GYNIDI-PGTLFRTLGIIFGSIGSVGG--LA L TGACIASLGQGGSTKYHLNSRWP : 221
Rat_CLC-5 : TLSFII-RGYLGKWTLIITTLVLAVSS--LS L VACCCGNLCH----CFN------ : 230
Hum_CLC-5 : TLSFII-RGYLGKWTLIITTLVLAVSS--LS L VACCCGNLCH----CFN------ : 230
Rat_CLC-3 : TLSFII-RGYLGKWTLIITTLVLAVAS--LS L VACCCGNLFSY----LFP------ : 243
Hum_CLC-3 : TLSFII-RGYLGKWTLIITTLVLAVAS--LS L VACCCGNLFSY----LFP------ : 243
Rat_CLC-k1 : TMLSVVL-EDYLDIKNFGAVVGLSCTLATGSTLFL V F LSVMISAYGRVRAKTIG------ : 189
Rat_CLC-2 : TLLTVIL-EDYLDIKNFGAVVGLSCTLATGSTLFL L F LSVMIAAYGRVRTKTVG------ : 189
Hum_CLC-k2 : TMLAVVL-EDYLDIKNFGAVVGLSCTLACGSTLFL V F LSVMMAAYGRVRTTTIG------ : 189
Rabit_CLC- : TLRSVVL-KEYLTLKTFVAVIGLTCALGSG--MPL E F HIASMCAALSKFLSLFGG------ : 231
Hum.placen : TLRSVVL-KEYLTLKTFIAVIGLTCALGSG--MPL E F HIASMCAALSKFLSLFGG------ : 228
T.californ : TIRGAVL-HEYLTLRTFVAVTVGLTVALSAG--FPL E F HIASICATLNQLLCFISG------ : 189
T.marmorat : TIRGAVL-HEYLTLRTFVAVTVGLTVALSAG--FPL E F HIASICATLNQLLCFISG------ : 189
Ra.muscle_ : TIRSVVL-KEYLTLKAFVAVVALTAGLGSG--MP E F HIASICAAMSKFMSMFSG------ : 255
Rat.muscle : TIRSVVL-KEYLTLKAFVAVVALTAGLGSG--MP E F HIASICAAMSKFMSMFSG------ : 255
Hum.muscle : TIRSVVL-KEYLTMKAFVAVVALTAGLGSG--MP E F HIASICAAMSKFMSVFCG------ : 255
Hum_CLC-1 : TIRSVVL-KEYLTMKAFVAVVALTAGLGSG--MP E F HIASICAAMSKFMSVFCG------ : 255
S.cerevisi : IVWVSSFEYNKEFLGLLTLVIFSVALPLAISSG--LS E HYATCCGYLTK-----WLL----- : 249
26K 6 G K 6 G 6GKeGP 6H

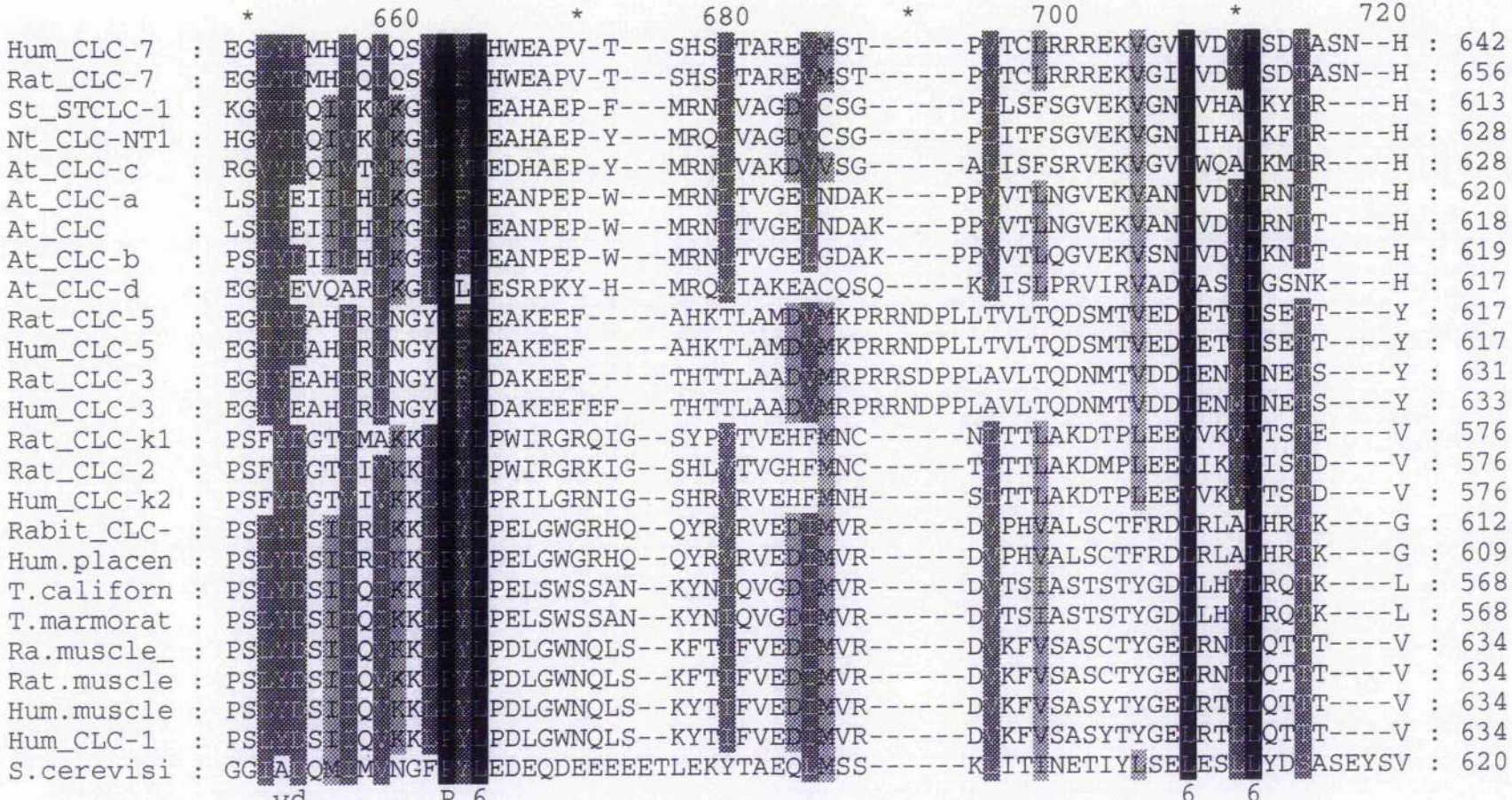

* 300 * 320 * 340 * 360
Hum_CLC-7 : 330
Rat_CLC-7 : 344
St_STCLC-1 : 297
Nt_CLC-NT1 : 312
At_CLC-c : 311
At_CLC-a : 302
At_CLC : 302
At_CLC-b : 301
At_CLC-d : 291
Rat_CLC-5 : 296
Hum_CLC-5 : 296
Rat_CLC-3 : 309
Hum_CLC-3 : 309
Rat_CLC-k1 : 257
Rat_CLC-2 : 257
Hum_CLC-k2 : 257
Rabit_CLC- : 299
Hum.placen : 296
T.californ : 258
T.marmorat : 258
Ra.muscle_ : 323
Rat.muscle : 323
Hum.muscle : 323
Hum_CLC-1 : 323
S.cerevisi : 317
6 a G6 F P gGVLF 6E 5 W4 5

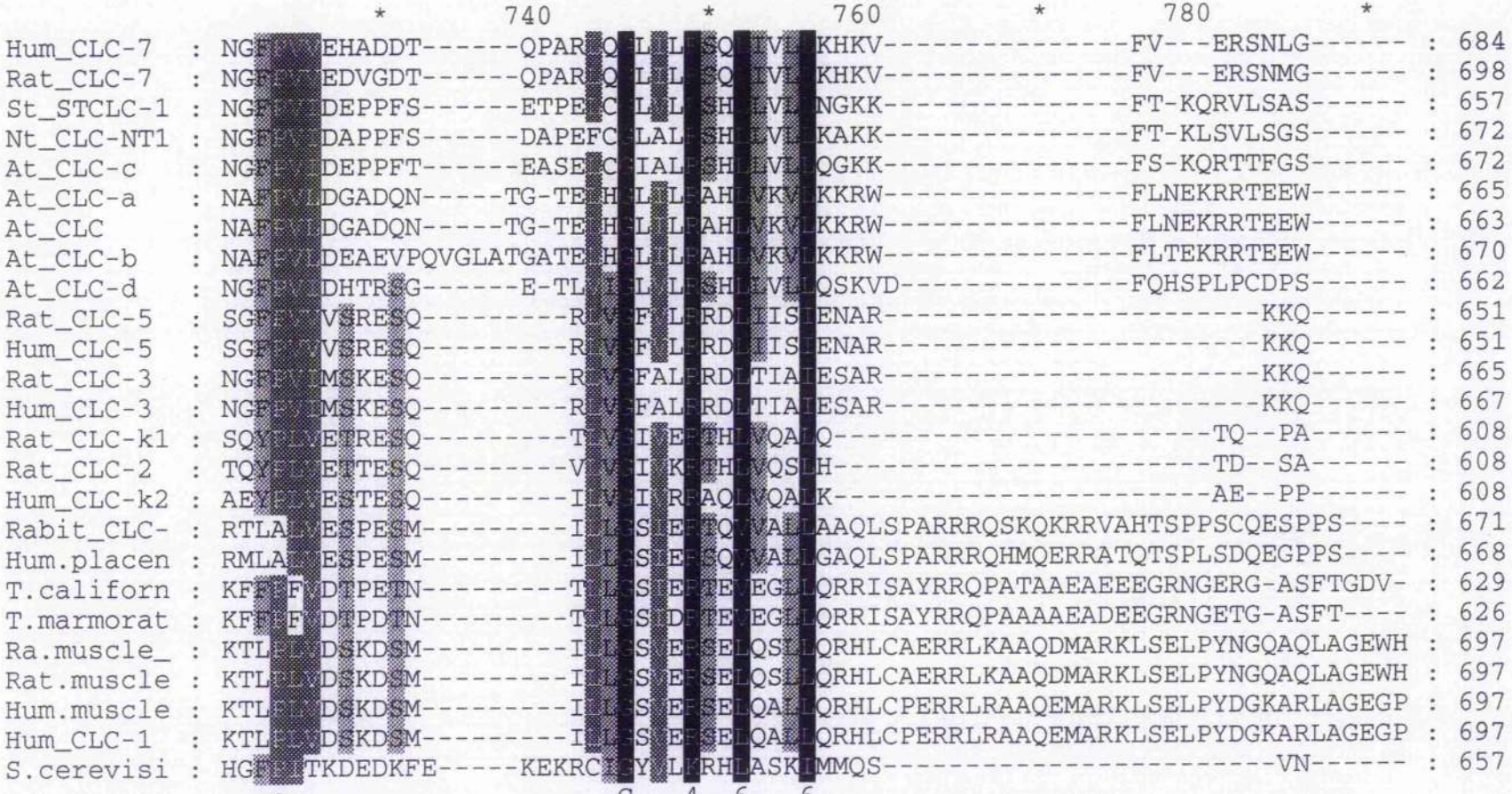

* 380 * 400 * 420 *
Hum_CLC-7 : -GNMWDLSSPGLIN GRFDSEKMAY THE PVF A VG VL AV NAL NYWL TMFRI--RY HR--PCLQ : 397
Rat_CLC-7 : -GNMWDLSSPGLIN GRFDSEKMAY THE PVF A VG VL AV NAL NYWL TMFRI--RY HR--PCLQ : 411
St_STCLC-1 : GGNCGLFGQGGLI DVNSGVSNYNTL DV ALIF- LG LL SL NYL VDKV RTY---AV NERGPAF : 365
Nt_CLC-NT1 : SGKCGLFGQGGLI DVNSGAPNYNTL DV AVLI- LG LL SL NYL VDKV RTY---SI NERGPAF : 380
At_CLC-c : SGRCGLFGKGGLI DVNSGPVLYSTPDL AIVF- IG VL SL NYL VDKV RTY---SI NEKGPRF : 379
At_CLC-a : SGKCGLFGSGGLI DVSHVEVRYHAAHI PVTL- FG LL SL NHL LHKV RLY---NL NQKGKIH : 370
At_CLC : SGKCGLFGSGGLI DVSHVEVRYHAADI PVTL- FG LL SL NHL LHKV RLY---NL NQKGKIH : 370
At_CLC-b : SGKCGLFGKGGLI DVSHVTYTYHVTDI PVML- IG LL SL NHL LHKV RLY---NL NEKGKIH : 369
At_CLC-d : SGICGHFGGGGFI DVSDGQDDYYFKEL PMAV- IG LL AL NQI TLYMTSWRR--NS HKKGNRV : 360
Rat_CLC-5 : -----PFGNSRLV YVE HTP-WHLFEL PFIV- FG LW AL IRTNIAWCRKR-----KTTQLGKYP : 356
Hum_CLC-5 : -----PFGNSRLV YVE HTP-WHLFEL PFIL- FG LW AL IRTNIAWCRKR-----KTTQLGKYP : 356
Rat_CLC-3 : -----PFGNSRLV YVE HTP-WYLFELF PFIL- FG LW AF IRANIAWCRRR-----KSTKFGKYP : 369
Hum_CLC-3 : -----PFGNSRLV YVE HTP-WYLFELF PFIL- FG LW AF IRANIAWCRRR-----KSTKFGKYP : 369
Rat_CLC-k1 : -----SEQETITS KTR RVDVP DLPE FFFVA FIC VLSCA LFCQRTF RFIKTNRYTSRLLATSK : 324
Rat_CLC-2 : -----SEQETITS KTS PVDIP DLPE FFFVA AIC LLSCG NYCQRTS FFLKSNGFTSKLLATSK : 324
Hum_CLC-k2 : -----SEQETITS KTS RVDVP DLPE FFFVV GLC LLSA LFCQRIF FGFIRNNRFSSKLLATSK : 324
Rabit_CLC- : -----RDEETITA KTR RLDFP DLQE PAFAV AS FG AL VYLNRK VQVMRKQKT NRFLMRKR : 366
Hum.placen : -----RDEETITA KTR RLDFP DLQE PAFAV AS FG AL VYLNRK VQVMRKQKT NRFLMRKR : 363
T.californ : -----KDTVTLTA KTN RGDIP DLQE PAFAI AS FF AL VYLNRQ IVFMRKKNF VTKILKKQR : 325
T.marmorat : -----KDTVTLTA KTN RGDIP DLQE PAFAI AS FF AL VYLNRQ IVFMRKKNF VTKILKKQR : 325
Ra.muscle_ : -----KDAVTITA RTN RMDFP DLKE PAFAV CC FL AV VYLHRQVMLGVRKHKAL SQFLAKHR : 390
Rat.muscle : -----KDAVTITA RTN RMDFP DLKE PAFAV CC FL AV VYLHRQVMLGVRKHKAL SQFLAKHR : 390
Hum.muscle : -----KDAVTITA RTN RMDFP DLKE PAFAA CC LL AV VYLHRQVMLGVRKHKAL SQFLAKHR : 390
Hum_CLC-1 : -----KDAVTITA RTN RMDFP DLKE PAFAA CC LL AV VYLHRQVMLGVRKHKAL SQFLAKHR : 390
S.cerevisi : -----PFRNGRVI NVT DRD--WKVQE PIF AL FG LY KYISKWNINF HFR------KMYLSSWP : 376
5 6G G g

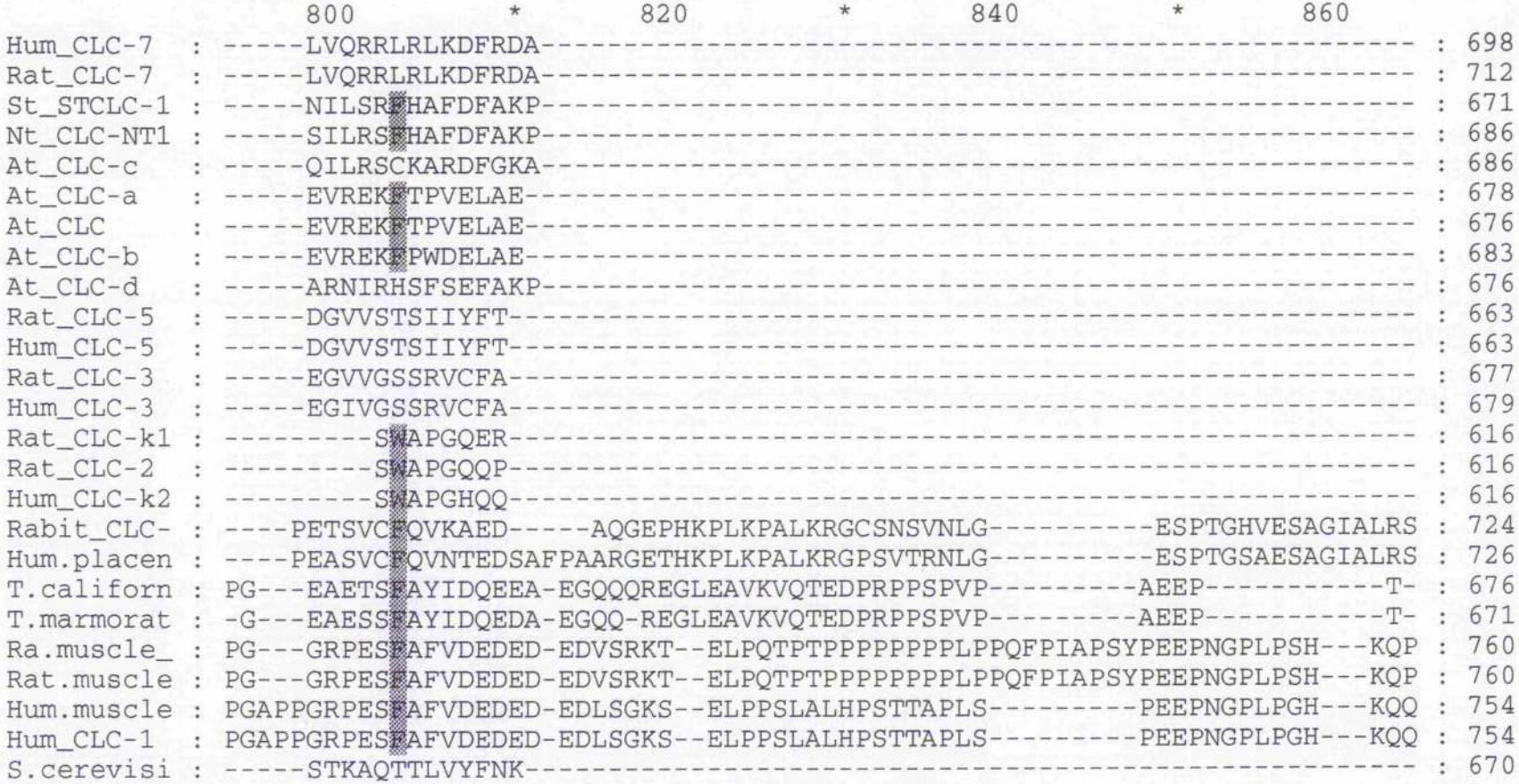

440 * 460 * 480 * 500
Hum_CLC-7 : LEAVLAAATATVAVLIYSSR--DC-Q---------PLQ-GGSMSYPLQLFCADG-EYNSMAAAFFNTPE : 455
Rat_CLC-7 : TEAMLAAATATVAVLIYSSR--DC-Q---------PLQ-GSSMSYPLQLFCADG-EYNSMAAAFFNTPE : 469
St_STCLC-1 : LLVMSSIITSCCSGLPWFAGCIPCPVGLEE---KCPTI-GRSGNY-KNFQCPAG-HYNDLASLFLNTND : 431
Nt_CLC-NT1 : LLVMTSIISSLCSGLPWFATCTPCPVGLED---KCPTI-GRSGNY-KNFQCPAG-HYNDLASLFMNTND : 446
At_CLC-c : LLVMASIISSCCAFGLPWLSQCTPCPIGIEEG--KCPSV-GRSSIY-KSFQCPPN-HYNDLSSLLLNTND : 446
At_CLC-a : LLSLGSLFTSVCLFGLPFLAECKPCDPSIDE---ICPTN-GRSGNF-KQFNCPNG-YYNDLSTLLLTTND : 436
At_CLC : LLSLGSLIH-QLLFGLPFLAN-EACDPTIDE---ICPTN-GRSGNF-KQFNCPNG-YYNDLSTLLLTTND : 434
At_CLC-b : LLSLTSLFTSVCLFGLPFLAKCKPCDPSIDE---ICPTN-GRSGNF-KQFHCPKG-YYNDLATLLLTTND : 435
At_CLC-d : LEACISCITSAISFGLPLLRKCSPCPESVPDSGIECPRPPGMYGNY-VNFFCKTDNEYNDLATIFFNTQD : 431
Rat_CLC-5 : EVLIITAATAILAFPNEYTRMSTSELIS---------ELFNDCGLLDSSKLCDYENHFNTSKGGE-LPDR : 418
Hum_CLC-5 : LEVLVITAATAILAFPNEYTRMSTSELIS---------ELFNDCGLLDSSKLCDYENRFNTSKGGE-LPDR : 418
Rat_CLC-3 : LEVIIAAATAVIAFPNPYTRLNTSELIK---------ELFTDCGPLESSSLCDYRNDMNASKIVDDIPDR : 432
Hum_CLC-3 : LEVIIAAATAVIAFPNPYTRLNTSELIK---------ELFTDCGPLESSSLCDYRNDMNASKIVDDIPDR : 432
Rat_CLC-k1 : PSYAALVALVLASITTPPGVGRFMASRLS----------MAE-HLHSLFDNNSWALMTRNSSPPWPAEP : 382
Rat_CLC-2 : PEYSALAAVVLASITTPPGVGRFMASRLS----------MSE-YLETLFDNNSWALMTKNSSPPWSAEP : 382
Hum_CLC-k2 : PEYSALATLVLASITTPPSAGRFLASRLS----------MKQ-HLDSLFDNHSWALMTQNSSPPWPEEL : 382
Rabit_CLC- : LFPALVTLLISTLTPPGFGQFMAGQLS----------QKE-TLVTLFDNRTWVRQG------LVEEL : 418
Hum.placen : LFPALVTLLISTLTPPGFGQFMAGQLS----------QKE-TLVTLFDNRTWVRQG------LAEEL : 415
T.californ : LYPAVVTFVLATLRPPGVGQFFGAGLM----------PRE-TINSLFDNYTWTKT--------I-DP : 374
T.marmorat : LYPAVVTFVLATLRPPGVGQFFGAGLM----------PRE-TINSLFDNYTWTKT--------I-DP : 374
Ra.muscle_ : LYPGIVTFVIASLTPPGMGQFMAGELM----------PRE-AISTLFDNNTWVKH--------IGDP : 440
Rat.muscle : LYPGIVTFVIASLTPPGMGQFMAGELM----------PRE-AISTLFDNNTWVKH--------IGDP : 440
Hum.muscle : LYPGIVTFVIASFTPPGMGQFMAGELM----------PRE-AISTLFDNNTWVKH--------AGDP : 440
Hum_CLC-1 : LYPGIVTFVIASFTPPGMGQFMAGELM----------PRE-AISTLFDNNTWVKH--------AGDP : 440
S.cerevisi : QEVLFATVTALISVFNEFLKLDMTESMG----------ILFHECVKNDNTSTFSHR-----LCQL : 428
5

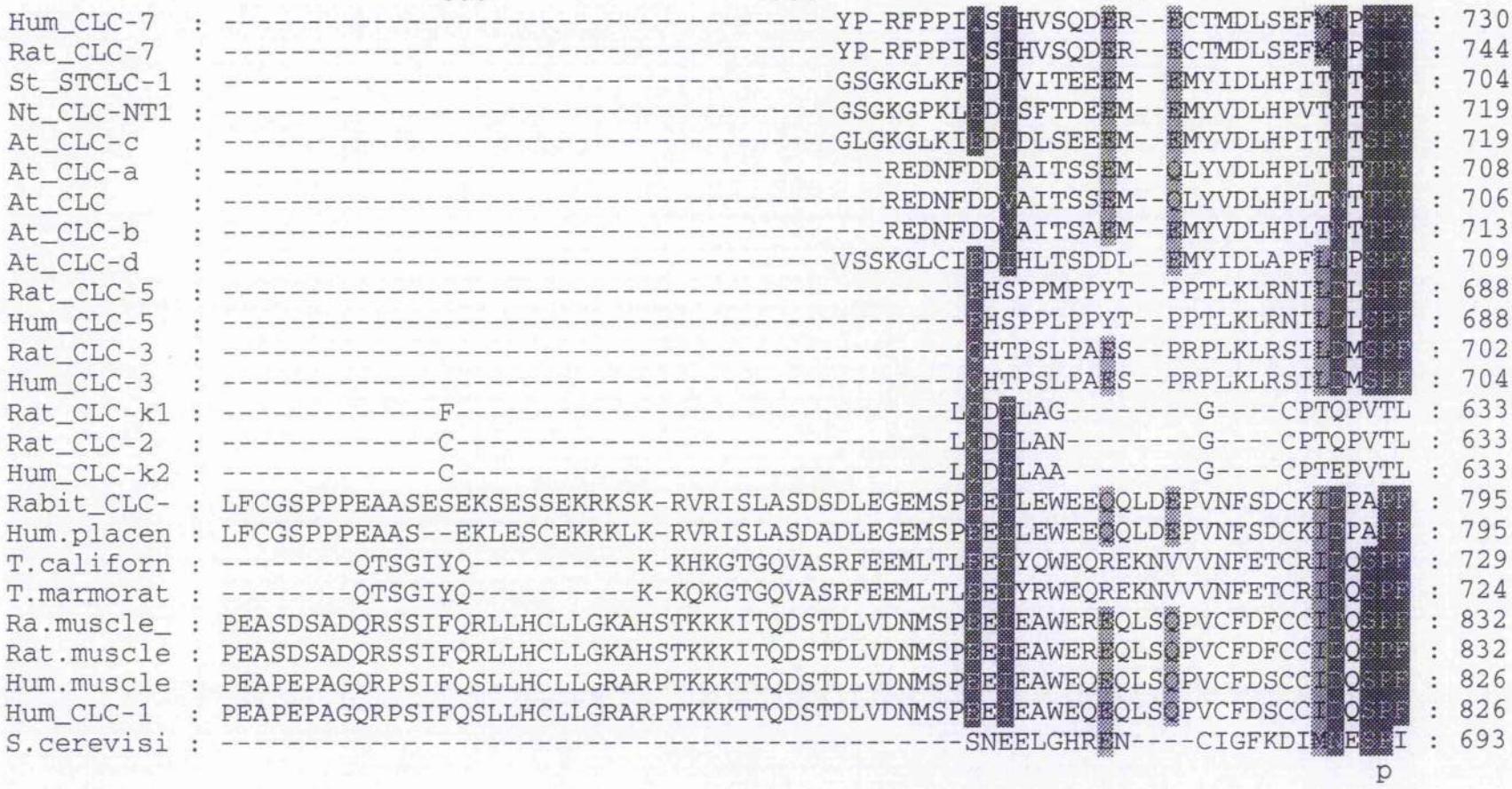

520
540
560
Hum_CLC-7 : 521
Rat_CLC-7 : 535
St_STCLC-1 : 497
Nt_CLC-NT1 : 512
At_CLC-c : 512
At_CLC-a : 502
At_CLC : 500
At_CLC-b : 501
At_CLC-d : 499
Rat_CLC-5 : 483
Hum_CLC-5 : 483
Rat_CLC-3 : 497
Hum_CLC-3 : 497
Rat_CLC-k1 : 451
Rat_CLC-2 : 451
Hum_CLC-k2 : 451
Rabit_CLC- : 487
Hum.placen : 484
T.californ : 443
T.marmorat : 443
Ra.muscle_ : 509
Rat.muscle : 509
Hum.muscle : 509
Hum_CLC-1 : 509
S.cerevisi : 491

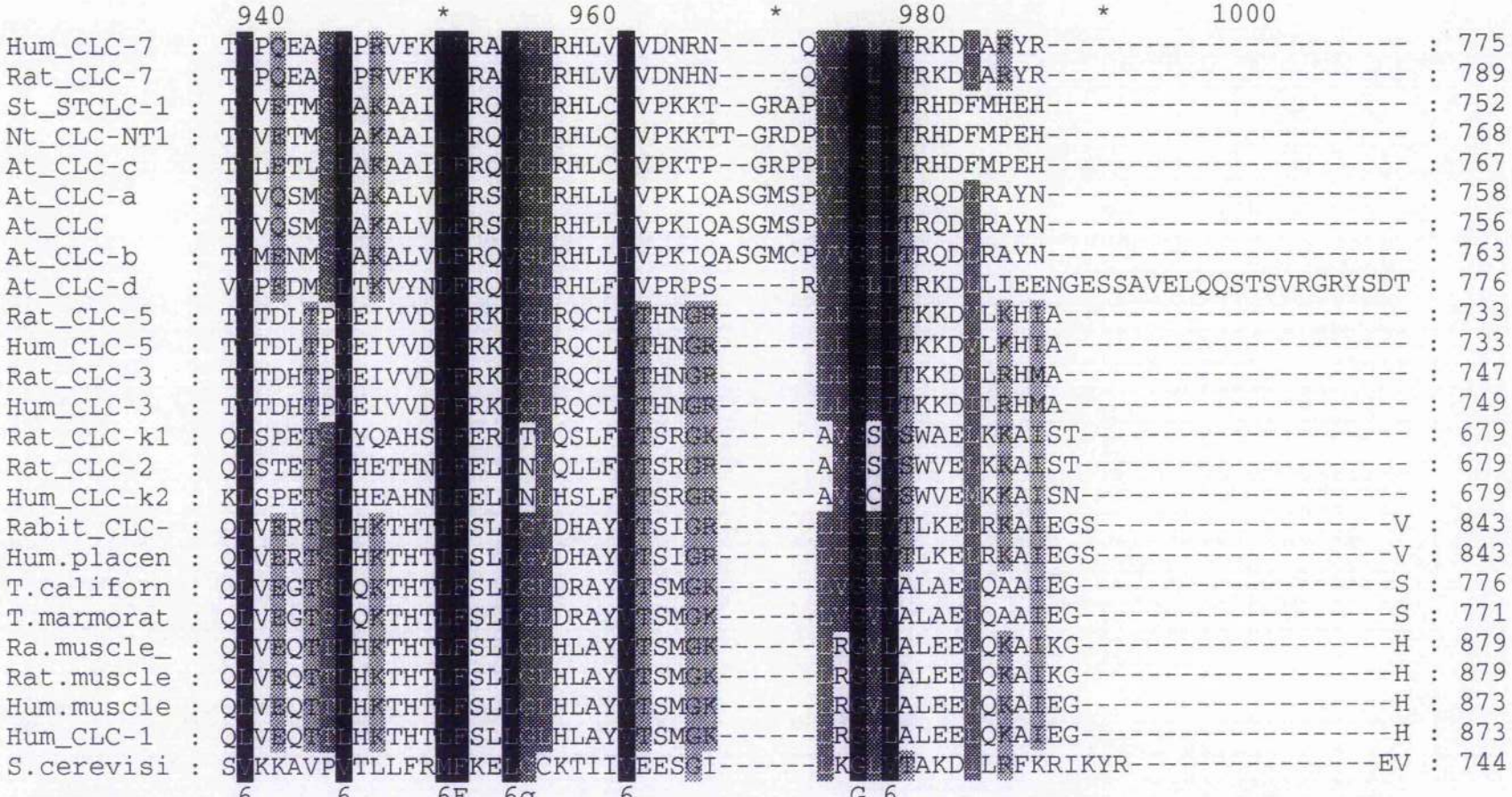

580
600
620
640
Hum_CLC-7 : 582
Rat_CLC-7 : 596
St_STCLC-1 : 555
Nt_CLC-NT1 : 570
At_CLC-c : 570
At_CLC-a : 560
At_CLC : 558
At_CLC-b : 559
At_CLC-d : 558
Rat_CLC-5 : 554
Hum_CLC-5 : 554
Rat_CLC-3 : 568
Hum_CLC-3 : 568
Rat_CLC-k1 : 516
Rat_CLC-2 : 516
Hum_CLC-k2 : 516
Rabit_CLC- : 552
Hum.placen : 549
T.californ : 508
T.marmorat : 508
Ra.muscle_ : 574
Rat.muscle : 574
Hum.muscle : 574
Hum_CLC-1 : 574
S.cerevisi : 554

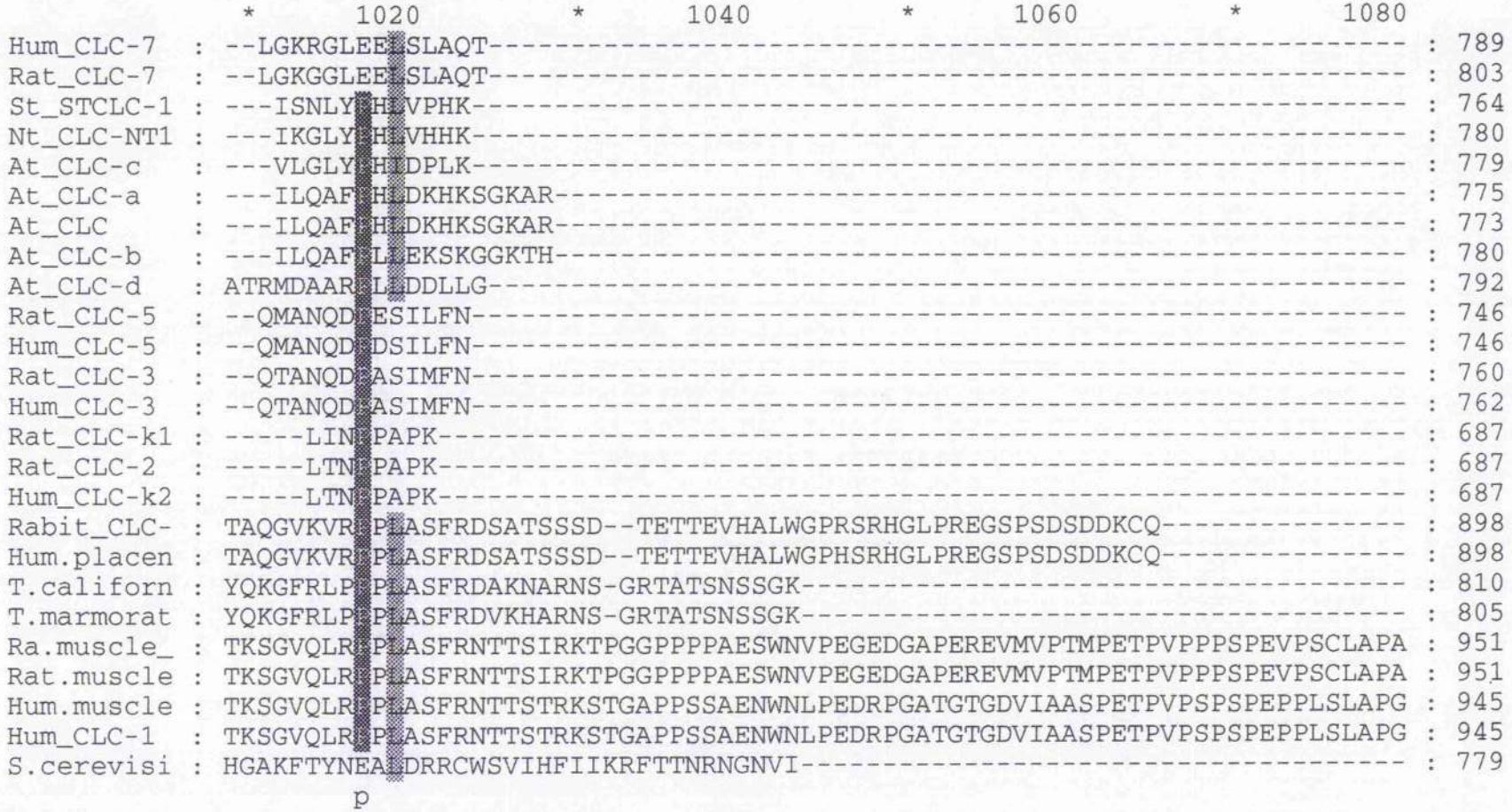

660
680
700
720
Hum_CLC-7 : 642
Rat_CLC-7 : 656
St_STCLC-1 : 613
Nt_CLC-NT1 : 628
At_CLC-c : 628
At_CLC-a : 620
At_CLC : 618
At_CLC-b : 619
At_CLC-d : 617
Rat_CLC-5 : 617
Hum_CLC-5 : 617
Rat_CLC-3 : 631
Hum_CLC-3 : 633
Rat_CLC-k1 : 576
Rat_CLC-2 : 576
Hum_CLC-k2 : 576
Rabit_CLC- : 612
Hum.placen : 609
T.californ : 568
T.marmorat : 568
Ra.muscle_ : 634
Rat.muscle : 634
Hum.muscle : 634
Hum_CLC-1 : 634
S.cerevisi : 620


740
760
780
Hum_CLC-7 : 684
Rat_CLC-7 : 698
St_STCLC-1 : 657
Nt_CLC-NT1 : 672
At_CLC-c : 672
At_CLC-a : 665
At_CLC : 663
At_CLC-b : 670
At_CLC-d : 662
Rat_CLC-5 : 651
Hum_CLC-5 : 651
Rat_CLC-3 : 665
Hum_CLC-3 : 667
Rat_CLC-k1 : 608
Rat_CLC-2 : 608
Hum_CLC-k2 : 608
Rabit_CLC- : 671
Hum.placen : 668
T.californ : 629
T.marmorat : 626
Ra.muscle_ : 697
Rat.muscle : 697
Hum.muscle : 697
Hum_CLC-1 : 697
S.cerevisi : 657


800 * 820 * 840 * 860
Hum_CLC-7 : -----LVQRRLRLKDFRDA---------- : 698
Rat_CLC-7 : -----LVQRRLRLKDFRDA---------- : 712
St_STCLC-1 : -----NILSRFHAFDFAKP---------- : 671
Nt_CLC-NT1 : -----SILRSFHAFDFAKP---------- : 686
At_CLC-c : -----QILRSCKARDFGKA---------- : 686
At_CLC-a : -----EVREKFTPVELAE---------- : 678
At_CLC : -----EVREKFTPVELAE---------- : 676
At_CLC-b : -----EVREKFPWDELAE---------- : 683
At_CLC-d : -----ARNIRHSFSEFAKP---------- : 676
Rat_CLC-5 : -----DGVVSTSIIYFT---------- : 663
Hum_CLC-5 : -----DGVVSTSIIYFT---------- : 663
Rat_CLC-3 : -----EGVVGSSRVCFA---------- : 677
Hum_CLC-3 : -----EGIVGSSRVCFA---------- : 679
Rat_CLC-k1 : ---------SWAPGQER---------- : 616
Rat_CLC-2 : ---------SWAPGQQP---------- : 616
Hum_CLC-k2 : ---------SWAPGHQQ---------- : 616
Rabit_CLC- : ----PETSVCFQVKAED-----AQGEPHKPLKPALKRGCSNSVNLG----------ESPTGHVESAGIALRS : 724
Hum.placen : ----PEASVCFQVNTEDSAFPAARGETHKPLKPALKRGPSVTRNLG----------ESPTGSAESAGIALRS : 726
T.californ : PG---EAETSFAYIDQEEA-EGQQQREGLEAVKVQTEDPRPPSPVP---------AEEP----------T- : 676
T.marmorat : -G---EAESSFAYIDQEDA-EGQQ-REGLEAVKVQTEDPRPPSPVP---------AEEP----------T- : 671
Ra.muscle_ : PG---GRPESFAFVDEDED-EDVSRKT--ELPQTPTPPPPPPPPLPPQFPIAPSYPEEPNGPLPSH---KQP : 760
Rat.muscle : PG---GRPESFAFVDEDED-EDVSRKT--ELPQTPTPPPPPPPPLPPQFPIAPSYPEEPNGPLPSH---KQP : 760
Hum.muscle : PGAPPGRPESFAFVDEDED-EDLSGKS--ELPPSLALHPSTTAPLS---------PEEPNGPLPGH---KQQ : 754
Hum_CLC-1 : PGAPPGRPESFAFVDEDED-EDLSGKS--ELPPSLALHPSTTAPLS---------PEEPNGPLPGH---KQQ : 754
S.cerevisi : -----STKAQTTLVYFNK---------- : 670


* 880 * 900 * 920 *
Hum_CLC-7 : 730
Rat_CLC-7 : 744
St_STCLC-1 : 704
Nt_CLC-NT1 : 719
At_CLC-c : 719
At_CLC-a : 708
At_CLC : 706
At_CLC-b : 713
At_CLC-d : 709
Rat_CLC-5 : 688
Hum_CLC-5 : 688
Rat_CLC-3 : 702
Hum_CLC-3 : 704
Rat_CLC-k1 : 633
Rat_CLC-2 : 633
Hum_CLC-k2 : 633
Rabit_CLC- : 795
Hum.placen : 795
T.californ : 729
T.marmorat : 724
Ra.muscle_ : 832
Rat.muscle : 832
Hum.muscle : 826
Hum_CLC-1 : 826
S.cerevisi : 693
p

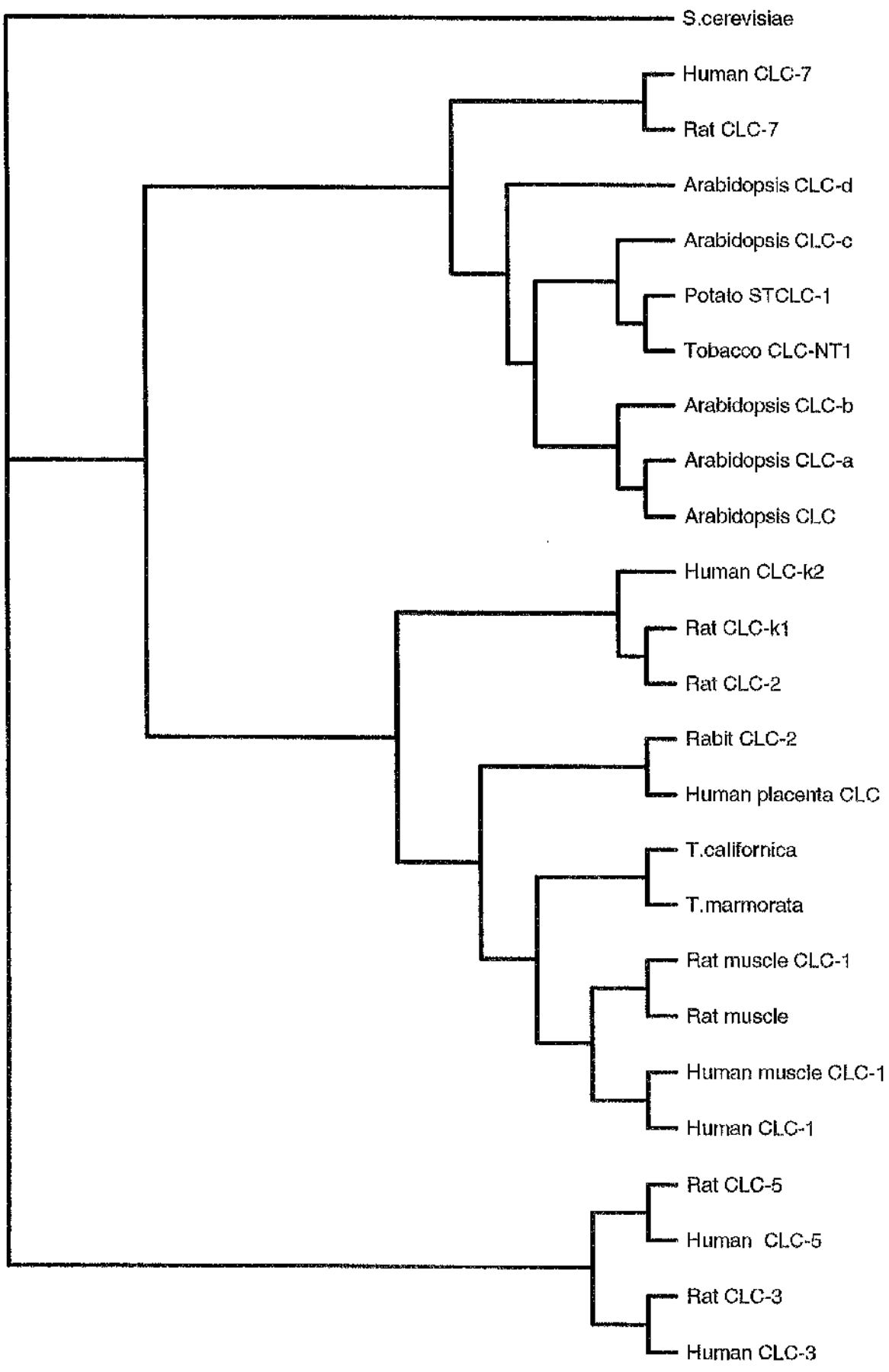

940
960
980
1000
Hum_CLC-7 : 775
Rat_CLC-7 : 789
St_STCLC-1 : 752
Nt_CLC-NT1 : 768
At_CLC-c : 767
At_CLC-a : 758
At_CLC : 756
At_CLC-b : 763
At_CLC-d : 776
Rat_CLC-5 : 733
Hum_CLC-5 : 733
Rat_CLC-3 : 747
Hum_CLC-3 : 749
Rat_CLC-k1 : 679
Rat_CLC-2 : 679
Hum_CLC-k2 : 679
Rabit_CLC- : 843
Hum.placen : 843
T.californ : 776
T.marmorat : 771
Ra.muscle_ : 879
Rat.muscle : 879
Hum.muscle : 873
Hum_CLC-1 : 873
S.cerevisi : 744


* 1020 * 1040 * 1060 * 1080
Hum_CLC-7 : --LGKRGLEELSLAQT---------- : 789
Rat_CLC-7 : --LGKGGLEELSLAQT---------- : 803
St_STCLC-1 : ---ISNLYTHLVPHK---------- : 764
Nt_CLC-NT1 : ---IKGLYTHLVHHK---------- : 780
At_CLC-c : ---VLGLYTHLDPLK---------- : 779
At_CLC-a : ---ILQAFTHLDKHKSGKAR---------- : 775
At_CLC : ---ILQAFTHLDKHKSGKAR---------- : 773
At_CLC-b : ---ILQAFTLLEKSKGGKTH---------- : 780
At_CLC-d : ATRMDAARTLLDDLLG---------- : 792
Rat_CLC-5 : --QMANQDTESILFN---------- : 746
Hum_CLC-5 : --QMANQDTDSILFN---------- : 746
Rat_CLC-3 : --QTANQDTASIMFN---------- : 760
Hum_CLC-3 : --QTANQDTASIMFN---------- : 762
Rat_CLC-k1 : -----LINTPAPK---------- : 687
Rat_CLC-2 : -----LTNTPAPK---------- : 687
Hum_CLC-k2 : -----LTNTPAPK---------- : 687
Rabit_CLC- : TAQGVKVRTPLASFRDSATSSSD--TETTEVHALWGPRSRHGLPREGSPSDSDDKCQ---------- : 898
Hum.placen : TAQGVKVRTPLASFRDSATSSSD--TETTEVHALWGPHSRHGLPREGSPSDSDDKCQ---------- : 898
T.californ : YQKGFRLPTPLASFRDAKNARNS-GRTATSNSSGK---------- : 810
T.marmorat : YQKGFRLPTPLASFRDVKHARNS-GRTATSNSSGK---------- : 805
Ra.muscle_ : TKSGVQLRTPLASFRNTTSIRKTPGGPPPPAESWNVPEGEDGAPEREVMVPTMPETPVPPPSPEVPSCLAPA : 951
Rat.muscle : TKSGVQLRTPLASFRNTTSIRKTPGGPPPPAESWNVPEGEDGAPEREVMVPTMPETPVPPPSPEVPSCLAPA : 951
Hum.muscle : TKSGVQLRTPLASFRNTTSTRKSTGAPPSSAENWNLPEDRPGATGTGDVIAASPETPVPSPSPEPPLSLAPG : 945
Hum_CLC-1 : TKSGVQLRTPLASFRNTTSTRKSTGAPPSSAENWNLPEDRPGATGTGDVIAASPETPVPSPSPEPPLSLAPG : 945
S.cerevisi : HGAKFTYNEALDRRCWSVIHFIIKRFTTNRNGNVI---------- : 779
p

```
                        *      1100          *      1120
Hum_CLC-7   : ---------------------------------------------- :    -
Rat_CLC-7   : ---------------------------------------------- :    -
St_STCLC-1  : ---------------------------------------------- :    -
Nt_CLC-NT1  : ---------------------------------------------- :    -
At_CLC-c    : ---------------------------------------------- :    -
At_CLC-a    : ---------------------------------------------- :    -
At_CLC      : ---------------------------------------------- :    -
At_CLC-b    : ---------------------------------------------- :    -
At_CLC-d    : ---------------------------------------------- :    -
Rat_CLC-5   : ---------------------------------------------- :    -
Hum_CLC-5   : ---------------------------------------------- :    -
Rat_CLC-3   : ---------------------------------------------- :    -
Hum_CLC-3   : ---------------------------------------------- :    -
Rat_CLC-k1  : ---------------------------------------------- :    -
Rat_CLC-2   : ---------------------------------------------- :    -
Hum_CLC-k2  : ---------------------------------------------- :    -
Rabit_CLC-  : ---------------------------------------------- :    -
Hum.placen  : ---------------------------------------------- :    -
T.californ  : ---------------------------------------------- :    -
T.marmorat  : ---------------------------------------------- :    -
Ra.muscle_  : RVEGELEELEMVGNLGPEEDLADILHGPSLRSTDEEDEDELIL : 994
Rat.muscle  : RVEGELEELEMVGNLGPEEDLADILHGPSLRSTDEEDEDELIL : 994
Hum.muscle  : KVEGELEELELVESPGLEEELADILQGPSLRSTDEEDEDELIL : 988
Hum_CLC-1   : KVEGELEELELVESPGLEEELADILQGPSLRSTDEEDEDELIL : 988
S.cerevisi  : ---------------------------------------------- :    -
```

Figure A.5. Phylogram of *CLC*-type Cl^- Channels.

The putative *clc* sequences voltage-gated Cl^- channels are deposited in the databases. The translations of these sequences were aligned using the CLUSTALW programe at the EMBL European Bioinformatics Institute, Cambridge. The phylogram of theses sequence was done to represent the molecular bases of evolution for the putative gene encoding chloride channels in the following: Human CLC-7, Rat CLC-7, Human CLC-5, Rat CLC-5, Human CLC-3, Rat CLC-3, Human CLC-k2, Rat CLC-k2, Rat CLC-k1, Rabit CLC, Human placenta CLC, *T. califoria*, *T. marmorata*, Rat. Muscle CLC, Human muscle CLC, Human CLC-1, *S. cerevisiae* GEF1, Nt CLC-NT1 (*Nicotiana tabaccum*) St STCLC1, (*Solanum tuberosum*), At CLC a-d (*Arabidopsis thaliana CLC-a, CLC-b, CLC-c* and *CLC-d*).


S.cerevisiae
Human CLC-7
Rat CLC-7
Arabidopsis CLC-d
Arabidopsis CLC-c
Potato STCLC-1
Tobacco CLC-NT1
Arabidopsis CLC-b
Arabidopsis CLC-a
Arabidopsis CLC
Human CLC-k2
Rat CLC-k1
Rat CLC-2
Rabit CLC-2
Human placenta CLC
T.californica
T.marmorata
Rat muscle CLC-1
Rat muscle
Human muscle CLC-1
Human CLC-1
Rat CLC-5
Human CLC-5
Rat CLC-3
Human CLC-3

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