The characterisation of barley and wheat oxalate oxidases expressed in transgenic plants

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ABSTRACT

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Oxalate oxidase is a water soluble, thermolabile, homo-oligomeric glycoprotein the synthesis of which marks the onset of germination in wheat and barley embryos. The protein is also highly abundant in barley roots. The enzyme has an average oligomer molecular mass of about 115 kDa and about 22.8 kDa for the monomers, as determined by mass spectrometry. The oligomeric cereal oxalate oxidases are resistant to dissociation in SDS containing media and to digestion by pepsin. The cereal organs produce two oxalate oxidase isoforms (G and G') which possess the same apoprotein but are differentially glycosylated. The oligosaccharide side chain(s) has a molecular mass of about 2-3 kDa. Barley root also contains a third active oxalate oxidase isoform with a mass of about 22.5 kDa, which was not detected in germinating embryos of the same cultivar. All of the cereal oxalate oxidases were shown to have identical N-terminal amino acid sequences and almost identical kinetic properties

This thesis describes the characterisation of oxalate oxidases isolated from three transgenic plants lines, expressing chimeric CaMV 35S-oxalate oxidase genes. SGS5 tobacco was expressing a gene with the native oxalate oxidase signal peptide and 3S1 oilseed rape and C26 tobacco were expressing a gene containing a foreign extensin signal peptide. Transgenic SGS5 tobacco produced an oxalate oxidase which was almost indistinguishable from the native cereal protein, in terms of its structure, stability, enzyme activity and resistance to dissociation in SDS containing media and digestion by pepsin. This work illustrated the ability of a dicotyledonous plant (tobacco) to recognised and correctly process a transgenic monocotyledon protein (wheat).

Transgenic 3S1 oilseed rape and C26 tobacco were shown to produce active oligomeric oxalate oxidases, which did not exhibit any of the unusual resistance properties normally associated with these proteins. Instead the 3S1 and C26 oxalate oxidases were unstable and exhibited significantly altered kinetic properties compared with the native cereal and transgenic SGS5 enzymes. The instability was thought to have arisen from the incorrect processing of the 3S1 and C26 oxalate oxidases, resulting in the partial cleavage of the extensin signal peptide, which in turn gave rise to a mature oxalate oxidase with an altered N-terminal sequence compared with the native cereal enzyme.

The use of vacuum infiltration confirmed the association of the transgenic enzymes with the extracellular spaces, although the majority of the enzyme was shown to be intracellular. The main objective for producing the transgenic oilseed rape expressing oxalate oxidase was to improve fungal pathogen resistance against oxalic acid secreting pathogens. The results described in this thesis are concerned with a direct comparison of the structure, stability and kinetics between the native cereal and transgenic oxalate oxidases and the possible consequences for pathogen resistance in plants expressing unstable yet active transgenic enzymes.

STATEMENT.

No part of this thesis has been previously submitted for a degree in this or any other University. I declare that, unless otherwise stated, the work presented herein is entirely my own.

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ABBREVIATIONS.

ABA	abscisic acid
Bis-acrylamide	Bis (N,N'-methylene-bis-acrylamide)
bp	base pair
Bq	bequerel
BSA	bovine serum albumin
°C	degrees centigrade
Ca Mg-TBS	calcium, magnesium-Tris buffered saline
CaMV 35S	cauliflower mosaic virus 35S promoter
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
CD	circular dichroism
cDNA	complementary DNA
Ci	Curie
ConA	concanavalin A
cpm	counts per minute
DMS	dimethyl suberimidate
DNA	dioxyribonucleic acid
DSC	differential scanning calorimetry
DTT	ditheothreitol
EDTA	ethylene diamine tetraacetic acid
ER	endoplasmic reticulum
g	gram
G	wheat germin 136 kDa isoform
G'	wheat germin 120 kDa isoform
hr	hours
^{125}I	^{125}iodine
IAA	indoyl acetic acid
IgG	immunoglobulin G
INF	intercellular fluid
Kan	kanamycin
Kb	kilobase pair
kDa	kilodalton
l	litre
m	metre
M	molar (concentration)
MALDI-ToF	matrix assisted laser desorption ionisation-time of flight
M_r	molecular weight
mRNA	messenger RNA
Nos	nopaline synthase
OD	optical density

PAGE	polyacrlyamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
rpm	revolutions per minute
RUBISCO	ribulose bis-phosphate carboxylase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
SSC	saline sodium citrate
Taq	*Thermus aquaticus*
TBS	Tris buffered saline
Tris-HCl	Tris (hydroxymethyl)methylamine hydrochloride
v/v	volume for volume
v/w	volume for weight

NUCLEIC AND AMINO ACID ABBREVIATIONS.

Code	Nucleic acid
A	adenosine
C	cytosine
G	guanine
I	inositol
N	unknown
T	thymidine
U	uracil

Codes		Protein
A	ala	alanine
C	cys	cysteine
D	asp	aspartic acid
E	glu	glutamic acid
F	phe	phenylalanine
G	gly	glycine
H	his	histidine
I	ile	isoleucine
K	lys	lysine
L	leu	leucine
M	met	methionine
N	asn	asparagine
P	pro	proline
Q	gln	glutamine
R	arg	arginine
S	ser	serine
T	thr	threonine
V	val	valine
W	trp	tryptophan
Y	try	tyrosine

TABLE OF CONTENTS.

LIST OF FIGURES.

LIST OF TABLES.

1. INTRODUCTION.

1.1. Improving fungal pathogen resistance in plants.

1.1.1. Pathogen inducible responses in plants.

Plants have evolved a variety of responses to enable them to cope with abiotic and biotic stresses in the local environment, these are collectively termed stress or defence responses [Wojtaszek 1997]. The invasion of plants by incompatible pathogens (e.g. viral, bacterial, fungal and nematodes) results in the activation of a number of inducible responses that have been implicated as playing active roles in disease resistance [Bowles 1990; Lamb *et al.* 1989]. Plant defence responses are generally induced by the presence of elicitors which are signal molecules secreted by the pathogen (e.g. glycans [Lamb *et al.* 1989]; oxalic acid [Davis *et al.* 1992]; salicylic acid [Bi *et al.* 1995]), released by plant cell walls [Bowles 1990; Lamb *et al.* 1989; Wojtaszek 1997] or as a result of the physical damage caused by the infection process [Gross 1993]. The disease resistance response in plants can involve the induction of one or many defence mechanisms. The spatial and temporal expression of these responses determines whether the plant will develop the disease symptoms (susceptibility) or whether it will restrict growth and destroy the pathogen (resistance) [Wojtaszek 1997].

The first responses to be initiated by the host plant involve the production of vast quantities of reactive oxygen species (superoxide radical, hydrogen peroxide and hydroxyl radical) in the plants extracellular compartment resulting from the one-electron reductions of molecular oxygen, this response is termed the oxidative burst [Wojtaszek 1997]. The production of reactive oxygen species in some plants depends on an influx of calcium into the cells, which activates Ca^{2+}-dependent protein kinases and ultimately NADPH-dependent $O_2^{\cdot}$-generating oxidases [Mehdy 1994]. In cultured soybean cells, extracellular H_2O_2 is generated within < 5 min of elicitation of the defence response [Apostol *et al.* 1989]. Milosevic and Slusarenko [1996] reported an increase in activity of an acidic peroxidase, xanthine oxidase and glutathione reductase, and a reduction in catalase activity at the site of infection in bean. H_2O_2 produced during the oxidative burst has also been linked with the initiation of other defence responses in the host plant [Tenhaken *et al.* 1995]. This includes the rapid transcription of defence genes and the cellular targeting of the resulting proteins and compounds generated through the activation of specific defence-related enzyme pathways (e.g. phytoalexin production). Defence-related proteins and compounds which accumulate at the site of infection, include phytoalexins (low molecular weight antibiotic compounds) [VanEtten *et al.* 1989], lytic enzymes, such as chitinases and glucanases [Dixon and Lamb 1990] and oxalate oxidase, a H_2O_2-generating enzyme [Zhang *et al.* 1995]. The accumulation of transcription-dependent defences may take several hours following initial elicitor induction [Yoshikawa *et al.* 1978; Brisson *et al.* 1994].

Bradley *et al.* [1992] and Brisson *et al.* [1994] reported the H_2O_2-mediated oxidative insolubilisation and cross-linking of pre-existing proline-rich and tryosine-rich cell wall proteins (within 2 to 5 min of induction by H_2O_2) resulting in the rapid strengthening of the cell wall, which precedes the expression of transcription-dependent defences. H_2O_2 produced from the

oxidative burst initiates a rapid and local triggering of programmed cell death of pathogen infected cells, as a means of limiting the spread of infection. This process is known as the hypersensitive response, which results in the localised formation of lesions around the site of infection [Levine *et al.* 1994]. H_2O_2 generated during the oxidative burst is utilised by a variety of extracellular peroxidases [Apostol *et al.* 1989]. Kerby and Somerville [1992] and Thordal-Christensen *et al.* [1992] reported the increased transcription and activity of extracellular peroxidases following inoculation of barley with *Erysiphe graminis* sp. *hordei,* the increased activity of these peroxidases is thought to be involved with the deposition of lignin-like compounds to strengthen cell walls in the area of infection.

Pathogenesis-related (PR) proteins are synthesised in response to local and systemic resistance following pathogen infection [Niderman *et al.* 1995]. These proteins exhibit antifungal activity and are resistant to the acidic pH and proteolytic cleavage by pathogenic enzymes in the infected extracellular spaces of the host plant [Niderman *et al.* 1995]. Dixon *et al.* [1991] identified PR proteins in tobacco which are synthesised and accumulated in crystal idioblasts, these are specialised cells which also accumulate calcium oxalate crystals.

1.1.2. Engineering plants to improve fungal pathogen resistance.

Although plants have evolved mechanisms to defend themselves against fungal pathogen attack (many of which are transcription-dependent defences) scientists have identified a need to enhance this natural resistance in a number of agronomically important species, by means of genetic engineering. Many of the published strategies have involved identifying specific enzymes and pathways which can be targeted to the invading pathogen and the expression of genes in the potential host plant which will produce proteins (e.g. enzymes and inhibitors) against these targets.

Chitinase catalyses the hydrolysis of chitin, a β-1,4-linked homopolymer of N-acetyl-D-glucosamine [Broglie and Broglie 1993]. Chitin is one of the major cell matrix components in many fungal pathogens, but it is not a component of plant cell walls. Plants already express chitinases as part of their natural defence mechanism against fungal pathogen ingress [Dixon and Lamb 1990]. Broglie and Broglie [1993] have expressed a bean chitinase 5B gene controlled by the cauliflower mosaic virus (CaMV) 35S promoter in tobacco. They found that the transformed plants exhibited increased chitinase activity and when challenged with *Rhizotonia solani* the infected plants showed near normal growth compared with the controls. The degree of resistance was shown to be linked to the levels of bean chitinase expression, i.e. the higher the expression of the transgene the higher the level of resistance. When these plants were infected with a pathogen which does not possess chitin in its cell wall (i.e. *Pythium aphanidermatum)* there was no detectable difference in survival between transgenic and control plants [Broglie and Broglie 1993]. Jach *et al.* [1995] demonstrated enhanced resistance to *R. solani* in transgenic tobacco plants expressing cDNAs encoding three different proteins from barley (a class-II chitinase, a class-II β-1,3-glucanase and a type-1 ribosome inactivating protein) under the control of separate CaMV 35S promoters. Transgenic plants expressing all three of these proteins exhibited significantly enhanced resistance

against fungal pathogen infection compared with plants which were only expressing the individual transgenes [Jach *et al.* 1995]. Wu *et al.* [1995] reported enhanced resistance to potato late blight caused by *Phytophthora infestans* in transgenic potato plants expressing a glucose oxidase PCR product from *Aspergillus niger,* under the control of the CaMV 35S promoter. This resistance was apparently mediated by the increased levels of H_2O_2 produced as a result of the oxidation of glucose by the transgene product [Wu *et al.* 1995]. Harding *et al.* [1997] produced transgenic tobacco cells expressing a dominant-acting calmodulin mutant gene (VU-3, lys to Arg 115) which hyperactivates NAD kinase in plants, resulting in increased production of NADPH and H_2O_2. The transgenic cells were shown to exhibit a stronger oxidative burst in response to biotic and abiotic stresses compared with control cells [Harding *et al.* 1997].

1.2. Oilseed rape, a major commercial crop and its fungal pathogens.

Oilseed rape has now become a major and important source of oilseed, the world production of which quadrupled between 1960 and 1980 (4 to 12 million tonnes) [Weiss 1983]. This increase in production resulted from the commercial release of so-called 'double-zero' varieties, which were low in erucic acid and glucosalinates, making them much more acceptable for human and livestock consumption [Weiss 1983]. Oilseed rape has become the most important arable crop in UK agriculture after wheat and barley, with a gross margin of 582 /ha and 443 /ha for winter and spring oilseed rape, respectively [Ward *et al.* 1985].

Commercial rapeseed is derived from *Brassica*, members of the Cruciferae. Roots, stems, leaves, inflorescences or seeds contribute to the useful plant parts of commercial interest, depending on the species in question. *Brassica napus* (oilseed rape) is one of the two most important rapeseed species in terms of oilseed production, which is mainly restricted to Europe and North America, the other being *Brassica campestris* [Weiss 1983]. Oilseed rape is a temperate crop which prefers daytime temperatures of below 25 °C, and is resistant to frost at all growth stages. Oilseed rape is also resistant to drought and salt stress, both very valuable characteristics in crop plants [Weiss 1983]. The nitrogen requirement of oilseed rape is high and shortage will retard leaf development and reduce yield [Weiss 1983].

Significant yield loss can be caused by a number of fungal pathogen diseases, the severity of which is generally dependent on climate and sowing regime. For example, if two *Brassica* species are grown in succession in an infected plot the damage and loss of yield to the second crop is likely to be much more severe [Weiss 1983]. Different fungal pathogens often produce similar symptoms on the plant, but vary in their importance in the damage caused and the final effect on yield [Ward *et al.* 1985]. Examples of some of the major and most widespread fungal pathogens of oilseed rape, include *Erysiphe polygoni* (mildew), *Leptosphaeria maculans* (blackleg), *Phytophthora cryptogea* (white-rot), *Pythium* sp. (damping-off) and *Sclerotinia sclerotiorum* (stem / white rot) [Weiss 1983].

L. maculans is probably the most important disease of oilseed rape, which is propagated by spores which have the capacity to be carried over many kilometres to infect neighbouring fields. The growing fungus can cause stem rot at the base of the stem and

significant loses can be sustained with major infestations. The spores can remain viable for up to 5 years [Weiss 1983]. Resistant rape varieties exist, but the best methods of reducing widespread damage, include crop rotation, deep ploughing and using non-infected seed [Weiss 1983]. *S. sclerotiorum* is also a major and widespread disease of oilseed rape which is becoming more prevalent in Britain.

The development of resistant cultivars to the pathogens of rape appears to be very much dependent on the development of resistance to local fungal pathogen populations in terms of pure breeding [Weiss 1983]. The breeding and genetic engineering of *Brassica* species resistant to fungal pathogen attack is probably the only viable option to combat diseases caused by these pathogens [Weiss 1983].

1.3. *Sclerotinia sclerotiorum* - an oxalic acid producing fungal pathogen.

1.3.1. Distribution and life cycle.

Sclerotinia sclerotiorum is a ubiquitous phytopathogenic fungus which has an unusually broad host range attacking a wide range of crop plants with almost 400 reported host species from 64 different plant families [Purdy 1979; Dickman and Mitra 1992], including *Brassicas*, beans, peas, celery, carrots, potatoes and sugar beet [Ward *et al.* 1985]. This pathogen has been found in countries located in all continents with hugely varied environments and climates [Purdy 1979]. *S. sclerotiorum* is probably one of the most non-specific and successful of plant pathogens, the effect on loss of yield has been reported to range from 0-100% with subsequent financial losses in the millions (US $) [Purdy 1979].

The majority of the life cycle of *Sclerotinia* species is spent as sclerotia, these are structures which allow *Sclerotinia* to survive for long periods of time under adverse conditions in the soil. The sclerotia germinate under certain environmental conditions (often dependent on moisture levels in the soil [Ward *et al.* 1985]) and these can either form mycelium or apothecium, which releases ascospores [Adams and Ayers 1979; Purdy 1979]. Healthy, uninjured plant tissue is generally resistant to penetration by the fungus. The ascospores which land on dead or morbid cells can usually germinate and then the mycelium passes to sound tissue (e.g. a well established route of infection involves infected petals which adhere to leaves and stems). The infected tissue is rapidly destroyed by the advancing hyphae as a result of enzymatic processes that affect the middle lamella between cells, producing soft rot [Brooks 1953; Adams and Ayers 1979; Purdy 1979; Jamaux *et al.* 1995]. Once the mycelium has penetrated the tissue (by mechanical pressure) the hyphae penetrate the sound tissue exclusively through the extracellular spaces [Purdy 1979; Lumsden 1979]. Eventually new sclerotia are formed in the host tissue which return to the soil environment [Adams and Ayes 1979]. In oilseed rape the sclerotia can exist at a similar density to the rapeseed, which can pose a threat to future crops if not properly separated during harvesting [Weiss 1983]. Some fungicides have shown promise, particularly if applied during apothecia development and petal fall, when the plant is most vulnerable [Ward *et al.* 1985]. Fungicides containing the active ingredients prochloraz, iprodione and vinclozolin have been used against *S. sclerotiorum* with varying degrees of pathogen control [Ward *et al.* 1985]. Fungicide application is only likely to

be a viable proposition where 10-20% of an untreated crop would become infected [Ward *et al.* 1985].

1.3.2. Oxalic acid - a phytotoxin of *Sclerotinia.*

Oxalic acid, a necrosis phytotoxin produced by *S. sclerotiorum* acts predominantly in the pathogenesis of the fungus, permeating the tissue surrounding the site of infection [Magro 1984; Maxwell and Lumsden 1970; Marciano *et al.* 1983 1989; Noyes and Hancock 1981]. In *S. sclerotiorum* the production of oxalic acid occurs as a result of the cleavage of oxaloacetic acid [Maxwell 1973]. Oxalic acid acts in several ways in the host tissue, by reducing the pH of the plant tissue from 6.8 to 4 which favours pectinolytic enzyme activity [Bateman and Millar 1966]; by direct disintegration of pectic substances [Beckman *et al.* 1974]; by increasing the vulnerability of cell wall calcium-pectate complexes to hydrolysis [Bateman and Beer 1965; Maxwell and Lumsden 1970] and by the binding of oxalic acid to polyphenol oxidase to form a stable inactive complex [Sato 1980; Marciano *et al.* 1983; Magro *et al.* 1984], which reduces the plants ability to protect its pectic substances due to a reduction in the level of phenolic oxidation products [Beckman *et al.* 1974]. *S. sclerotiorum* produces the oxalic acid degrading enzyme oxalate decarboxylase [Magro *et al.* 1988]. Marciano *et al.* [1983] and Magro *et al.* [1984] demonstrated that a hypovirulent strain of *S. sclerotiorum* (SS41) is able to hydrolyse oxalic acid (with oxalate decarboxylase) at a faster rate than a virulent strain (B24) in infected sunflower and tomato stems, respectively. This results in lower levels of oxalic acid being present in stems infected by SS41, which would explain its reduced ability to establish an infection and spread through plant tissue compared with B24. Riou *et al.* [1992] reviewed the production and secretion of various endo- and exoenzymes by *S. sclerotiorum,* capable of degrading cellulosic, hemicellulosic and pectinolytic polysaccharides. These enzymes facilitate the maceration of tissue and the degradation of plant cell wall components [Riou *et al.* 1992]. Oxalic acid is also produced by a number of other fungal pathogens including, *Phanerochaete chrysosporium* (white-rot fungus) and *E. graminis* f. sp. *hordei* (powdery mildew) [Hurkman and Tanaka 1995].

1.3.3. Control of *Sclerotinia.*

Various strategies have been employed in the past in attempts to control the potentially damaging effects of *S. sclerotiorum.* These have included, biological control using fungal, bacterial and insect species (of which there are at least 30 species) which have been reported to be parasites or antagonists of *Sclerotinia* species, particularly with respect to the sclerotia [Adams and Ayers 1979; Steadman 1979]. Crop rotation and the selection and growth of crop species with increased disease tolerance (i.e. an increased ability to slow the spread of the infection), has often been used to control the effect and spread of *Sclerotinia* species. However, sclerotia generally remain viable in the soil for at least 3 years and so this method of control is not particularly effective [Steadman 1979]. Reduced susceptibility to *S. sclerotiorum* has been reported in a variety of species including white bean [Tu 1985] and sunflower [Noyes and Hancock 1981]. Tolerance to *S. sclerotiorum* is linked to tolerance to

oxalic acid [Tu 1989; Noyes and Hancock 1981]. Oxalic acid deficient mutants of *S. sclerotiorum* have been reported to be non-pathogenic in *Arabidobsis thaliana* [Dickman and Mitra 1992] and *Phaseolus vulgaris* [Godoy *et al.* 1990]. Several lines of *B. napus* [Bailey 1987] and sunflower [Huang and Dorrell 1978] have been screened for potential resistance to *Sclerotinia* with varying levels of susceptibility. Mouly *et al.* [1992] reported the oxalic acid induced expression of extensin mRNA transcripts in sunflower following infection with *S. sclerotiorum*. They suggested that the increased expression and accumulation of extensins (a major protein component of the plant cell wall) results in increased tolerance to *S. sclerotiorum*, because of the strengthening of the cell wall. Oxalic acid appears to have a dual role as the phytotoxic determinant of pathogenicity and as an elicitor of plant defence responses [Mouly *et al.* 1992].

One strategy which has been under serious investigation for some time has involved the production of genetically engineered plants expressing genes coding for proteins which improve the resistance of normally sensitive plants. The ultimate aim being a reduction in the rate of establishment or complete inhibition of infection in species of major economic importance, including oilseed rape and sunflower [Thompson *et al.* 1995].

1.4. The metabolism and distribution of oxalic acid.

1.4.1. Oxalic acid, calcium oxalate and crystal idioblasts in plants.

Oxalic acid is generally considered to be an inert end product of metabolism which is toxic to the majority of plants. Oxalic acid has relatively strong acidic properties (pKa = 1.23 and 3.83), it is a reducing agent and a very strong chelating agent (particularly of calcium ions) [Franceschi and Horner 1980; Libert and Franceschi 1987]. Oxalic acid is probably present in all plants, but in the majority it is only present in very small quantities [Bennet-Clark 1933; Olsen 1939; Libert and Franceschi 1987]. For example, in wheat [Nelson and Hasselbring 1931], barley, maize, oats and rye oxalic acid has been found at concentrations of between 0.019-0.048% of fresh weight material [Nelson and Mottern 1931]. There are only a few plants which produce and accumulate large quantities of dissolved oxalic acid in the cell sap of their leaves, including plants of the genus, *Atriplex, Begonia, Geranium, Mesembryanthemum, Oxalis, Pelagonium* and *Rumex* [Bennet-Clark 1933; Olsen 1939]. Oxalic acid is readily formed in plants (as by-products of respiration and photosynthesis) from the oxidation of a number of compounds including, malic acid, tartaric acid, acetic acid, glycollic acid and glyoxylic acid, which is the predominant direct precursor of oxalic acid [Bennet-Clark 1933], catalysed by glycolate oxidase and lactate dehydrogenase [Davies and Asker 1983; Franceschi 1987]. Oxalic acid is also produced from the cleavage of oxaloacetic acid [Jakoby and Bhat 1958] and L-ascorbic acid [Nuss and Loewus 1978]. Information on the localisation of oxalic acid biosynthesis and transport in plants is scarce. Biosynthesis may possibly taking place in the cytosol, glyoxysomes and peroxisomes, considering the pathways involved [Leek *et al.* 1972; Franceschi and Horner 1980; Libert and Franceschi 1987]. Osmond [1968], Joy [1968] and Smith [1972] suggested that the accumulation of oxalic acid in *Atriplex spongiosa, Beta vulgaris* and *Setaria sphacelata* leaves is connected with the assimilation of nitrate and

the preservation of the cation-anion balance of the plants. Oxalic acid may also play a role in the oxidative biochemistry of the extracellular matrix of higher plants [Lane 1994].

Oxalic acid occurs in the insoluble (non-toxic) form, as crystals of calcium oxalate in the majority of plants [Olsen. 1939]. Olsen [1933] reported the quantitative relationship between oxalic acid production and the amount of calcium absorbed by oxalic acid producing plants, including *Fagus silvatica*, in which 62% of total calcium was present as calcium oxalate. *Beta vulgaris* contains about 12.4% oxalic acid on a dry weight basis, about 25% of total oxalic acid exists in the soluble form [Baker and Eden 1954]. Calcium oxalate crystals (in the monohydrate or dihydrate forms) are usually associated with membranes or as inclusions within cell vacuoles. These cells are called crystal idioblasts, which form in different tissues in different plants and as different crystalline structures [Franceschi and Horner 1980; Franceschi 1984 1989]. Trees in the genera Casuarinacea possess a calcium oxalate secreting system where by the epidermal cells deposit crystals in the cell walls of the branchlet surface [Berg 1994]. Various functions for the formation of calcium oxalate crystals and crystal idioblasts have been suggested, including the conversion of oxalic acid to non-toxic calcium oxalate, involvement in cell ionic balance, structural support, protection against foraging animals and in gas exchange by regulating calcium ions in the vicinity of stomatal guard cells [Franceschi and Horner 1980; Ruiz and Mansfield 1994]. A storage function for crystal idioblasts has also been suggested as there is evidence to indicate the reabsorbance of calcium during periods of calcium shortage [Franceschi and Horner 1980; Franceschi 1989]. Webb *et al.* [1995] recently demonstrated that the matrix associated with calcium oxalate crystals in the leaves of grape consisted of two phases. A water soluble matrix phase which surround the crystal chambers and membrane chambers which enclose the developing crystals.

1.4.2. Oxalic acid degrading enzymes.

Two enzymes are known to catalyse the breakdown of oxalic acid. Oxalate oxidase (E.C. 1.2.3.4), which produces two moles of carbon dioxide and one mole of hydrogen peroxide from two mole of oxalic acid and one mole of oxygen (Equation A). Oxalate decarboxylase (E.C. 4.1.1.2) converts two moles of oxalic acid to one mole of formic acid and one mole of carbon dioxide (Equation B).

A $2COOH + O_2 \xrightarrow{\text{oxalate oxidase}} H_2O_2 + 2CO_2$

B $2COOH \xrightarrow{\text{oxalate decarboxylase}} HCOOH + CO_2$

Oxalate oxidase has been identified in an increasing number of plants, including barley [Chiriboga 1966; Pietta *et al.* 1982; Dumas *et al.* 1993; Lane *et al.* 1993], *Sorghum vulgare* [Pundir and Nath 1984; Pundir and Kuchhal 1989; Pundir 1991; Kuchhal *et al.* 1993; Satyapal and Pundir 1993] and the soil bacterium *Pseudomonas* sp. OX-53 [Koyama 1988].

Oxalate decarboxylase has been identified as the oxalic acid degrading enzyme in a number of plants, including pea seeds [Giovanelli and Tobin 1964] and spinach [Hitomi *et al.* 1992]. Oxalate decarboxylase is also the major oxalic acid degrading enzyme in the micro-organisms *Collyvia veltipes* [Shimazono and Hayaishi 1957; Mehta and Datta 1991], and *Neurospora crassa* which can utilise oxalate oxidase as the sole carbon and energy source [Jakoby and Bhat 1958].

1.5. Distribution of oxalate oxidases.

The nascent synthesis of increasing amounts of a protein designated as G and its mRNA marks the onset of growth in germinating wheat embryos. This protein was first identified in 1979 by Thompson and Lane who were studying cell free translation of total mRNA isolated from dry and imbibing wheat embryos. This protein was shown to be prominent by 24 hr postimbibition but was absent in immature and mature quiescent wheat embryo. Protein G begins to appear as a conspicuous polypeptide spot in two-dimensional O'Farrell-type gels between 5 and 10 hr after the onset of water uptake by dry wheat embryos [Grzelczak *et al.* 1982; Grzelczak and Lane 1983]. Protein G was later renamed germin because of its conspicuous appearance in wheat embryos during imbibition and germination [Grzelczak and Lane 1984; Grzelczak *et al.* 1985]. Maximum concentrations of germin were detected at 30-35 hr postimbibition, with higher levels of the protein detected in embryos cultured in 5% sucrose as opposed to water [Lane *et al.* 1986]. Germin was originally thought to be a rare growth-related protein [Grzelczak and Lane 1984 1985].

In 1993 work carried out by Lane *et al.* showed that wheat germin possessed 96% homology over the 201 amino acid residues of the mature protein of barley seedling oxalate oxidase and were therefore essentially identical (Fig. 1.1). Enzyme activity staining of SDS-PAGE gel electroblots confirmed that germin was actually a oxalate oxidase expressed in germinating wheat embryos, with a specific activity of 8 - 9 units / mg of protein [Lane *et al.* 1993]. Chiriboga [1966] was one of the earliest researchers to publish data concerning the isolation and characterisation of oxalate oxidase from barley seedlings. Many other groups have also isolated and studied barley oxalate oxidase [Sugiura *et al.* 1979; Pietta *et al.* 1982; Schmitt 1991; Dumas *et al.* 1993]. Oxalate oxidases have also been isolated, purified and characterised from moss [Suzuki and Meeuse 1965], sugar beet [Leek *et al.* 1972; Obzansky and Richardson 1983], *Bourgainvillea spectabilis* [Shivastava and Krishnan 1962], *Sorghum vulgare* [Pundir and Nath 1984; Pundir and Kuchhal 1989; Pundir 1991; Kuchhal *et al.* 1993; Satyapal and Pundir 1993], banana [Inamdar *et al.* 1986; Raghaven and Tarachand 1986; Inamdar *et al.* 1989] and *Pseudomonas* OX-53 [Koyama 1988].

1.6. Expression of oxalate oxidases.

Oxalate oxidase probably accounts for less than 0.1% of soluble proteins in germinating wheat embryos [Grzelczak and Lane 1984] with an estimated 2×10^6 oligomeric molecules in each cell [Lane *et al.* 1986]. Oxalate oxidase is expressed in roots, leaves and stems of wheat seedlings between 1.5 and 7 days postimbibition [Grzelczak *et al.* 1985]. The

Figure 1.1. Amino acid sequence alignment of wheat embryo and barley root oxalate oxidases.

WEOX, wheat embryo oxalate oxidase [Lane *et al.* 1991] (Accession no. P15290); BROX, barley root oxalate oxidase [Lane *et al.* 1993] (Accession no. P45850). The numerals designate the amino acid sequence. The characters in bold represent differences between the two amino acid sequences.

```
BROX SDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFLFSSKLTKAGNTSTPNGSAVTELD  60
WEOX TDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFLFSSKLAKAGNTSTPNGSAVTELD  60

BROX VAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIGMVMKGELLVGILGSLDSGNKLYS 120
WEOX VAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIGIVMKGELLVGILGSLDSGNKLYS 120

BROX RVVRAGETFVIPRGLMHFQFNVGKTEAYMVVSFNSQNPGIVFVPLTLFGSDPPIPTPVLT 180
WEOX RVVRAGETFLIPRGLMHFQFNVGKTEASMVVSFNSQNPGIVFVPLTLFGSNPPIPTPVLT 180

BROX KALRVEAGVVELLKSKFAGGS                                         201
WEOX KALRVEARVVELLKSKFAAGF                                         201
```

quantity of oxalate oxidase in seedlings (about 1 mg / 100 g) keeps pace with growth from embryo to seedling in spite of the 100-fold increase in fresh weight [Grzelczak *et al.* 1985]. Pulse-labelled oxalate oxidase is equally distributed between the pellet (insoluble) and soluble fractions of homogenates of growing wheat embryos, which suggests a possible association of some of the oxalate oxidase with the cell wall / plasma membrane [Lane *et al.* 1986]. At day 7 the concentration of oxalate oxidase is greater in roots and stems compared with leaves on a per weight basis [Grzelczak *et al.* 1985]. The calculated oxalate oxidase concentrations in the soluble fractions of homogenates are 1, 2, 0.2 and 0.8 mg / 100 g fresh weight for roots, stems and leaves of seedlings and germinated embryos, respectively [Grzelczak *et al.* 1985]. In roots, stems and leaves oxalate oxidase accounts for approximately 0.5, 0.3 and 0.02% of soluble proteins [Grzelczak *et al.* 1985]. Measurable levels of oxalate oxidase are not detected in flower buds [Lane *et al.* 1986]. Grzelczak *et al.* [1985] also detected oxalate oxidase in barley, oat and rye seedlings, but not in dicotyledonous plants, including pea, lettuce or radish seedlings, lower plants (moss) or higher animals (e.g. fibroblasts in tissue culture). However, Suzuki and Meeuse [1965] had reported the presence of oxalate oxidase in moss. Oxalate oxidase has been detected in the stems of 37 day old wheat plants using dye-staining techniques [Lane *et al.* 1986]. An antigenically related oxalate oxidase isoform has also been identified associated with the cell walls of barley seedlings [Lane *et al.* 1992]. Hurkman and Tanaka [1996] demonstrated that barley oxalate oxidase is expressed at maximum levels by 2 days postimbibition.

Pseudogermin, a protein related to wheat embryo oxalate oxidase was discovered by Lane *et al.* [1992] as a protein expressed between 20 - 25 days postanthesis (a stage of embryo development where cell enlargement is occurring at its maximum rate) in the wash fraction of mature embryo or in soluble extracts of immature wheat embryos. Chiriboga [1966] identified barley roots as containing the highest levels of oxalate oxidase activity (2.55) followed by hypocotyls of 10 day old plants (0.234) and seeds of barley (0.021). Like the wheat embryo protein, barley oxalate oxidase is strongly expressed and accumulates in barley embryos during germination [Dumas *et al.* 1993]. Antibodies raised against barley root oxalate oxidase expressed in *E. coli* cross-react with wheat embryo oxalate oxidase [Dumas *et al.* 1993]. Kotsira and Clonis [1997] demonstrated that when barley was grown in the presence of NaCl the oxalate oxidase activity content and specific activity decreased compared with barley grown in water only. However, the presence of NaCl in the growth media caused significant inhibition of seedling growth; by day 4 seedling growth was inhibited by about 68% [Hurkman and Tanaka 1996]. NaCl treatment prolonged the expression of oxalate oxidase by an additional day [Hurkman and Tanaka 1996].

Barley oxalate oxidase mRNA levels are highest in roots compared with the shoots where an additional oxalate oxidase transcript is expressed [Hurkman and Tanaka 1996]. Oxalate oxidase expression increases as a result of treating barley roots with plant growth regulators (abscisic acid (ABA) and indole acetic acid (IAA)) and compounds which activate PR proteins, such as salicylic acid, methyl salicylic acid and methyl jasmonic acid [Hurkman and Tanaka 1996]. Oxalate oxidase is expressed in barley seedlings following infection with

powdery mildew (*E. graminis* f. sp. *hordei*) but not wounding [Dumas *et al.* 1995; Zhang *et al.* 1995] and the levels of oxalate oxidase are 10 fold higher in infected compared with uninoculated plants [Zhang *et al.* 1995]. Trinchant and Riguad [1996] have reported the occurrence of oxalate oxidase in bacteriods (detected activity assays), the name given to the symbiotic partnership between rhizobia and legumes, in this case faba bean.

1.7. Localisation of oxalate oxidases.

Srivastava and Krishnan [1962] identified a plastid bound oxalate oxidase in leaves of *B. spectabilis*. Meeuse and Campbell [1959] found evidence to suggest a mitochodrial association of oxalate oxidase in the roots of sugar beet. Whereas, Leek *et al.* [1972] observed oxalate oxidase activity with the chloroplast fraction of sugar beet leaf homogentates, which contained peroxisomes, following subcellular fractionation. Schmitt [1991] reported high activity for oxalate oxidase in the particulate fraction of root homogenates. Lane *et al.* [1992] used subcellular localisation and immunological techniques to demonstrate the association of wheat oxalate oxidase with the cell walls of germinating embryos. Approximately 40% of total wheat oxalate oxidase is reported to be in the cell wall-associated form at 2 days postimbibition, which remains associated with the pellet (insoluble) fraction from a total extract of embryos. Wheat oxalate oxidase was not present in the nuclei or mitochondria of the pellet fraction of embryo homogenates [Grzelczak *et al.* 1982]. The exact location of the other 60% of the intracellular wheat embryo oxalate oxidase has yet to be determined [Lane *et al.* 1986 1992].

Caliskan and Cuming [1997] demonstrated that oxalate oxidase is expressed and initially accumulates within the coleorhiza of barley (Fig. 1.2.). Oxalate oxidase is detected in roots but not in the apical meristem 48 hr after germination and it is only detected in the coleoptile of older seedlings (7 days postimbibition) in the epidermal cell layer, the vascular bundles and the bundle sheath cells [Caliskan and Cuming 1997]. In 3 day germinated barley seedlings oxalate oxidase is located in the epidermal cells of the mature region of the primary roots and the coleorhiza [Caliskan and Cuming 1997]. The coleoptile, elongation zones and tips exhibit no oxalate oxidase activity [Caliskan and Cuming 1997]. By day 10 activity is only detected in the coleorhiza of barley seedlings [Dumas *et al.* 1995]. In powdery mildew infected barley, oxalate oxidase expression was mainly localised along the vascular bundles of infected leaves [Dumas *et al.* 1995].

1.8. Nucleic and amino acid sequence homology of oxalate oxidases.

Rahman *et al.* [1985 1988] produced a cDNA library from mRNA of germinating wheat embryos, which enabled the cloning of a full length oxalate oxidase-specific cDNA (about 1200 nucleotides). Dratewka-Kos *et al.* [1989] deduced the polypeptide structure of wheat embryo oxalate oxidase from a cDNA clone which they isolated and sequenced (Fig. 1.3.). The 1075 nucleotide germin mRNA contained a 69 nucleotide sequence encoding a 23 amino acid residue signal peptide and a 603 nucleotide sequence encoding the 201 mature protein sequence. The N-terminal sequence of wheat embryo oxalate oxidase is acidic (1-87) and the

Figure 1.2. Diagram representing a mature barley caryopsis.

Taken from Salisbury and Ross [1992].

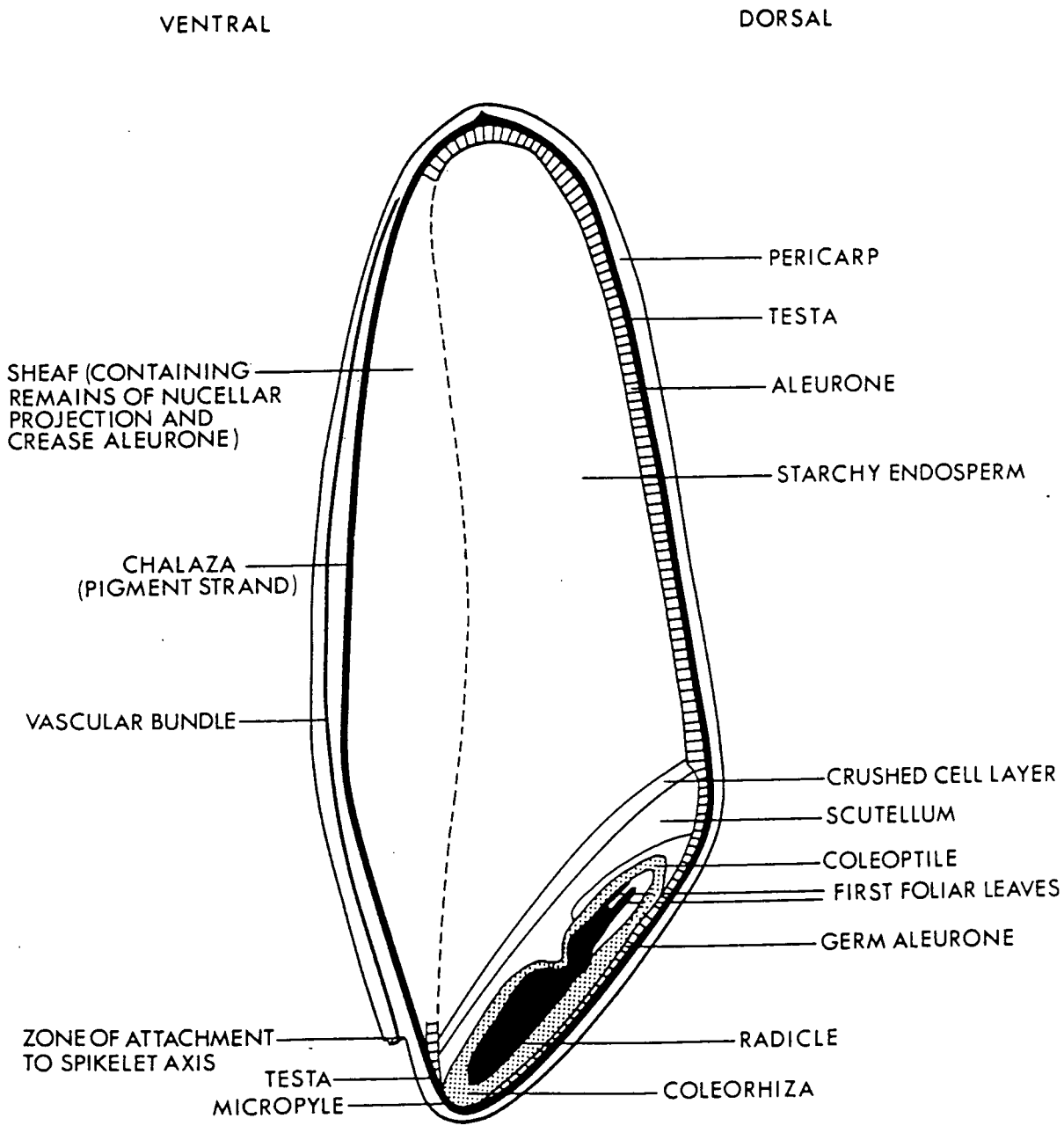


Figure 1.3. The wheat embryo gf-2.8 oxalate oxidase cDNA sequence and translation product.

Lower case lettering designates nucleotides in 'untranslated' regions. Upper case lettering designates nucleotides in the 'translated' regions. The large numerals designate positions in the nucleotide sequence and the superscript numerals designate positions in the amino acid sequence. The characters in bold represent the stretch of 34 uncharged residues and the underlined characters represent the strongly conserved decapeptide sequence. The diagram is modified from that presented by Dratewka-Kos *et al.* [1990] and Lane *et al.* [1991].

5'-untranslated sequence: 85

```
gcagcagcaaccaccagtgccatagacactctccatcaacaaactctagctgatcaatcccta   63
gctaagcttattacatagcaagc                                            85
```

signal peptide coding sequence: 69

```
ATGGGGTACTCCAAAACCCTAGTAGCTGGCCTGTTCGCAATGCTGTTACTAGCTCCGGCCGTC  148
 M  G  Y  S  K  T  L  V  A  G  L  F  A  M  L  L  L  A  P  A  V21
TTGGCC                                                             154
 L  A23
```

mature protein coding sequence: 603

```
ACCGACCCAGACCCTCTCCAGGACTTCTGTGTCGCCGACCTCGACGGCAAGGCGGTCTCGGTG  217
 T  D  P  D  P  L  Q  D  F  C  V  A  D  L  D  G  K  A  V  S  V21
AACGGGCACACGTGCAAGCCCATGTCGGAGGCCGGCGACGACTTCCTCTTCTCGTCCAAGTTG  280
 N  G  H  T  C  K  P  M  S  E  A  G  D  D  F  L  F  S  S  K  L42
GCCAAGGCCGGCAACACGTTCACCCCGAACGGCTCCGCCGTGACGGAGCTCGACGTGGCCGAG  343
 A  K  A  G  N  T  S  T  P  N  G  S  A  V  T  E  L  D  V  A  E63
TGGCCCGGTACCACCACGCTGGGTGTGTCCATGAACCGCGTGGACTTTGCTCCCGGAGGCACC  406
 W  P  G  T  N  T  L  G  V  S  M  N  R  V  D  F  A  P  G  G  T84
ACCCCACCACACATCCACCCGCGTGCCACCGAGATCGGCATCGTGATGAAAGGTGAGCTTCTC  469
 N  P  P  H  I  H  P  R  A  T  E  I  G  I  V  M  K  G  E  L  L105
GTGGAAATCCTTGGCAGCCTCGACTCCGGGAACAAGCTCTACTCGAGGGTGGTGCGCGCCGGA  532
 V  G  I  L  G  S  L  D  S  G  N  K  L  Y  S  R  V  V  R  A  G126
AGACGTTCCTCATCCCACGGGGCCTCATGCACTTCCAGTTCAACGTCGGTAAGACCGAGGCCA  595
 E  T  F  L  I  P  R  G  L  M  H  F  Q  F  N  V  G  K  T  E  A147
TCCATGGTCGTCTCCTTCAACAGCCAGAACCCCGGCATTGTCTTCGTGCCCCTCACGCTCTTC  658
 S  M  V  V  S  F  N  S  Q  N  P  G  I  V  F  V  P  L  T  L  F168
GGCTCCAACCCGCCCATCCCAACGCCGGTGCTCACCAAGGCACTCCGGGTGGAGGCCAGGGTC  721
 G  S  N  P  P  I  P  T  P  V  L  T  K  A  L  R  V  E  A  R  V189
GTGGAACTTCTCAAGTCCAAGTTTGCCGCTGGGTTT                              757
 V  E  L  L  K  S  K  F  A  A  G  F201
```

3'-untranslated sequence: 318

```
taatttctaggagccttccctgaaatgataattatataattccatatatgcatgctagcaaaa  820
tttaataatcctcaccagaagacatgtattcaagtttcaggttaatctcgcatgtagtcgtgt  883
aataagattgaacaagttagcctcatggtgtagccttcgatcagaaccaatatgaggaattga  946
atgtactacttttttattgtcgtctttgttcttttcactgaacggaatatataataagcatttt 1006
cgta                                                              1010
```

C terminal sequence is basic (88-201). A stretch of 34 uncharged amino acid residues may mediate formation of the oligomeric structure of wheat embryo oxalate oxidase and its unusual structural and stability properties [Dratewka-Kos *et al.* 1989]. The amino acid sequence of oxalate oxidase is likely to be heterogenous since wheat is hexaploid and is the product of three progenitor species [Dratewka-Kos *et al.* 1989]. Two full length oxalate oxidase genomic clones (gf-2.8 and gf-3.8) were isolated from a wheat genomic DNA library and sequenced by Lane *et al.* [1991]. The sequence of gf-2.8 was identical to that previously identified by Dratewka-Kos *et al.* [1989]. The coding sequences of gf-2.8 and gf-3.8 do not contain introns and exhibited 87% homology with one another, it was suggested that these genes might encode different oxalate oxidase isoforms [Lane *et al.* 1991]. Wheat embryo total mRNA contained at least a 10-fold higher concentration of gf-2.8 transcripts compared with gf-3.8 [Lane *et al.* 1991]. The mature protein coding regions of the two genes exhibited a difference of 8% in the nucleotide sequence and a difference of 7% in the amino acid residues of the corresponding proteins (201 residues) [Lane *et al.* 1991]. The central core of the two proteins are strongly conserved and they contain the decapeptide sequence PH(I/T)HPRATEI, which is also 50% similar to spherulins 1a and 1b from *Physarum polycephalum* plasmodium [Bernier *et al.* 1987; Lane *et al.* 1991]. Plasmodium is a giant multinucleated diploid cell, which is one of the stages observed in the life cycle of *Physarum.* Spherulins 1a and 1b are specifically expressed during spherulation, a process which involves the encystment, desiccation and developmental arrest of plasmodium during various forms of environmental stress [Bernier *et al.* 1987; Lane *et al.* 1991]. Two sequences characteristic of auxin responsive genes have been identified in the 5'-flanking region of the gf-2.8 gene [Lane *et al.* 1991]. Wheat oxalate oxidase is encoded by a multigene family of 15-20 copies of the gene per hexaploid genome (chromosomes 4A ~5 copies, 4B ~3 copies and 4D ~9 copies) [Lane *et al.* 1991]. Lagudah *et al.* [1991] demonstrated that oxalate oxidase segregated as a single locus on chromosome 4D of *Triticum tauschii*, the D-genome donor to hexaploid wheat. This single locus contained the gene gf-2.8 [Lane *et al.* 1991]. No differences between the amino acid composition of G and G' were detected and N-terminal sequencing of the two wheat embryo oxalate oxidase isoforms showed no differences over the first 22 residues examined [Lane *et al.* 1987]. Schmitt [1991] identified two monomeric oxalate oxidases in barley root which have the same N-terminal amino acid sequence, through the first 18 residues.

1.9. Structural properties of oxalate oxidases.

1.9.1. Protein molecular mass.

Peptide mapping illustrated that the two closely size related wheat embryo oxalate oxidases observed on SDS-PAGE gels were virtually indistinguishable although there was a size difference between the proteins of about 1-2 kDa [Grzelczak and Lane 1984; Lane *et al.* 1986]. The two water soluble germin isoforms are present at similar concentrations in the soluble fraction of wheat embryo homogenates [Grzelczak and Lane 1984]. The molecular weights (M_r) of oligomeric oxalate oxidase isoforms have been calculated to be approximately 126 kDa and 130 kDa by several methods, including sedimentation equilibrium ultra

centrifugation, SDS-PAGE (12.5%), sedimentation coefficient and Stokes radius [Grzelczak *et al.* 1985; McCubbins *et al.* 1987]. There is no difference between the M_r values of the oxalate oxidase isoforms expressed in roots, leaves or stems of wheat seedlings [Grzelczak *et al.* 1985].

The two isoforms of wheat embryo oxalate oxidase identified were named G and G'. G is the dominant species in the water soluble fraction of homogenates of germinated embryos and G', is the dominant species in a fraction obtained by rinsing growing embryos in an aqueous buffer [Lane *et al.* 1986; McCubbins *et al.* 1987]. The G isoform is larger than G' and the two isoforms have oligomer M_r values of about 136 kDa and 120 kDa, respectively [Lane *et al.* 1986]. The G and G' oligomers are converted by brief heat treatment (2 min at 100 °C) to their corresponding monomers (about 26.2 and 23.5 kDa, respectively). Only about 5% of total protein was retrieved from the wash fraction of germinated wheat embryos and of this more than 50% was oxalate oxidase [Lane *et al.* 1986]. Crystals (prolate ellipsoid) of wheat embryo oxalate oxidase (> 90% G') were reported to have dimensions of 9.5 X 7.8 X 5.0 nm [McCubbins *et al.* 1987]. The proportion of G' to G increased (> 80% G' : < 20% G) when the embryos were cultured in 5% sucrose compared with water [Lane *et al.* 1986]. The presence of salts in the wash buffer appeared to be necessary for the release and recovery of G' when washing intact embryos [Lane *et al.* 1986]. Saturated solutions of NaCl and KCl did not cause any dissociation of the oligomer to monomer at room temperature [Lane *et al.* 1986]. The wheat oxalate oxidase isoforms are unusually stable and resistant to dissociation in sodium dodecyl sulphate (SDS) containing solutions [Grzelczak and Lane 1983 1984 1985]. Cross-linking of the G' isoform of wheat oxalate oxidase with dimethyl suberimidate confirmed that the protein was pentameric forming a series of cross-linked heat stable bands of the various oligomeric forms on SDS-PAGE gels, following heat treatment of the protein in the presence of SDS [McCubbins *et al.* 1987].

Pseudogermin exhibits different physical properties to the oxalate oxidase isoforms expressed during wheat embryo germination (G and G'). Pseudogermin has an oligomer M_r value mass of about 105 kDa and a monomer M_r value of about 25.3 kDa, which suggests that this protein may be a tetramer and not a pentamer like G and G' [Lane *et al.* 1992]. Pseudogermin has unprecedented disulphide-independent thermostability with only limited dissociation to the monomer if heat treated in the presence of SDS at 100 °C ($t_{½}$ ≈6 min), compared with G and G' ($t_{½}$ <20 sec) which fully dissociate [Lane *et al.* 1992].

Sugiura *et al.* [1979] showed that barley seedling oxalate oxidase is a dimer with an M_r value of 150 kDa consisting of two 75 kDa monomers, a result confirmed by Pietta *et al.* [1982]. Schmitt [1991] later reported the presence of an oxalate oxidase in barley roots which appears to exist as an active monomer, with a M_r value of 25-29 kDa. Several forms of the enzyme have been identified following reverse phase HPLC of purified protein [Schmitt 1991]. Peptide mapping of these different forms highlighted only minor differences in their profiles, suggesting that they may be isoforms [Schmitt 1991]. Oxalate oxidase purified from *Pseudomonas* sp. OX-53 has an oligomer M_r value of about 320 kDa and a monomer M_r value of 38 kDa, this enzyme may exist as an octamer [Koyama 1988]. *Sorghum vulgare* leaf

oxalate oxidase has an M_r value of 120.2 kDa for the dimer and 62 kDa for the monomer, as determined by gel filtration chromatography and SDS-PAGE, respectively [Pundir 1991; Satyapal and Pundir 1993].

Lane *et al.* [1993] and Kotsira and Clonis [1997] subsequently demonstrated that barley seedling oxalate oxidase exists as a homopentameric protein with an M_r value for the oligomer of about 125 kDa and 25 kDa for the monomer, which is obtained by brief heat treatment (2 min at 100 °C). Dumas *et al.* [1993] also isolated an oxalate oxidase from barley root which has an oligomer M_r value of about 100 kDa and a monomer M_r value of about 26 kDa, which suggests that this enzyme is a homotetramer. Both of these barley root oxalate oxidase enzymes are water soluble and resistant to dissociation in SDS containing solutions [Lane *et al.* 1993; Dumas *et al.* 1993]. Zhang *et al.* [1995] have identified an active, SDS stable, oxalate oxidase in barley induced as a result of powdery mildew infection. The protein has an M_r value of about 100 kDa, which consist of subunits with M_r values of about 27 kDa. A minor band with an M_r value of about 95 kDa was also observed on SDS-PAGE gels. Zhang *et al.* [1995] isolated an oxalate oxidase from uninoculated barley plants which has an M_r value of about 97 kDa and an oxalate oxidase obtained from a commercial source is reported as having an M_r value of about 80 kDa. Considerable variability in the size of the oxalate oxidase subunits and the degree of oligomerisation for the enzymes isolated from different barley organs has been reported.

There are two reports of the pI of barley seedling oxalate oxidase pH 2.8 [Sugiura *et al.* 1979] and pH 6.5-7.5 [Schmitt 1991]. Koyama [1988] found *Pseudomonas* oxalate oxidase has a pI of pH 4.7.

1.9.2. Glycosylation.

Lane *et al.* [1987 1992] initially demonstrated that the wheat embryo oxalate oxidase isoforms and pseudogermin are glycoproteins, as determined by staining with Schiff reagent. Treatment of wheat embryo oxalate oxidase with TFMS (trifluoromethane sulphonic acid) resulted in the deglycosylation and dissociation of the oligomer and the formation of monomeric polypeptides with increased mobility on SDS-PAGE gels, corresponding to loss of molecular mass of about 1 kDa [Lane *et al.* 1987]. The wheat embryo oxalate oxidase isoforms are absorbed onto concanavalin A (ConA)-sepharose confirming the presence of glycan(s) [Jaikaran *et al.* 1990]. Lane *et al.* [1987] cultured wheat embryos in the presence of tritiated glycans (mannose, glucosamine or fucose) and found that the G and G' isoforms of oxalate oxidase are differentially labelled which suggests that the observed size difference between the two isoforms may result from differential glycosylation of otherwise identical apoproteins. Lane *et al.* [1987] speculated that the difference in molecular weight of the two oxalate oxidase isoforms might have resulted from the presence of a greater number of N-acetylglucosamine residues in G compared with G'. The oxalate oxidase apoprotein has two possible site of N-glycosylation (at positions 47 and 52, relative to the N-terminus) and the apoprotein itself has a molecular weight of about 20 kDa, as calculated from the primary amino acid sequence [McCubbins *et al.* 1987]. Tunicamycin treatment of germinating wheat

embryos resulted in the production of a 100 kDa oligomer for oxalate oxidase, corresponding to a monomer of about 20 kDa. McCubbins *et al.* [1987] suggested that the presence of a 2.5 kDa oligosaccharide chain at each of the two glycosylation sites would account for the molecular weight of wheat embryo oxalate oxidase (i.e. about 25 kDa). Glycosylation of the monomers was necessary for the assembly and maintenance of the pentameric structure of wheat embryo oxalate oxidase [Jaikaran *et al.* 1990].

Jaikaran *et al.* [1990] demonstrated that wheat embryo oxalate oxidase contained N-linked but not O-linked glycans. The oxalate oxidase isoform G is converted to a protein which has the same molecular weight as G' following treatment with β-N-acetylglucosaminidase, which suggests the presence of anntenary N-acetylglucosamines in the N-glycans of G but not G'. The only N-acetyl glucosamine in G' is confined to the N, N-diacetylchitobiosyl core [Jaikaran *et al.* 1990]. Following sub-lethal tunicamycin treatment of germinating wheat embryo a 'ladder' of seven oxalate oxidase oligomers is detected by SDS-PAGE replacing the usual doublet [Jaikaran *et al.* 1990]. The heptad of oxalate oxidase oligomers consists of pentamers containing different ratios of glycosylated and unglycosylated monomers. The lowest molecular weight oligomers do not bind to ConA-sepharose and consist entirely of unglycosylated monomers [Jaikaran *et al.* 1990]. Incorporation of tritiated glycans into oxalate oxidase synthesised in germinating wheat embryos confirmed the presence of fucose, glucosamine, xylose and mannose in the polysaccharide attachments (G monomer, $(GlcNAc_2(Man)_2(Man\text{-}Xyl)(GlcNac)(GlcNAc\text{-}Fuc))$; G' monomer $((Man)_2(Man\text{-}Xyl)(GlcNAc)(GlcNAc\text{-}Fuc)))$ [Jaikaran *et al.* 1990]. Jaikaran *et al.* [1990] calculated the oligosaccharide component of pentameric oxalate oxidase to have an M_r value of about 2.5 kDa (i.e. about 0.5 kDa per monomer). The additional 13.5 kDa difference in the M_r value of G (136 kDa) and G' (120 kDa) could be attributed to the retarding effect on protein mobility of the additional antennary N-acetylglucosamine residues in G on SDS-PAGE gels and / or the reduced net binding of SDS to the glycoprotein [Jaikaran *et al.* 1990]. Psuedogermin has also been shown to be glycosylated [Jaikaran *et al.* 1990]. Schmitt [1991] found no evidence to suggest that the isolated 25-29 kDa barley root oxalate oxidase is glycosylated. However, Dumas *et al.* [1993] reported that their isolated barley root oxalate oxidase absorbed onto a column of sepharose ConA, providing strong evidence that this enzyme is also glycosylated.

1.9.3. Secondary structure.

Ten cysteine residues are present per oxalate oxidase homopentamer [Kotsira and Clonis 1997]. Kotsira and Clonis [1997] suggested that disulphide bond formation between the cysteines may help to maintain the integrity of the monomer subunits and help to preserve catalytic activity. McCubbins *et al.* [1987] examined the CD spectra of wheat embryo oxalate oxidase under various experimental conditions. Resuspending the protein in water or 50 mM sodium phosphate (pH 7.0) had little effect on the CD spectra. The native protein contained approximately 10 - 20% α-helix, 33 - 38% β-sheet and 19% β-turn. Heat treatment of the protein (73 °C for 10 min or 100 °C for 2 min) did not appear to cause any change in the levels of different secondary structure compared with untreated protein. However, in the presence of

SDS (0.8% (w/v)) the secondary structures altered considerably when the proteins were heat treated giving rise to increased α-helix and decreased β-sheet content. Low pH (2.0) had a similar effect to heat treatment in the presence of SDS.

1.9.4. Resistance to proteolysis.

Grzelczak and Lane [1984 1985] first reported the use of proteolysis for the isolation and purification of wheat embryo oxalate oxidase. Oxalate oxidase is completely resistant to the proteolytic conditions used to degrade nucleoplasmin (0.1 µg pepsin for 15 min at 37 °C), the most abundant protein in the nucleus of *Xenopus laevis* oocytes [Digwall *et al.* 1982; Grzelczak and Lane 1984 1985]. Wheat oxalate oxidase also exhibits a degree of stability towards the action of broad-specificity proteases [Grzelczak and Lane 1984 1985]. There is no evidence to suggest that proteolysis physically alters the wheat oxalate oxidase compared with the untreated protein [Lane *et al.* 1987]. The protease-resistance property of wheat oxalate oxidase has been utilised to enable the purification of this protein by digesting the majority of the soluble wheat embryo proteins [Grzelczak and Lane 1984 1985; Lane *et al.* 1987]. 90 - 100% yields oxalate oxidase are obtained using ammonium sulphate precipitation and gel filtration of the pepsin-treated soluble wheat embryo proteins [Lane *et al.* 1987]. Dumas *et al.* [1993] demonstrated that barley root oxalate oxidase exhibits no loss of activity following digestion with trypsin (for 4 hr).

Antibodies raised against the wheat embryo oxalate oxidase isoforms (G and G') and pseudogermin oligomers cross-reacted well with the other oligomers and to the a lesser extent to the corresponding monomers. Antibodies raised against the monomers cross-reacted well with all the monomers but not with the oligomers [Lane *et al.* 1992]. Antibodies against barley and wheat oxalate oxidases cross-react, showing that these proteins have common epitopes [Dumas *et al.* 1993 1995; Lane *et al.* 1993]. The anti-barley oxalate oxidase antibodies were raised against *E. coli* expressed oxalate oxidase apoproteins which meant that cross-reactions did not result from glycan binding [Dumas *et al.* 1995]. Lane *et al.* [1993] reported that staining of electroblots for enzyme activity for the detection of oxalate oxidase was superior to detection by anti-oxalate oxidase antibodies.

1.10. Enzyme kinetic of oxalate oxidases.

Considerable variations are reported in the K_m values for oxalate oxidases isolated and purified from different sources, which are listed in table 1.1. Substrate inhibition was observed at high concentrations of oxalic acid with many of the oxalate oxidases studied, including *S. vulgare* leaf (> 250 µM) [Pundir and Nath 1984],barley root (≥ 4 mM) [Kotsira and Clonis 1997] and bacteriod (> 2.5 mM) [Trinchant and Rigaud 1996].

There is a degree of variation in the reported pH optima for the different oxalate oxidases which have been studied, the results of which are listed in table 1.2.

Enzyme source	K_m value (mM)	Reference
barley seedling	0.42	Chiriboga 1966
barley root	0.20 0.27	Schmitt 1991 Kotsira and Clonis 1997
sugar beet peroxisomal preparation	0.8	Leek *et al.* 1972
S. vulgare leaf	0.024 0.078	Pundir and Nath 1984 Satyapal and Pundir 1993
bacteriod	1.4	Trinchant and Rigaud 1996
Pseudomonas	9.5	Koyama 1988

Table 1.1. Oxalate oxidase Michaelis constant (K_m) values.

Enzyme source	pH optimum	Reference
barley seedling	3.5	Chiriboga 1966; Sugiura *et al.* 1979
barley root	3.5-3.8 2.4-3.4	Schmitt 1991; Kotsira and Clonis 1997 Saka Amini and Vallon 1994
sugar beet peroxisomal preparation	4.0	Leek *et al.* 1972
sugar beet stem	5.2	Obzansky and Richardson 1983
S. vulgare leaf	4.3 5.0	Pundir 1991; Pundir and Nath 1984; Kuchhal *et al.* 1993; Satyapal and Pundir 1993
S. vulgar root	5.0	Pundir and Kuhhal 1989;
banana peel	5.2	Inamdar *et al.* 1986; Raghavan and Tarachand 1986; Inamdar *et al.* 1986
B. spectabilis	6.8-7.0	Shivastava and Krishnan 1962
Pseudomonas	4.8	Koyama 1988

Table 1.2. Oxalate oxidase pH optima.

Oxalate oxidases from various plant sources have broad activity in the acid pH range, including barley seedling (pH 2-4) [Sugiura *et al.* 1979], *S. vulgare* leaf (pH 3-6) [Pundir and Nath 1984; Pundir 1991], *S. vulgare* root (pH 2-6) [Pundir and Kuchhal 1989] and bacteriod (pH 8) [Trinchant and Rigaud 1996].

Oxalate oxidase isolated from different sources (mainly from plant origin) have been reported to exhibit differing degrees of stability when subjected to high temperatures, as shown in table 1.3.

Enzyme source	% total activity	Reference
barley seedling	49% activity after 3 min at 89°C 80% activity after 30 min at 75°C	Chiriboga 1966 Sugiura *et al.* 1979
barley root	20% activity after 30 min at 75°C 100% activity after 10 min at 75°C	Schmitt 1991 Dumas *et al.* 1993
S. vulgare leaf	48% activity after 10 min at 50°C 20% activity after 3 min at 80°C	Pundir 1991 Kuchhal *et al.* 1993
B. spectabilis	40% activity after 15 min at 60°C	Shivastava and Krishnan 1962
Pseudomonas	9% activity after 10 min at 65°C	Koyama 1988

Table 1.3. Oxalate oxidase thermal stability.

Suzuki and Meeuse [1965] reported no significant loss in oxalate oxidase activity when an extract prepared from moss was heat treated (15 min at 100 °C) to eliminate catalase activity. All the oxalate oxidases tested have been found to exhibit optimum enzyme activity at temperatures between 35 and 45 °C. The reported optimum temperatures for oxalate oxidases of plant origin are listed in table 1.4.

Enzyme source	Temperature optimum (°C)	Reference
barley seedling	37	Sugiura *et al.* 1979
barley root	35	Saka Amini and Vallon 1994
S. vulgare root	40	Pundir and Kuchhal 1989
S. vulgare leaf	40-45	Pundir and Nath 1984; Pundir 1991; Kuchhal *et al.* 1993

Table 1.4. Oxalate oxidase temperature optima

Many of the oxalate oxidases also appear to be stable at low temperatures with little or no loss of enzyme activity, including barley root (2 weeks at -80, -20, 4 °C and room temperature) [Schmitt 1991] and *Pseudomonas* (6 months at -20 °C) [Koyama 1988]. Lane *et al.* [1987] reported that wheat embryo oxalate oxidase is structurally stable with no evidence of deterioration when stored for more than a year at -70 C.

The majority of oxalate oxidases studied have almost complete substrate specificity towards oxalic acid. Oxalate oxidases isolated from *Pseudomonas* oxidised glyoxylic and malic acid at low rates compared with oxalic acid as substrate (3.7 and 2.1%, respectively) [Koyama 1988] and *S. vulgare* leaf oxalate oxidase caused low levels of maleic acid oxidation (4.8%) [Satyapal and Pundir 1993].

Some of the oxalate oxidases studied are activated by flavins (e.g. FAD and FMN), including barley seedling [Chiriboga 1966], *S. vulgare* leaf [Pundir and Nath 1984, Pundir 1991; Satyapal and Pundir 1993] and *Pseudomonas* [Koyama 1988]. Inhibition of enzyme activity of various oxalate oxidases (barley root, *S. vulgare* and *Pseudomonas*) by iodoacetate suggests the presence of -SH groups at the active site [Chiriboga 1966; Pundir and Nath 1984; Koyama 1988]. Thiocyanate and diethyldithiocarbamate cause strong inhibition of some oxalate oxidases, which suggests the presence of heavy metal ions in the enzyme system, such as moss [Suzuki and Meeuse 1965] and *S. vulgare* root and leaf [Pundir and Kuchhal 1989; Kuchhal *et al.* 1993]. Thiocyanate and diethyldithiocarbamate are both strong chelators and inhibitors of metal ions, particularly iron and copper. EDTA caused the activation of some oxalate oxidases (moss) [Suzuki and Meeuse 1965] and the inhibition of others (*B. spectabilis*) [Srivastava and Krishnan 1962], (*S. vulgare* leaf and root) [Pundir and Nath 1984; Pundir and Kuchhal 1989; Kuchhal *et al.* 1993; Satyapal and Pundir 1993], which suggests that these enzymes have a metal ion requirement (in particular Cu^{2+} and Fe^{2+}). Several groups have reported the inhibition of oxalate oxidase by nitrate and chlorate [Srivastava and Krishnan 1962; Chiriboga 1966; Inamdar *et al.* 1986; Varalakshmi *et al.* 1995], which is reversible by

dialysis [Suzuki and Meeuse 1965]. Considerable loss of activity of barley root oxalate oxidase was reported by Pietta *et al.* [1982] following ammonium sulphate precipitation.

Oxalate oxidase is widely used as a medical tool for the measurement of oxalic acid in urea for the diagnosis of hyperoxaluria, a condition in which oxalic acid accumulates in body fluids, due to a lack of oxalate metabolising enzymes in man. Oxalic acid retention also results in urinary stone formation, which leads to other renal disorders [Hodgkinson 1977]. Many of the oxalate oxidases identified from different plant sources have been examined with respect to their suitability for measuring urinary oxalic acid, including moss [Laker *et al.* 1980], beet stem [Obzansky and Richardson 1983; Varalakshmi *et al.* 1995] and barley root [Ichiyama *et al.* 1985; Buttery and Pannall 1987; Saka Amini and Vallon 1994]. Immobilised oxalate oxidase is of particular interest because of the broader pH range and a shift in the optimal pH observed for several of these bound protein to a more physiological pH (about 7.0-7.4) [Raghavan and Tarachand 1986; Varalakshmi *et al.* 1995]. As well as exhibiting an enhanced specific activity and detection range for oxalic acid [Saka Amini and Vallon 1994].

Several reports have been published regarding altered physicochemical properties of oxalate oxidases immobilised on various solid supports. Varalakshmi and Richardson [1992] and Varalakshmi *et al.* [1995] reported increased storage, thermostability, resistance to proteolytic activity and increased tolerance to heavy metal inactivation of ConA and polyethylene glycol (PEG) linked beet stem oxalate oxidases. Similar properties are reported for banana fruit peel oxalate oxidase immobilised on different supports, including acrylamide [Inamdar *et al.* 1989] and hydrazine beads [Lathika *et al.* 1995]. ConA immobilised beet stem oxalate oxidase showed a broad optimum pH at 3.5-5.0 and an apparent 3-fold increase in the apparent K_m value [Varalakshmi and Richardson 1992]. The PEG-linked beet stem enzyme exhibited a marked shift in optimum pH from 4.5 to 6.5 without any significant change in K_m [Varalakshmi *et al.* 1995] Immobilised banana peel oxalate oxidase has a higher K_m values compared with the enzyme in solution (220-660 µM and 160 µM, respectively) Immobilisation of barley root oxalate oxidase on collagen or nylon resulted in a narrowing of the optimum pH to 2.4-2.6 and greatly increased K_m values compared with the native enzyme [Saka Amini and Vallon 1994]. Antibody complexed banana peel oxalate oxidase retained activity following storage at 4 °C for two years [Lathika *et al.* 1995].

1.11. Germin-like proteins in plants.

In the last few years, particularly since the advent of more accessible computer databases many proteins have been identified which have significant protein sequence homology to wheat germin (wheat embryo oxalate oxidase), but only a small minority of these proteins exhibit oxalate oxidase enzyme activity. These proteins have been termed germin or oxalate oxidase-like proteins. The majority of germin-like protein sequences which have been identified are derived from translated cDNA sequences and the functions of these proteins are unknown.

A number of germin-like proteins have been identified in a variety of plants whose synthesis and accumulation occurs during salt stress. Michalowski and Bohnert [1992]

isolated a nucleotide sequence from a root cDNA library of 6 hr salt stressed *Mesembryanthemum crytstallinum,* which shares 38.5% homology and 75.5% similarity (taking into account conserved replacements) with wheat germin and about 57% similarity with spherulins 1a and 1b. Several prominent proteins have been identified in barley roots, whose expression and accumulation increases during salt stress, in salt-tolerant (CM 72) but not salt-sensitive cultivars (Prato) [Hurkman and Tanaka 1987 1988; Hurkman *et al.* 1989 1991 1994]. Expression did not occur as a result of heat shock [Hurkman and Tanaka 1987] or drought stress [Hurkman and Tanaka 1988]. The levels of expression of these proteins fell when the plants were transferred to nutrient solution without NaCl [Hurkman and Tanaka 1987]. Two of these proteins Gs1 and Gs2, had M_r values of about 26 and 27 kDa and calculated pI's of 6.3 and 6.5, respectively [Hurkman *et al.* 1991]. Both of these glycoproteins showed high amino acid sequence homology to wheat germin (83% and 75% for Gs1 and Gs2, respectively) and shared similar resistance to proteolysis [Hurkman *et al.* 1991]. Gs1 and Gs2 were expressed in roots and coleoptiles but not in leaves and the levels of expression decline in coleoptiles during salt stress and ABA treatment resulted in the accumulation of Gs2 [Hurkman *et al.* 1991]. A third protein (M_r 25.5 kDa) which is antigenically related to Gs1 has also been identified, the status of which is unknown [Hurkman *et al.* 1991]. A cDNA sequence isolated from a library prepared from salt stressed barley roots showed 98.5% sequence similarity to wheat germin (gf-2.8). The protein (oligomer M_r value of 125 kDa and a monomer M_r value of 25 kDa) exhibits oxalate oxidase activity on electroblots and cross-reacted with antibodies raised against Gs1 and Gs2 [Hurkman *et al.* 1994; Hurkman and Tanaka 1996]. Hurkman and Tanaka [1995] have also demonstrated significantly increased levels of wheat germin gene transcription during powdery mildew infection of wheat, as well as in response to methyl salicylic acid and methyl jasmonic acid treatment (activators of PR protein expression). Singh *et al.* [1987] have identified a 26 kDa protein in cultured tobacco cells which constitutes 10% of the total cellular protein when the cells are subjected to salt stress.

Heintzen *et al.* [1994] have identified a cell wall associated, germin-like protein (*Sa*gIp) in the long day plant *Sinapis alba* (white mustard), a non-oxalic acid producing plant [Olsen 1933]. The levels of the *Sa*gIp mRNA undergo circadian oscillations in light / dark cycles with maximum expression at about 14 hr from the beginning of the light period. The mature protein has a predicted M_r value of 22 kDa (pI 5.6) which shares 40% amino acid homology with wheat germin [Heintzen *et al.* 1994]. A fusion protein produced in *E. coli* from the coding region of *Sa*gIp had an M_r value of 22 kDa. *In situ* hybridisation with an *Sa*gIp antisense RNA probe demonstrated that *Sa*gIp is expressed in the epidermis and spongy parenchyma in young leaves and in the epidermis and outer cortical cell layers of petioles and stems [Heintzen *et al.* 1994]. Immunological localisation identified the predominant association of *Sa*gIp with the borders of the cell walls towards the intercellular spaces [Heintzen *et al.* 1994]. Ono *et al.* [1996] recently reported the detection of a germin-like protein in the short day plant *Pharbitis nil* (Choisy c.v. violet) (*Pn*gIp). This protein is specifically expressed in cotyledons and leaves and it reaches maximum accumulation at about 10 hr from the beginning of the dark period [Ono *et al.* 1996]. The protein has a predicted M_r value of about 20 kDa (pI 7),

which shares 33.6% amino acid homology with wheat germin (gf-3.8) [Ono *et al.* 1996]. Oxalate oxidase activity has not yet been observed with either of these germin-like proteins.

Domon *et al.* [1995] identified three extracellular proteins (including GP111) expressed by embryogenic (but not non-embryogenic) pine cell cultures, which have significant amino acid sequence homology to wheat germin. These proteins cross-reacted with antibodies raised against wheat and barley oxalate oxidases [Domon *et al.* 1995]. GP111 is oligomeric with a M_r value of about 125 kDa and a monomer M_r value of about 25 kDa. The protein undergoes partial dissociation in the presence of 0.1% SDS without heat treatment, indicating differences in stability from oxalate oxidase [Domon *et al.* 1995]. Oxalate oxidase activity has not been associated with any of the three pine germin-like proteins [Domon *et al.* 1995].

Membré *et al.* [1997] have reported the identification of three differentially expressed *A. thaliana* cDNAs (*At*Ger1 ,2 and 3) and one *B. napus* (*Bn*Ger1) cDNA corresponding to germin-like proteins. *At*Ger1 and *At*Ger2 were expressed at similar developmental stages to germin and pseudogermin expression in wheat, respectively. *At*Ger3 showed 94% amino acid homology to *Sa*gIp and is more closely related to *At*Ger1 than *At*Ger2 [Membré *et al.* 1997]. The four germin-like proteins identified share about 40% amino acid sequence homology to wheat germin, they contained signal peptides and one or two putative N-glycosylation site(s) [Membré *et al.* 1997]. *At*Ger2 contains a putative C-terminal peroxisomal targeting sequence not present in the other *At*Ger or *Bn*Ger sequences [Membré *et al.* 1997]. *Bn*Ger1 is expressed in leaves, cotyledons and floral buds, *At*Ger1 in young seedlings and rosette leaves, and *At*Ger2 in stems, siliques during seed maturation and germination [Membré *et al.* 1997]. Mapping of the *A. thaliana* genes indicates that *At*Ger1 and *At*Ger3 are present on chromosome 5 and *At*Ger2 is present on chromosome 1 [Membré *et al.* 1997]. Again no oxalate oxidase activity has so far been detected for any of these germin-like proteins [Membré *et al.* 1997].

Recent molecular taxonomic studies have suggested that legumin-like and vicilin-like seed storage proteins may have originated from a common evolutionary origin as the oxalate oxidases (germin) and spherulins [Bäumlein *et al.* 1995; Shutov *et al.* 1995; Braun *et al.* 1996]. In angiosperms and gymnosperms legumin-like 11S and vicilin-like 7S globulins are the major storage proteins in seeds. Legumin and vicilin are thought to have arisen from a common ancestral gene [Shutov *et al.* 1995]. In spermatophytes these proteins are specifically expressed and accumulated during embryogenesis and seed development and they share significant amino acid similarity to oxalate oxidases and spherulins [Bäumlein *et al.* 1995]. Cycads have been used in these studies because this plant family has undergone little evolutionary change in over 200 million years and a vicilin-like protein from this group of plants has been identified which exhibits homology to wheat embryo oxalate oxidase [Braun *et al.* 1996]. It has been suggested that the genes for seed globulins, oxalate oxidases, spherulins and sucrose-binding proteins may form a super family of related genes all of which have common ancestry [Bäumlein *et al.* 1995; Braun *et al.* 1996]. The 7S and 11S seed storage proteins contain two domains, whereas oxalate oxidases and spherulins only contain single

domains. This change in structure may have occurred only after the evolutionary divergence of these proteins [Bäumlein *et al.* 1995; Braun *et al.* 1996].

1.12. Function of oxalate oxidases and germin-like proteins.

The initial speculation regarding the function of wheat germin involved a possible role in promoting water uptake in imbibing embryos by increasing cell wall plasticity, plasmalemmal water conductance or vesicle formation [Lane 1991] and the cellular uptake of metabolites transported from the leaf (e.g. sucrose) [Grzelczak *et al.* 1985]. Wheat germin was thought to promote water uptake by driving peroxisome-mediated tissue remodelling [Lane *et al.* 1992 1993]. The presence of adventitious polysaccharides in wheat germin suggested a possible involvement with the cell wall [Jaikaran *et al.* 1990] and wall expansion [Lane *et al.* 1992]. Jaikaran *et al.* [1990] suggested that germin may accumulate with polysaccharide(s) (arabinoxylans) in the extracellular spaces under stress conditions. Germin may act as a transporter of highly substituted glucuronoarabinoxylans during cell wall expansion to the apoplast or as a mediator of intercalation between nascent hemicelluloses and other cell-wall polysaccharides [Lane *et al.* 1992]. A role in the transport of H^+ ions into the apoplast is compatible with the stability of the germin pentamer at extreme pH values [Lane *et al.* 1992]. The fact that wheat germin is an oxalate oxidase with catalytic activity suggests an involvement in developmental processes in higher plants [Lane *et al.* 1993].

The dissociation of calcium oxalate to oxalic acid and Ca^{2+} and the subsequent hydrolysis of oxalic acid by cell wall associated oxalate oxidase to produce H_2O_2 provides evidence for a function in cell wall expansion, where H_2O_2 and Ca_{2+} are important prerequisites for various processes, including cross-linking of cell wall polymers. Lignification, for example, involves lignin peroxidase an H_2O_2-requiring enzyme which mediates the cross-linking of carboxylated sugars in pectins and hemicellulosic glucuronogalactoarabinoxylans [Halliwell 1978; Darvill *et al.* 1980; Carpita and Gilbeaut 1988; Cassab and Varner 1988; Showalter 1993; Olsen and Varner 1993; Lane *et al.* 1994]. H_2O_2 generated by the oxidation of oxalic acid may also be involved in lignin degradation [Valli *et al.* 1990].

Oxalate oxidase expression resulting from fungal pathogen infection infers that this protein may play a role in enhancing the levels of H_2O_2-mediated oxidative cross-linking of the cell wall to establish a physical barrier to reduce the rate of infection and spread of pathogens [Dumas *et al.* 1995; Hurkman and Tanaka 1995 1996; Zhang *et al.* 1995]. Keates *et al.* [1995] demonstrated the synthesis and secretion of oxalate oxidase from bean leaf cells in response to inoculation with *S. sclerotiorum*. H_2O_2 generated during fungal pathogen infection may also act as a second messenger molecule [Neuenschwander *et al.* 1995] and an inhibitor of fungal spore germination [Peng and Kuc 1992].

Bacteriod oxalate oxidase may play a role in nitrogen fixation by rhizobia in faba bean root nodules. The peribacteriod membrane prevents the influx of oxalic acid which would result in substrate inhibition of the enzyme. Water stress reduces the activity of acetylene reduction activity in the nodules, which correlates with a marked decline in the levels of oxalic

acid, the oxidation of which could provide energy for nitrogen fixation during periods of water stress [Trinchant and Rigaud 1996].

The role of germin-like proteins in plants is much less clear, several suggestions for the potential role of these proteins have been proposed, although at present no definitive functions have been associated with any of these proteins. Many of the germin-like proteins so far identified may exhibit similar characteristics (i.e. some exist as oligomers which are resistant to dissociation by SDS) and varying degrees of amino acid sequence similarity to oxalate oxidase but they do not exhibit oxalate oxidase enzyme activity. It is possible that these homologous proteins may have a variety of functions in plants, which are highlighted by their differential expression during different developmental stages or during environmental stress and pathogen infection.

*Sa*glp like wheat germin could be involved in the maturation process of the walls of growing cells because of its localisation within the primary cell wall [Heintzen *et al.* 1994]. During periods of water shortage and / or salt stress, germin-like proteins most abundant in roots may be controlling plant growth by acting as sensors of water availability. These proteins are therefore thought to have a stress protection / amelioration function in certain plants, including barley and *M. crystallinum* [Hurkman and Tanaka 1987 1988; Mickalowski and Bohnert 1992]. The H_2O_2 produced in barley during salt stress may act as a signal to activate the expression of proteins which have a protective role in the plant [Hurkman and Tanaka 1996]. Ono *et al.* [1996] suggest that *Pn*glp may play a role in photoperiodic floral induction.

1.13. Strategies for the production of transgenic plants expressing cereal oxalate oxidases.

Berna and Bernier [1997] produced transgenic tobacco which were expressing either wheat embryo oxalate oxidase (gf-2.8) promoter-GUS fusions or the intact wheat embryo oxalate oxidase (gf-2.8) gene. The pattern of developmental expression in germinating tobacco was almost the same as that in germinating wheat embryos. GUS expression was also shown to increase in response to auxin treatment presumably resulting from the presence of three putative auxin-responsive elements in the gf-2.8 promoter. The transgenic tobacco plants expressing the intact gf-2.8 gene produced both the G and G' oxalate oxidase isoforms, confirming that these two isoforms were synthesised from the single gf-2.8 gene. The synthesis of the two isoforms also confirmed that the processing mechanisms in tobacco responsible for protein folding, glycosylation and oligomerisation are likely to be very similar if not identical between wheat and tobacco [Berna and Bernier 1997]. Like the native wheat oxalate oxidase in germinating embryo about 40% of the transgenic tobacco oxalate oxidase was associated with the pellet fraction of a total homogenate and about 30% of the protein was recovered from the apoplast by vacuum infiltration [Berna and Bernier 1997].

Oxalate oxidase has been identified as a potential candidate enzyme for expression in transgenic plants (tobacco, oilseed rape and sunflower) to improve resistance towards *S. sclerotiorum*, an oxalic acid-producing fungal pathogen. Transgenic plants have been produced at Zeneca Seeds (Jealott's Hill Research Station, Berkshire, U.K.), which are

expressing a chimeric gene consisting of a *Nicotiana plumbaginifolia* extensin signal peptide linked to the barley root oxalate oxidase gene under the control of the CaMV 35S promoter (pSR2) [Thompson *et al.* 1995]. The signal peptide was included in the construct to ensure efficient entry of the nascent protein into the rough endoplasmic reticulum (ER) where it will undergo glycosylation, folding and oligomerisation. Transgenic tobacco plants were initially prepared to ensure that the monocotyledonous oxalate oxidase gene was being correctly expressed and processed, giving rise to an active enzyme in the dicotyledonous plant environment prior to transformation of oilseed rape with the extensin-oxalate oxidase chimeric gene. Oilseed rape is a major host crop for *S. sclerotiorum*, which has been transformed with the Zeneca Seeds construct so that glasshouse and field trials can be performed with the pathogen. Basic characterisation of the transgenic lines by Southern and western blotting and enzyme activity assays showed that the transformations were successful and that the transgenic oilseed rape plants were expressing active oxalate oxidase [Thompson *et al.* 1995]. It was hoped that the transgenic oxalate oxidase would be expressed at sufficient levels resulting in the localisation and accumulation of the enzyme in the extracellular spaces of the transgenic plants. Localisation of the transgenic enzyme to the extracellular would equip the transgenic plants with a mechanism for degrading the oxalic acid produced by the invading fungal pathogen. As previously stated oxalic acid is the primary phytotoxin produced by the target fungal pathogen *S. sclerotiorum*, which penetrates the plant tissues resulting in a reduction in the pH of the plants apoplast. The oxalic acid has several direct effects on plant tissues as well as lowering the pH to a level which results in optimum activity of the various lytic enzymes produced by the pathogen. It was hoped that the extracellular localisation of oxalate oxidase would slow the rate of spread and the development of stem rot sufficiently, so that the plants can set seed (i.e. the yield from the transgenic plants should be greater than that from control plants where loss of yield resulting from *S. sclerotiorum* infection can be significant). The overall objective of the work carried out at Zeneca Seeds has involved the expression of the barley root oxalate oxidase gene in transgenic sunflower. *S. sclerotiorum* causes stem and head rot in this species leading to heavy lose of yield. The final transformation into sunflower has not yet been performed. A second line of transgenic tobacco (SGS5) has been produced by François Bernier (Institute de Botanique, Universite de Louis Pasteur, Strasbourg, France) expressing the wheat embryo oxalate oxidase (gf-2.8) gene identified by Lane *et al.* [1991] from a genomic DNA library of 3 day isolated and germinated wheat embryos, under the control of the CaMV 35S promoter. This gene contains the sequences for the wheat embryo oxalate oxidase signal peptide, the entire mature protein coding region and terminator.

The barley root oxalate oxidase and wheat embryo oxalate oxidase genes expressed in the different transgenic lines code for proteins which have 96% amino acid sequence identity with 4% conserved residue changes. Therefore, the only significant difference between the oxalate oxidase genes of the two constructs used in the production of these transgenic plants involves the signal peptides. Zeneca Seeds used the foreign *N.*

plumbaginifolia extensin signal peptide and Bernier retained the native wheat embryo oxalate oxidase signal peptide.

1.14. Objectives of this research.

During the course of this work Thompson *et al.* [1995] confirmed that when infected the transgenic oilseed rape plants expressing oxalate oxidase exhibit increased resistance to *S. sclerotiorum*. The overall aim of this project was therefore to study the biochemistry of the oxalate oxidases expressed in the transgenic oilseed rape and tobacco plants and to contribute original findings to the area of oxalate oxidase and transgenic research. Transgenic plants expressing the mature barley root oxalate oxidase and wheat embryo oxalate oxidase genes produced by Zeneca Seeds and François Bernier were to provide the basis for further investigation into the biochemistry of foreign genes expressed in plants. Wherever possible the characteristics of the transgenic proteins were compared against those of the native cereal proteins from which the transgenes were derived. During the course of the work it was hoped that the following questions could be addressed.

1. Are there any difference in the temporal, spatial and organ expression of the transgenes and accumulation of the active enzyme in the transgenic plants.
2. Where are the oxalate oxidases located in the transgenic plants and is there evidence for accumulation of the enzyme in association with the cell wall / extracellular spaces.
3. Do the N-terminal amino acid sequences of the native and transgenic proteins show complete homology, which would indicate the correct cleavage of the signal peptide from the mature protein during processing in the rough ER.
4. Have the transgenic protein been correctly processed in the transgenic plants to produce oxalate oxidases which exhibit similar structural, stable and kinetic properties in comparison with the native cereal oxalate oxidases.

The efficacy of this strategy for producing transgenic plants with improved fungal pathogen resistance relies on the correct expression and processing of the nascent oxalate oxidase polypeptide in the transgenic plants, to produce active and stable enzymes, which will accumulate in the extracellular spaces at sufficient levels to retard the establishment and growth of *S. sclerotiorum*. In oilseed rape, the leaves and stems are the main organs of attack and so high levels of expression and accumulation of oxalate oxidase are necessary, particularly during flowering when the plants are most prone to infection.

Initial work to characterise oxalate oxidase was carried out on transgenic 3S1 oilseed rape, expressing the chimeric extensin-barley root oxalate oxidase gene controlled by the CaMV 35S promoter. However, it soon became clear that this transgenic line was not expressing an oxalate oxidase with the stable properties characteristic of barley root oxalate oxidase. At this time François Bernier had producing a transgenic tobacco line (SGS5) expressing the native wheat embryo oxalate oxidase gene controlled by the CaMV 35S promoter. The decision was made to obtain the SGS5 transgenic tobacco to allow a direct comparison of the two transgenic lines essentially expressing genes containing the same mature protein coding region but different signal peptides. The SGS5 transgenic tobacco

produced an active oxalate oxidase with the same structural stability as the native wheat enzyme. The C26 tobacco line expressing the extensin-oxalate oxidase gene was also included in this work to allow the comparison of the enzymes produced from the expression of the different transgenes in the same species. Like the enzyme expressed in 3S1 oilseed rape the C26 transgenic oxalate oxidase exhibited the same unstable properties, suggesting that the gene sequence introduced and expressed in these two transgenic lines was not producing an enzyme identical to the native barley root oxalate oxidase. It was hoped that by comparing expression, localisation, structure, stability and the enzyme kinetics of these three transgenic proteins (i.e. 3S1, C26 and SGS5) it would be possible to establish why the transgenic 3S1 oilseed rape and C26 tobacco plants were producing active yet unstable oxalate oxidases compared with the stable enzyme produced by transgenic SGS5 tobacco.

2. MATERIALS.

2.1. Glassware and plasticware.

Glassware used for DNA and protein manipulations was washed with distilled water, dried, sealed in aluminium foil and autoclaved (20 min, 121 °C, 15 psi). Plasticware for DNA and protein manipulations was purchased sterile or was autoclaved as before. Microcentrifuge tubes, pipette tips, cuvettes and 96 well microtitre plates were obtained from Sarstedt Ltd (Leicester, U.K.). Petri dishes were supplied by Bibby Sterilin Ltd (Staffordshire, U.K.).

2.2. Plant material.

Non-transgenic and transgenic *Brassica napus* (c.v. Westar) (line 3S1) and non-transgenic and transgenic *Nicotiana tabacum* (c.v. samson) (line C26) seeds expressing an extensin-oxalate oxidase chimeric gene were supplied by Zeneca Seeds (Jealotts Hill Research Station, Berkshire, U.K.). Transgenic *N. tabacum* (c.v. xanthi) (line SGS5) seeds expressing the wheat oxalate oxidase (*gf*-2.8) gene [Lane *et al.* 1991] were kindly provided by Professor François Bernier (Institute de Botanique, Universite de Louis Pasteur, Strasbourg, France).

Barley (c.v. Sundance) seeds were kindly provided by Peter Shewry (Horticulture Research International, Warwickshire, U.K.) and wheat (c.v. Winter Genesis) seeds were provided by G. Bainbridge (Department of Biological Sciences, University of Durham, U.K.).

2.3. Bacterial strains.

E. coli strain DH5α was available within the Department of Biological Sciences, (University of Durham, U.K.). The strain genotype and source references are listed in Sambrook *et al.* [1989].

2.4. Nucleic acids.

Plasmid pNEXTOX was obtained from Zeneca Seeds (Jealotts Hill Research Station, Berkshire, U.K.). Fig. 2.1. illustrates the procedure used by Zeneca Seeds for the production of transgenic oilseed rape (3S1) and tobacco (C26) expressing barley root oxalate oxidase. A partial clone of oxalate oxidase isolated from a barley root cDNA library and synthetic oligonucleotides containing the *Nicotiana plumbaginifolia* extensin signal peptide were used to obtain the full-length extensin-oxalate oxidase clone (EXTOX, 771 bp) by PCR. The EXTOX PCR product was cloned into the CaMV 35S-Nos expression cassette in the correct orientation in the *E. coli* vector pJR1Ri to give pNEXTOX. The CaMV 35S-EXTOX-Nos expression cassette (Fig. 2.2.) was then inserted into a binary *Agrobacterium* vector (pBin19) to give plasmid pSR2 by standard techniques and introduced into *A. tumefaciens* strain LBA4404 by triparental mating. Cotyledon petioles of oilseed rape were transformed using a modified version of the method of Moloney and Gulya [1989] and the transformed material was selected on the basis of resistance to kanamycin [Thompson *et al.* 1995].

Figure 2.1. Preparation of transgenic 3S1 oilseed rape and C26 tobacco plants.

Diagrammatic representation of the preparation of the chimeric CaMV 35S-extensin-oxalate oxidase gene and transformation into oilseed rape and tobacco. See text in section 2.4. for details. Abbreviations: OXSP, oxalate oxidase signal peptide; ESP, *N. plumbaginifolia* extensin signal peptide; CaMV 35S, cauliflower mosaic virus 35S promoter; NosT, nopaline synthase terminator; NosP, nopaline synthase promoter; NPTII, neomycin phosphotransferase II gene; Kan, kanamycin resistance gene; TDNA, transfer DNA; LB, left border; RB, right border.

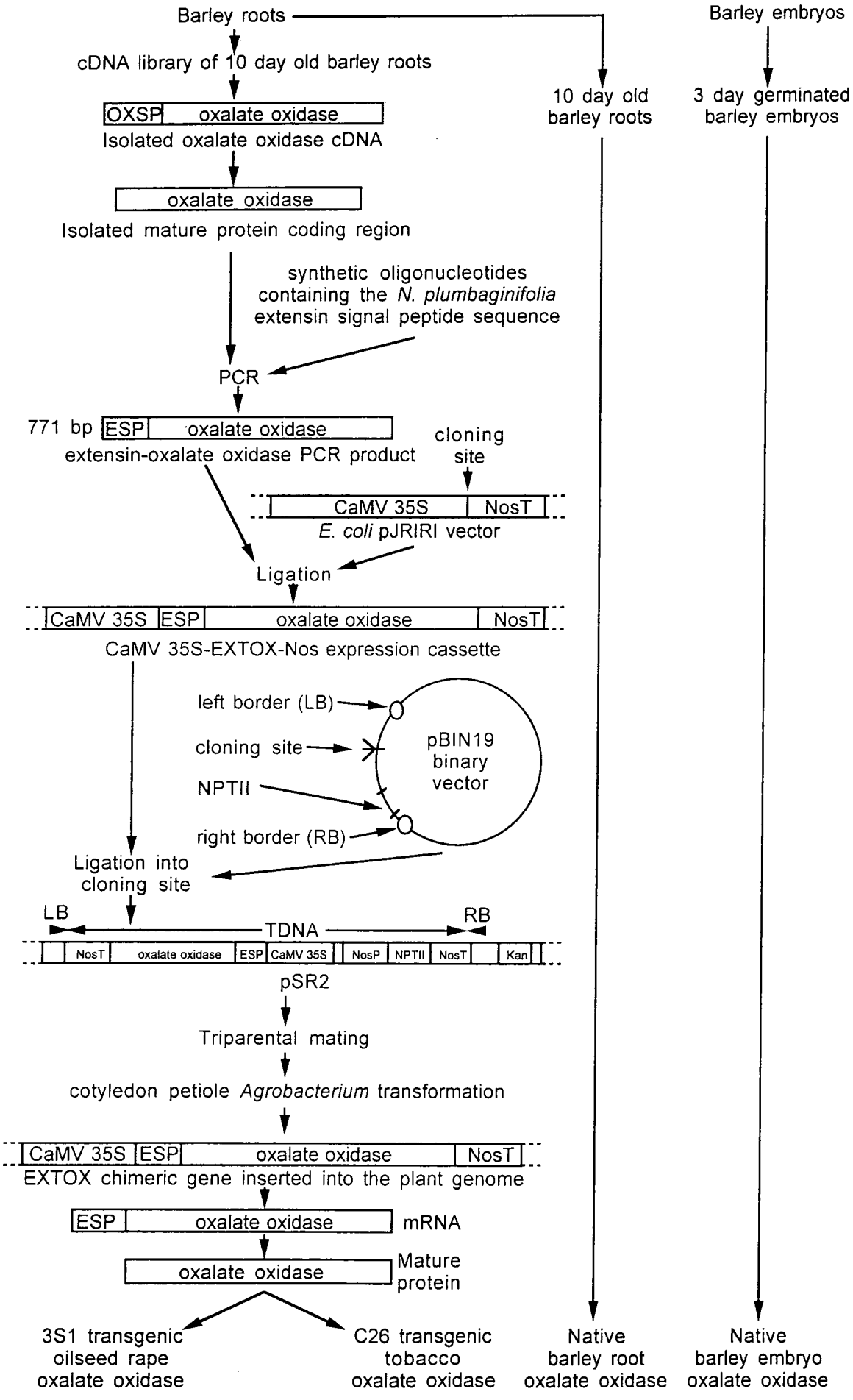

Barley roots
Barley embryos
cDNA library of 10 day old barley roots
10 day old barley roots
3 day germinated barley embryos
OXSP
oxalate oxidase
Isolated oxalate oxidase cDNA
oxalate oxidase
Isolated mature protein coding region
synthetic oligonucleotides containing the N. plumbaginifolia extensin signal peptide sequence
PCR
771 bp
ESP
oxalate oxidase
extensin-oxalate oxidase PCR product
cloning site
CaMV 35S
NosT
E. coli pJRIRI vector
Ligation
CaMV 35S
ESP
oxalate oxidase
NosT
CaMV 35S-EXTOX-Nos expression cassette
left border (LB)
cloning site
NPTII
right border (RB)
pBIN19 binary vector
Ligation into cloning site
LB
TDNA
RB
NosT
oxalate oxidase
ESP
CaMV 35S
NosP
NPTII
NosT
Kan
pSR2
Triparental mating
cotyledon petiole Agrobacterium transformation
CaMV 35S
ESP
oxalate oxidase
NosT
EXTOX chimeric gene inserted into the plant genome
ESP
oxalate oxidase
mRNA
oxalate oxidase
Mature protein
3S1 transgenic oilseed rape oxalate oxidase
C26 transgenic tobacco oxalate oxidase
Native barley root oxalate oxidase
Native barley embryo oxalate oxidase

Figure 2.2. The extensin-oxalate oxidase chimeric gene.

DNA sequence and translation protein of the extensin-oxalate oxidase gene construct expressed in the transgenic 3S1 oilseed rape and C26 tobacco. CaMV 35S promoter (nucleotides 1-536), *N. plumbaginifolia* extensin signal peptide (556-627), barley oxalate oxidase open reading frame encoding the mature protein (628-1227), Nos 3' region (1314-1573). The signal peptide consists of 23 amino acid residues (in italics) and the mature barley root oxalate oxidase polypeptide contains 201 amino acid residues. The two putative glycosylation sites are in bold and the KpnI restriction sites are underlined. *, stop codon (TAA).

```
GAATTCCCATGGAGTCAAAGATTCAAATAGAGGACCTAACAGAACTCGCCGTAAAGACTG   60
GCGAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAAATCTTCGTCAACA  120
TGGTGGAGCACGACACGCTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACC  180
AAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATT  240
GCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAAT  300
GCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCA  360
AAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTT  420
CAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACT  480
ATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACAGGTA  540
CCATTTGTTTCAAAGATGGGAAAAATGGCTTCTCTATTTGCCACATTTTTAGTGGTTTTA  600
                 M  G  K  M  A  S  L  F  A  T  F  L  V  V  L

GTGTCACTTAGCTTAGCTTCTGAAACCGACCCAGACCCACTCCAGGACTTCTGCGTCGCG  660
 V  S  L  S  L  A  S  E  T  D  P  D  P  L  Q  D  F  C  V  A

GACCTCGATGGCAAGGCGGTCTCGGTGAACGGGCATACGTGTAAGCCCATGTCGGAGGCC  720
 D  L  D  G  K  A  V  S  V  N  G  H  T  C  K  P  M  S  E  A

GGCGACGACTTCCTCTTCTCGTCCAAGCTGACCAAGGCCGGCAACACGTCCACCCCGAAC  780
 G  D  D  F  L  F  S  S  K  L  T  K  A  G  N  T  S  T  P  N

GGCTCGGCCGTGACGGAGCTCGACGTGGCCGAGTGGCCCGGTACGAACACGCTGGGCGTG  840
 G  S  A  V  T  E  L  D  V  A  E  W  P  G  T  N  T  L  G  V

TCCATGAACCGTGTGGACTTCGCGCCGGGGGGCACCAACCCGCCGCACATCCACCCGCGT  900
 S  M  N  R  V  D  F  A  P  G  G  T  N  P  P  H  I  H  P  R

GCAACCGAGATCGGCATGGTGATGAAAGGTGAGCTCCTCGTTGGAATCCTCGGCAGCCTT  960
 A  T  E  I  G  M  V  M  K  G  E  L  L  V  G  I  L  G  S  L

GACTCCGGAAACAAGCTCTACTCCAGGGTGGTGCGTGCCGGAGAGACTTTCGTCATCCCG 1020
 D  S  G  N  K  L  Y  S  R  V  V  R  A  G  E  T  F  V  I  P

CGCGGCCTCATGCACTTCCAGTTCAACGTTGGTAAGACGGAAGCCTACATGGTTGTGTCC 1080
 R  G  L  M  H  F  Q  F  N  V  G  K  T  E  A  Y  M  V  V  S

TTCAACAGCCAGAACCCTGGCATCGTCTTCGTGCCGCTCACACTCTTCGGCTCCGACCCT 1140
 F  N  S  Q  N  P  G  I  V  F  V  P  L  T  L  F  G  S  D  P

CCCATCCCCACGCCCGTGCTCACCAAGGCTCTCCGGGTGGAGGCCGGAGTCGTGGAACTT 1200
 P  I  P  T  P  V  L  T  K  A  L  R  V  E  A  G  V  V  E  L

CTCAAGTCCAAGTTCGCCGGTGGGTCTTAACTTCCATGAGCCCCAAATGATCAATATGAA 1260
 L  K  S  K  F  A  G  G  S  *

TATGTAATTCTATATATCCAGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGTCGTTCA 1320
AACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATC 1380
ATATAATTTCTGTTGAATTACGAATTGCATGTAATAATTAACATGTAATGCATGACGTTA 1440
TTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAA 1500
AACAAAATATAGCGCGCAAACTACCATAAATTATCGCGCGCGGTGTCATCTATGTTACTA 1560
GATCGGGAAGCTT                                                 1573
```

Fig. 2.3. illustrates the procedure used by François Bernier for the production of transgenic tobacco (SGS5) expressing wheat embryo oxalate oxidase. The signal peptide, mature protein coding region and the terminator of the genomic clone *gf*-2.8 gene [Lane *et al.* 1991] was excised from pEMBL18 and introduced into the vector pBI101 downstream of the CaMV 35S promoter (Fig. 2.4.). The Nos terminator was removed from the construct. Triparental mating involving an *E. coli* stain carrying pRK2013 was used to transfer the pBI101 vector from *E. coli* to *A. tumefaciens* strain LBA4404. The method of Horsh *et al.* [1985] was used to transform leaf disks of *N. tabacum*. Transformed material was selected on the basis or resistance to kanamycin [François Bernier, personal communication].

2.5 Buffers and solutions.

All buffers, Milli Q (Ergastat, UHQPS, Elga Ltd. Buckinghamshire, U.K.) and distilled water were sterilised by autoclaving (20 min, 121 °C, 15 psi) unless they contained thermolabile components in which case the solutions were filter sterilised (0.2 µm, Whatman Ltd, Maidstone, Kent, U.K.).

2.6. Antibodies.

Anti-barley root oxalate oxidase antibodies raised against the heat treated (monomeric) form of the protein were prepared and supplied by Zeneca Seeds (Jealotts Hill Research Station, Berkshire, U.K.). Anti-*Sinapis alba* germin-like protein antibodies were provided by Dr Dorthee Staiger (Institute of Plant Science, Swiss Federal Institute of Technology , Zurich, Switzerland). Anti-pea RUBISCO (ribulose bisphosphate carboxylase) antibodies were supplied by Dr Nick Harris (Department of Biological Sciences, University of Durham, U.K.).

2.7. Chemical and biological reagents.

Chemical reagents were supplied by BDH Ltd (Leicestershire, U.K.) and were of AnalaR grade. Other reagents were obtained from the following sources:

-Yeast extract, Bacto-agar - Difco (Detroit, Michigan, U.S.A.).

-Trypticase peptone - Becton Dickinson (Meylan, France).

-Restriction endonucleases - Boehringer Mannheim U.K. (East Sussex, U.K.).

-Alkaline phosphatase-conjugated donkey anti-rabbit antibodies, 4-aminoantipyrine, ampicillin, bovine serum albumin, bromophenol blue, concanavalin A-sepharose, Coomassie brilliant blue R250 CI 42660, ditheothreitol, DNase, ethidium bromide, glycerol, horseradish peroxidase (type VI-A, 1100 units/mg), α-mannopyranoside, molecular weight markers (MW-SDS-70L and MW-SDS-200), N,N-dimethylaniline, oxalic acid, RNase A, sodium dodecyl sulphate, succinic acid, Tween-20, xylene cyanol - Sigma (Poole, Dorset, U.K.).

-'kaleidoscope' prestained protein markers (Cat. no. 161-0324), prestained SDS-PAGE standards (Cat. no. 161-0318), Bradford protein assay kit, silver stain kit, TEMED, ammonium persulphate - Bio-Rad (Hemel Hempstead, Hertfordshire, U.K.).

Figure 2.3. Preparation of transgenic SGS5 tobacco plants.

Diagrammatic representation of the preparation of the CaMV 35S-wheat embryo oxalate oxidase gene and transformation into tobacco. See text in section 2.4. for details. Abbreviations: SP, gf-2.8 signal peptide; CaMV 35S, cauliflower mosaic virus 35S promoter; Term, gf-2.8 terminator; NosP, nopaline synthase promoter; NosT, nopaline synthase terminator; NPTII, neomycin phosphotransferase II gene; Kan, kanamycin resistance gene; TDNA, transfer DNA; LB, left border; RB, right border.

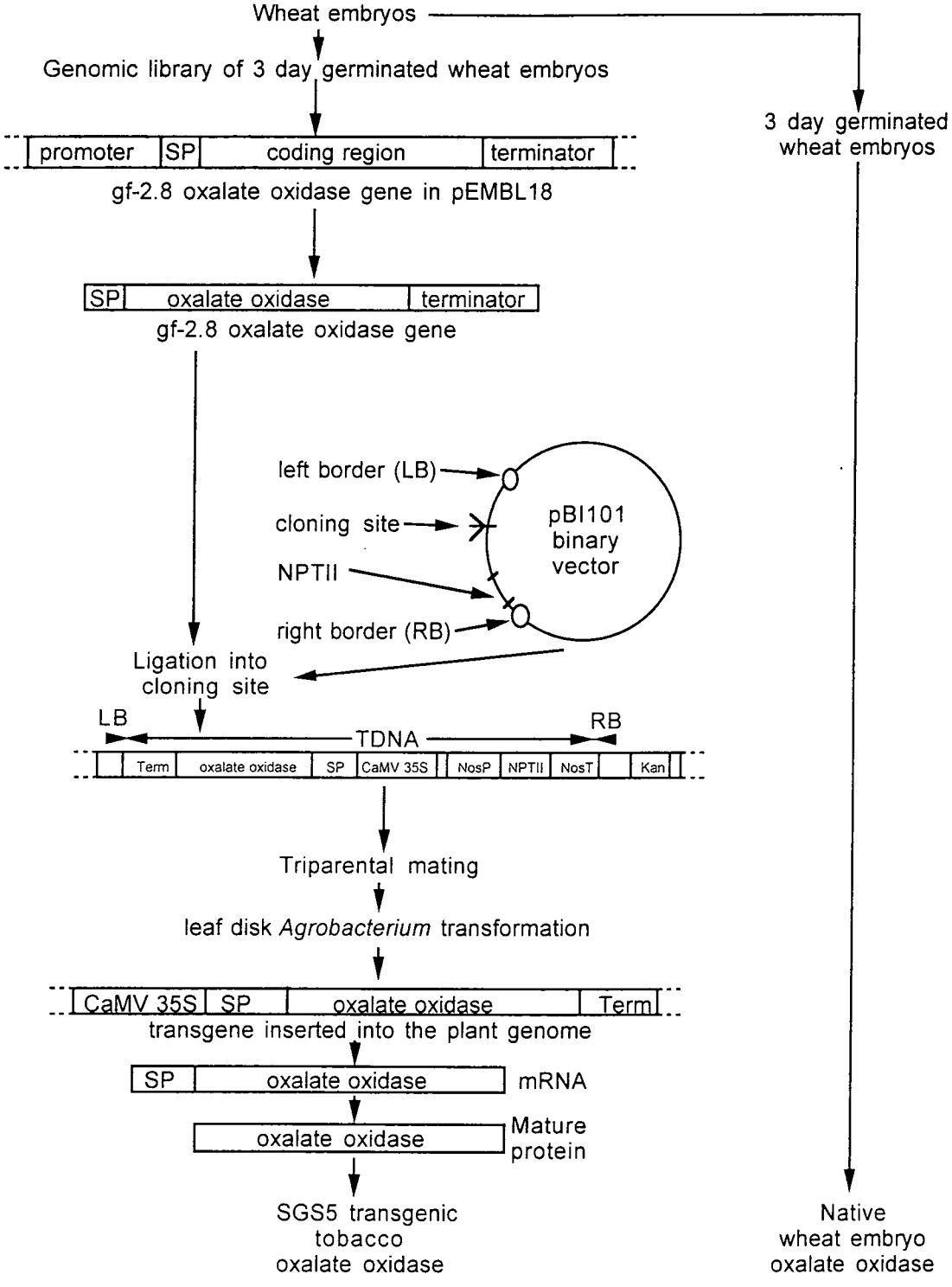

Wheat embryos
Genomic library of 3 day germinated wheat embryos
promoter
SP
coding region
terminator
gf-2.8 oxalate oxidase gene in pEMBL18
SP
oxalate oxidase
terminator
gf-2.8 oxalate oxidase gene
3 day germinated wheat embryos
left border (LB)
cloning site
NPTII
right border (RB)
pBI101 binary vector
Ligation into cloning site
LB
TDNA
RB
Term
oxalate oxidase
SP
CaMV 35S
NosP
NPTII
NosT
Kan
Triparental mating
leaf disk Agrobacterium transformation
CaMV 35S
SP
oxalate oxidase
Term
transgene inserted into the plant genome
SP
oxalate oxidase
mRNA
oxalate oxidase
Mature protein
SGS5 transgenic tobacco oxalate oxidase
Native wheat embryo oxalate oxidase

Figure 2.4. The CaMV-35S oxalate oxidase (gf-2.8) cassette of pBI101.

DNA sequence and translation protein of the CaMV 35S-gf-2.8 gene construct expressed in the transgenic SGS5 tobacco plants. CaMV 35S promoter (nucleotides 1-536), gf-2.8 signal peptide (562-622), wheat gf-2.8 oxalate oxidase open reading frame encoding the mature protein (623-1222), g.f-2.8 terminator (1321-1550). The signal peptide consists of 23 amino acid residues (in italics) and the mature barley root oxalate oxidase polypeptide contains 201 amino acid residues. The two putative glycosylation sites are in bold.

```
GAATTCCCATGGAGTCAAAGATTCAAATAGAGGACCTAACAGAACTCGCCGTAAAGACTG   60
GCGAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAAATCTTCGTCAACA  120
TGGTGGAGCACGACACGCTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACC  180
AAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATT  240
GCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAAT  300
GCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCA  360
AAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTT  420
CAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACT  480
ATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACAGCAG  540
CAGCAACCACCAGTGCCATAGACACTCTCCATCAACAAACTCTAGCTGATCAATCCCTAG  600
CTAAGCTTATTACATAGCAAGCATGGGGTACTCCAAAACCCTAGTAGCTGGCCTGTTCGC  660
                        M  G  Y  S  K  T  L  V  A  G  L  F  A

AATGCTGTTACTAGCTCCGGCCGTCTTGGCCACCGACCCAGACCCTCTCCAGGACTTCTG  720
  L  L  K  S  K  F  A  A  G  F  T  D  P  D  P  L  Q  D  F  C

TGTCGCCGACCTCGACGGCAAGGCGGTCTCGGTGAACGGGCACACGTGCAAGCCCATGTC  780
  V  A  D  L  D  G  K  A  V  S  V  N  G  H  T  C  K  P  M  S

GGAGGCCGGCGACGACTTCCTCTTCTCGTCCAAGTTGGCCAAGGCCGGCAACACGTTCAC  840
  E  A  G  D  D  F  L  F  S  S  K  L  A  K  A  G  N  T  S  T

CCCGAACGGCTCCGCCGTGACGGAGCTCGACGTGGCCGAGTGGCCCGGTACCACCACGCT  900
  P  N  G  S  A  V  T  E  L  D  V  A  E  W  P  G  T  N  T  L

GGGTGTGTCCATGAACCGCGTGGACTTTGCTCCCGGAGGCACCACCCCACCACACATCCA  960
  G  V  S  M  N  R  V  D  F  A  P  G  G  T  N  P  P  H  I  H

CCCGCGTGCCACCGAGATCGGCATCGTGATGAAAGGTGAGCTTCTCGTGGAAATCCTTGG 1020
  P  R  A  T  E  I  G  I  V  M  K  G  E  L  L  V  G  I  L  G

CAGCCTCGACTCCGGGAACAAGCTCTACTCGAGGGTGGTGCGCGCCGGAAGACGTTCCTC 1080
  S  L  D  S  G  N  K  L  Y  S  R  V  V  R  A  G  E  T  F  L

ATCCCACGGGGCCTCATGCACTTCCAGTTCAACGTCGGTAAGACCGAGGCCATCCATGGT 1140
  I  P  R  G  L  M  H  F  Q  F  N  V  G  K  T  E  A  S  M  V

CGTCTCCTTCAACAGCCAGAACCCCGGCATTGTCTTCGTGCCCCTCACGCTCTTCGGCTC 1200
  V  S  F  N  S  Q  N  P  G  I  V  F  V  P  L  T  L  F  G  S

CAACCCGCCCATCCCAACGCCGGTGCTCACCAAGGCACTCCGGGTGGAGGCCAGGGTCGT 1260
  N  P  P  I  P  T  P  V  L  T  K  A  L  R  V  E  A  R  V  V

GGAACTTCTCAAGTCCAAGTTTGCCGCTGGGTTTTAATTTCTAGGAGCCTTCCCTGAAAT 1320
  E  L  L  K  S  K  F  A  A  G  F  *

GATAATTATATAATTCCATATATGCATGCTAGCAAAATTTAATAATCCTCACCAGAAGAC 1380
ATGTATTCAAGTTTCAGGTTAATCTCGCATGTAGTCGTGTAATAAGATTGAACAAGTTAG 1440
CCTCATGGTGTAGCCTTCGATCAGAACCAATATGAGGAATTGAATGTACTACTTTTTATT 1500
GTCGTCTTTGTTCTTTTCACTGAACGGAATATATAATAAGCATTTTCGTA           1560
```

-Protogel, 30% (w/v) acrylamide: 2.7% (w/v) cross-linked with methylene bisacrylamide (37.5:1 ratio) in distilled water - National Diagnostics (Hull, U.K.).

-Agarose (electrophoresis grade), low-melting point agarose - GIBCO-BRL Ltd. (Paisley, Scotland, U.K.).

-^{125}Iodine conjugated donkey anti-rabbit antibodies (specific activity 28 TBq/mmol), [α-^{32}P] dCTP Redivue (specific activity about 15 TBq/ mmol), nylon "Hybond-N" membranes - Amersham International plc. (Buckinghamshire, U.K.).

-Nitrocellulose membrane (0.45 µm) - Schleicher and Schuell (Dassel, F.R.G.).

-ProBlott™ membrane (0.45 µm), Blott™ cartridges, Bioprene Plus - Applied Biosystems (Warrington, Cheshire, U.K.).

-3MM chromatography paper - Whatman Ltd (Maidstone, Kent, U.K.).

-Non-fat dried milk, nappies - Boots (Nottingham, U.K.).

-Fuji RX x-ray film - Fuji Photo Film Co. Ltd. (Tokyo, Japan).

-MiracleGro - Sterns Nursuries Inc. (Surrey, U.K.).

-Photographic fixative; Kodak (Cambridge, U.K.).

-Photographic developer; Ilford Ltd (Cheshire, U.K.).

-2' deoxyadenosine 5'-triphosphate (Cat. no. 27-2050-01), 2' deoxycytidine 5'-triphosphate (Cat. no. 27-2060-01), 2' deoxyguanine 5'-triphosphate (Cat. no. 27-2070-01), 2' deoxythymidine 5'-triphosphate (Cat. no. 27-2080-01) - Pharmacia Biotech (St. Albans, Hertfordshire, U.K.).

-Taq DNA polymerase, PCR buffer (Cat. no. 18038-026) - GibcoBRL (Paisley, U.K.). This enzyme was responsible for catalysing the amplification of DNA in the polymerase chain reaction.

-Wizard minipreps DNA purification system (Cat. no. A7 100) - Promega (Southampton, U.K.), used for the isolation and purification of plasmid DNA from *E. coli.*

-Shrimp alkaline phosphatase (Cat. no. 1758250) - Boehringer Mannheim (Lewes, East Sussex, U.K.), used for the dephosphorylation of restriction endonuclease digested DNA prior to ligation with vector DNA.

-Prime-It II random primer labelling kit (Cat. no. #300385) - Stratagene (Cambridge, U.K.). This kit was used for the radio-labelling ([α-^{32}P] dCTP) of DNA fragments, which were subsequently used to probe Southern blots.

-Qiaquick gel extraction kit (50) (Cat. no. 28704) - Qiagen GmbH (Hilden, Germany)

-Geneclean kit (Cat. no. #1001-000) - Bio 101 inc. (California, U.S.A.). Both of these kits were used for the isolation of PCR and restriction endonuclease digested DNA fragments from agarose gels.

3. METHODS.

3.1. Plant growth conditions.

The plants were all grown in a purpose built containment growth room illuminated by high-intensity fluorescent tubes under a cycle of 16 hr light and 8 hr dark at 22 °C.

Brassica napus (oilseed rape), *Nicotiana tabacum* (tobacco), and *Sinapis alba* (white mustard) seeds were sown in Fisons number 1 compost. After approximately one month the seedlings were potted up individually into Fisons number 3 compost in 10 cm pots. The plants were supplemented with 0.3 % (w/v) Miracle Gro every 14 days.

Barley grains were grown in vermiculite to generate seedlings for root tissue. Seeds were initially surface sterilised with 10% (v/v) sodium hypochlorite for 10 min. Then washed for 30 min in three changes of sterile distilled water. The grains were then imbibed in sterile water for 6 hr with gentle agitation (150 rpm). The soaked seeds were sown in seed trays containing vermiculite and initially grown in the dark at 22 °C. Following the appearance of the coleoptile (about day 3), the seeds were transferred to a 16 hr light regime at 22 °C. Roots were harvested 10 days after imbibition and were used immediately or frozen in liquid air and stored frozen at -80 °C.

3.2. Barley and wheat embryo isolation and germination.

Barley and wheat embryos were separated from field ripened grains using a modified version of the method of Johnson and Stern [1957]. 500 g of grain was incubated for 5 min with 250 ml of 2 cm^2 pellets of dry ice, ground in a model 530 Moulinex blender for 10 sec and passed through a 1.8 mm mesh-sieve (Baid and Tatlock Ltd, Essex, U.K.). The portion remaining in the 1.8 mm mesh-sieve was mixed with 50 ml of dry ice, re-homogenised for 10 sec and passed back through the 1.8 mm mesh-sieve. The material which passed through the 1.8 mm mesh-sieve was then sieved in a 1.2 mm mesh-sieve, the contents of which was vigorously mixed with 2 L of 2 M sucrose in a stainless steel tray. The freed embryos being less dense than the sucrose solution collected at the surface while the endosperm sank. The embryos were collected in a sidearm conical flask attached to a KNF Neuberger vacuum pump (8.465 x 10^4 Pa (Nm^{-2})). The collected embryos were then refloated on 1 L of 1.5 M sucrose to further remove any pieces of contaminating endosperm and in the case of barley, contaminating bran which floated in 2 M sucrose but sank in 1.5 M sucrose. The embryos were collected from the 1.5 M sucrose, placed in a 425 µm mesh-sieve and washed briefly with distilled water.

The isolated embryos were surface sterilised with 10% (v/v) sodium hypochlorite for 10 min followed by three 10 min washes in sterile distilled water. 100 mg of embryos (about 50-100 embryos) were germinated for 72 hr at 22 °C in darkness in a petri dish on a disk of moistened Whatman no. 1 filter paper (1.5 ml of sterile distilled water) containing 10 µg/ml chloramphenicol, to prevent the growth of micro-organisms. An additional 250 µl of distilled water was added to the germinating embryos at 24 hr intervals if necessary, to maintain sufficient moisture levels around the embryos.

3.3. Preparation of crude total protein extracts.

Crude total soluble protein extracts were prepared from isolated barley and wheat embryos. The 72 hr germinated embryos were dried briefly on paper towels, incubated with 20 ml of 2 cm^2 pellets of dry ice for 10 min, then ground to a fine powder in a Moulinex blender for 1 min. The resulting powder was allowed to thaw on ice and then homogenised with two volumes of extraction buffer (400 mM NaCl, 50 mM Na_2HPO_4, pH 7.5). The resulting extracts were filtered through Miracloth and centrifuged at 30,000 g for 30 min at 4 °C using a Beckman J2-HS centrifuge in a JA-14 rotor. The supernatants were transferred to fresh tubes and respun. Following centrifugation the supernatants were decanted and stored on ice until use. Care was taken to ensure that none of the low density lipid deposits which accumulated at the surface of the supernatant were transferred on the pipette tips.

Roots were harvested from 10 day old barley plants grown in vermiculite, frozen in liquid air and ground to a powder in a Moulinex blender for 1 min in the presence of 50 ml of dry ice. The powdered tissues were homogenised with two volumes of extraction buffer, filtered through Miracloth and centrifuged at 30,000 g for 30 min at 4 °C using a Beckman J2-HS centrifuge in a JA-14 rotor. The supernatants were transferred to fresh tubes and respun to obtain crude protein extracts.

Crude protein extracts were also prepared in a similar fashion from *B. napus* and *N. tabacum* leaves. The tissue was frozen in liquid air, ground with a mortar and pestle and homogenised with extraction buffer and a small amount of quartz sand. The weight to volume ratio used was 1 g tissue : 1 ml buffer. Homogenates were centrifuged at 14,000 g for 10 min, at 4 °C using a Beckman GS-1SR centrifuge in a F0850 rotor. Fresh total extracts were prepared just prior to use and kept on ice or stored frozen at -20 °C.

3.4. Preparation of protein extracts from freeze dried plant tissues.

Harvested plant tissues (roots, stems, leaves and petals) were placed in 50 ml screw cap tubes covered with a layer of parafilm, punctured with several small holes. The tissue was frozen in liquid air and freeze dried on a Flexi-Dry µP freeze drier (FTS Systems, New York, U.S.A.) for 48 hr. Once dry the caps were tightly replaced on the tubes which were then stored in a desiccation jar at 4 °C. The freeze dried tissues were ground with a mortar and pestle with a small amount of quartz sand and homogenised in four volumes of extraction buffer (400 mM NaCl, 50 mM Na_2HPO_4, pH 7.5) containing 1% (w/v) SDS. The homogenate was centrifuged at 14,000 g for 10 min at 4 °C using a Beckman GS-1SR centrifuge in a F0850 rotor. The supernatant was removed and stored frozen at -20 °C.

3.5. Differential protein extraction of leaf tissue.

Differential protein extraction was used to determine the efficiency with which different buffers could be used for optimal release of oxalate oxidase from leaves.

20 g of transgenic 3S1 oilseed rape leaf material was frozen in liquid air and ground with a mortar and pestle to a fine powder in the presence of quartz sand. The extract was then

sequentially extracted in a series of Milli Q quality sterile distilled water and buffers, as indicated in the following table.

Wash	Buffer	Wash	Buffer
1	Milli Q water	9	Milli Q water
2	Milli Q water	10	0.002 M DTT
3	Milli Q water	11	Milli Q water
4	Milli Q water	12	0.2 M sodium borate pH 7.5
5	Milli Q water	13	Milli Q water
6	0.2 M $CaCl_2$	14	1 M NaCl pH 7.5
7	Milli Q water	15	Milli Q water
8	0.1 M EDTA	16	0.035 M SDS (1% (w/v))

Table 3.1. Sequential extraction buffers used for the isolation of oxalate oxidase from homogenised leaf material.

The leaf extract was agitated in the presence of each buffer for 5 min at 4 °C and centrifuged at 18,000 g for 10 min at 4 °C using a Beckman GS-1SR centrifuge in a F0850 rotor. The supernatant was carefully removed and passed through a glass microfibre filter (0.45 µm). The extracts were stored frozen at -20 °C.

3.6. Isolation of extracellular proteins.

3.6.1. Preparation of intercellular fluid from leaf tissue.

Vacuum infiltration involves the initial removal of air from the apoplastic space by the application of a vacuum. The subsequent flooding of this space with buffer, achieved by the gradual release of the vacuum and the recovery of the infiltrated fluid from the tissue by low speed centrifugation. This fluid contains water soluble polysaccharides, extracellular proteins and other materials [Hammond-Kosack 1992].

The procedures described by Hammond-Kosack [1992] and Husted and Schjoerring [1995] were adapted for the preparation of intercellular fluid (INF) from leaves. Leaves from mature plants was cut into approximately 2 cm^2 pieces (avoiding major leaf veins) and gently washed for >15 min in distilled water. The leaf pieces were submerged in infiltration buffer (200 mM NaCl, 50 mM Na_2HPO_4 pH 7.5) prechilled to 4 °C, under wire mesh held in place with a 1 kg weight in a glass vacuum jar connected to a KNF Neuberger vacuum pump. Air was drawn from the apoplastic spaces by applying a vacuum of 8.465 x 10^4 Pa (Nm^{-2}) for 1 min. The vacuum jar was periodically shaken to dislodge air bubbles from the leaf surfaces. The vacuum was slowly released allowing buffer to be drawn into the apoplasm. The evacuation step was repeated twice for tobacco and 5 times for oilseed rape and white mustard leaves to ensure that the tissue was completely water-logged with buffer. Each leaf piece was briefly dried on a paper towel, gently rolled up taking care not to damage the leaf pieces and placed in a 0.5 ml microcentrifuge tube with a 2-3 mm hole cut in its base. This

tube was then placed into an intact 1.5 ml microcentrifuge tube and centrifuged using a Beckman GS-1SR centrifuge in a F3602 rotor. The conditions used for each species are summarised in the following table.

Plant species	Centrifugal force (g)	Time (min)	Temperature (°C)
oilseed rape	10,000	15	4
tobacco	2,500	15	4
white mustard	800	15	4

Table 3.2. Centrifugal forces applied to the different infiltrated leaves.

The clear INF drawn from the apoplastic spaces that collected in the bottom of each of the 1.5 ml microcentrifuge tube was pooled and either stored frozen at -20 °C or at 4 °C as a lyophilised powder (see section 3.9.). Any green coloured INF, indicative of leakage of intracellular contents, was discarded as it contained intracellular contaminants.

3.6.2. Repeated vacuum infiltration.

The concentration of oxalate oxidase isolated from the extracellular spaces was improved by repeating the entire infiltration process described in section 3.6.1. on the same leaf pieces. The infiltration process could be repeated up to four times with no significant increase in the levels of intracellular contamination detected.

3.6.3. Differential vacuum infiltration.

In order to determine the efficiency with which different buffers could be used to isolate extracellular oxalate oxidase, transgenic 3S1 oilseed rape leaf pieces were infiltrated in various buffers. Fresh leaf pieces were used with each different buffer following the vacuum infiltration method described in section 3.6.1. except for the use of a lower centrifugal force (800 g). Infiltration was performed using distilled water, PBS pH 7.4 [Sambrook *et al.* 1989] 100, 200 and 400 mM NaCl, 2 mM DTT, 200 mM $CaCl_2$, 100 and 200 mM sodium borate.

3.7. Malate dehydrogenase assay.

Intracellular contamination of isolated leaf INF was assessed according to a modified method of Ting [1968] and Husted and Schjoerring [1995]. The presence of malate dehydrogenase, a cytoplasmic enzyme was measured in the INF and then related to a total leaf protein extract prepared from the same leaf material that was used in the vacuum infiltration experiments. The assay followed the reduction of oxaloacetate spectrophotometrically at 340 nm.

10 µl of total leaf extract (1g / ml of extraction buffer) or 50 µl of leaf INF was added to 2.99 ml or 2.95 ml of malate dehydrogenase enzyme assay buffer (170 µM oxaloacetate, 94 µM NADH, 50 mM Tris-HCl pH 7.5) in a 3 ml quartz cuvette, respectively. The samples were

shaken to mix and the absorbance measured immediately at 340 nm in a UniCam UV2 spectrophotometer (ATI Unicam, Cambridge, U.K.). The initial rates of enzyme activity were used in the calculation of malate dehydrogenase enzyme activity.

3.8. Concentrating protein extracts.

Protein samples to be analysed on polyacrylamide gels were concentrated using one of the following methods:

3.8.1. Ammonium sulphate precipitation.

Ammonium sulphate precipitation was carried out using % relative saturation data from "Data for Biochemical Research" Ed.s Dawson *et al.* [1969]. The protein sample was chilled to 0 °C. Then 707g / L of ammonium sulphate was added to the solution to take it to 100% relative saturation at 0 °C. The solution was incubated on ice with gentle magnetic stirring for at least 2 hr and then centrifuged at 18,000 g for 30 min at 4 °C using a Beckman GS-1SR centrifuge in a F0850 rotor. The supernatant was removed entirely and the pellet was resuspended in distilled water and stored frozen at -20 °C.

3.8.2. Acetone precipitation.

Two volumes of 100% (v/v) acetone chilled to -20 °C was added to the protein solution in a 1.5 ml microcentrifuge tube, mixed by gentle agitation and then incubated for 1 hr at -20 °C. The precipitate was recovered by centrifugation at 18,000 g for 10 min at 4 °C using a Beckman GS-1SR centrifuge in a F2402 rotor. The supernatant was removed and the pellet was washed twice with 70% (v/v) acetone. The pellet was air dried, resuspended in distilled water and stored frozen at -20 °C.

3.9. Freeze drying protein extracts.

1 ml protein samples in 1.5 ml microcentrifuge tubes were frozen in liquid air and then lyophilised for 24 hr using a Flexi-Dry µP freeze drier (FTS Systems, New York, U.S.A.). Tubes had a small hole cut into the lid to prevent the loss of protein on release of the vacuum.

3.10. Estimation of protein concentration.

Relative protein concentrations in INF and total protein extracts were estimated according to a modified method of Bradford [1976] using the Bio-Rad protein assay kit. Bovine serum albumin (BSA) was used as a protein standard. Protein samples were diluted to a volume of 160 µl with distilled water. Blanks were prepared with distilled water in the first set of wells (lane A) in a microtitre plate. 40 µl of Bradford stock solution (Bio-Rad, Hemel Hempsted, Hertfordshire, U.K.) was added to each well. The plate was briefly shaken (900 rpm) on a microtitre plate shaker for 5 sec and the samples were measures at 570 nm using a Dynatech MT5000 microtitre plate reader (Dynatech Laboratories Ltd, West Sussex, U.K.).

3.11. Oxalate oxidase enzyme assay.

Several methods were used to measure oxalate oxidase activity in solution or following immobilisation of the enzyme onto a nitrocellulose membrane (e.g. dot blotting or electroblotting of oxalate oxidase separated by SDS-PAGE). The nitrocellulose membrane assay of SDS-PAGE separated proteins enabled the size determination of the active enzyme in non-heat treated protein samples and was an alternative to western blotting with antibodies for oxalate oxidase detection. The cuvette solution assay was originally used and then superseded by the microtitre plate assay which was more convenient as it allowed the rapid processing of a large number of samples and it only required one fifth of the solutions and enzyme preparation needed in the cuvette solution assay. The cuvette immobilised assay method was developed to allow enzyme activity measurement following protein treatment with chemicals which may otherwise have interfered with the assay, had they remained in the assay reaction mixture.

3.11.1. Nitrocellulose membrane assay.

A modified procedure described by Lane *et al.* [1993] was used for the detection of enzyme activity associated with oxalate oxidase immobilised onto a nitrocellulose membrane. This method was only used to confirm the presence of oxalate oxidase and not for the quantitative assessment of activity. Proteins were applied onto a nitrocellulose membrane by dot blotting or electroblotting as described in section 3.17 and 3.18, respectively. The nitrocellulose filter was then incubated at room temperature with 5 ml of substrate solution (1 mM oxalic acid in 100 mM succinic acid buffer pH 3.5) and 3 ml of developer (10 ml of stock solution contained 0.83 mg of 4-aminoantipyrine, 133 units of horseradish peroxidase (HRPO) and 2 µl of N, N-dimethylalinine in 100 mM sodium phosphate buffer pH 5.5) until sufficient colour had developed (about 2-5 min). The filter was then washed in several changes of distilled water.

3.11.2. Cuvette solution assay.

The nitrocellulose membrane assay (section 3.11.1.) was adapted for the detection of the enzyme in solution. A 500 µl aliquot of substrate solution (see section 3.11.1.) was preincubated at 37°C for 5 min in a 1 ml plastic cuvette, followed by the addition of enzyme solution or protein extract. After a 60 min incubation at 37 °C with agitation (200 rpm) 300 µl of developer (see section 3.11.1.) and 10 µl of 2.5 mM Tris-HCl buffer (pH 7.5) were added and the tube was inverted several times, which resulted in immediate and stable colour development. Tris-HCl buffer (pH 7.5) was added to stop the HRPO reaction and to stabilise the purple stain. The absorbance of the resulting solution was measured at 550 nm using a Unicam UV2 Spectrophotometer.

3.11.3. Cuvette immobilised assay.

The cuvette solution assay (section 3.11.2.) was adapted for the detection of oxalate oxidase on immobilised nitrocellulose membrane disks. The oxalate oxidase protein samples

were applied onto a nitrocellulose membrane, as described in section 3.18. Each protein dot on the filter was excised using a 5 mm diameter cork borer added to the substrate solution and assayed as described in section 3.11.2.

3.11.4. Microtitre plate solution assay.

The cuvette solution assay (section 3.11.2.) was also adapted for the detection of oxalate oxidase enzyme activity using a Dynatech MR5000 microtitre plate reader. Water blanks were prepared in the first row of 8 wells (lane A) of a 96 well microtitre plate. Enzyme solutions or protein extracts were added to distilled water to a volume of 50 µl per well. The protein solutions were pre-incubated at 37 °C for 5 min followed by the addition of 50 µl of 2 x substrate solution (2 mM oxalic acid in 200 mM succinic acid buffer pH 3.5), pre-incubated to 37 °C. After 60 min at 37 °C with agitation on a microtitre plate shaker (900 rpm) 60 µl of developer (see section 3.11.1.) and 2 µl of 2.5 mM Tris-HCl buffer (pH 7.5) were added. The microtitre plate was briefly shaken (1,500 rpm) and the absorbance of the resulting solution was measured at 570 nm using a Dynatech MR500 microtitre plate spectrophotometer. The samples were measured at 570 nm as opposed to 550 nm because the plate reader did not have a filter for 550 nm.

3.12. Leaf disk assay.

The leaf disk assay was a rapid and simple method which was developed for the monitoring and selection of high oxalate oxidase expressing plants. This method allowed the rapid screening of a large number of potentially expressing plants. Leaf disks from control and transgenic plants were isolated using a paper hole punch (avoiding leaf veins) to give a disk of about 4.5 mm diameter. Eight disks were isolated from the same leaf of each plant. Leaf disks of different plants were isolated from leaves at a similar stage of growth and from the same node position. The disks were carefully placed in the wells of a 96 well microtitre plate each containing 100 µl of distilled water. The blade of the hole punch was cleaned with 70% (v/v) ethanol between each disk isolation to prevent cross-contamination of samples. When all the leaf disks were harvested the microtitre plate was pre-incubated at 37 °C for 5 min. 50 µl of 2 x substrate solution (see section 3.11.4.), pre-incubated to 37 °C was added to each well and the plate was incubated at 37 °C for 60 min with agitation (900 rpm) on a microtitre plate shaker. 100 µl of the reacted solution was then removed from each well and pipetted into identical wells of a fresh microtitre plate and 60 µl of developer (see section 3.11.1.) and 2 µl of 2.5 mM Tris-HCl buffer (pH 7.5) were added. The microtitre plate was briefly agitated (1500 rpm) for 5 sec and the absorbance of the samples were measured at 570 nm using a Dynatech MT5000 microtitre plate reader.

3.13. SDS-Polyacrylamide Gel Electrophoresis (PAGE).

Protein samples were analysed and size fractioned by SDS-electrophoresis using a Bio-Rad Mini-Protean II dual slab cell according to the method of Laemmli [1970]. The apparatus was assembled and slab gels (0.75 mm thick) were cast according to the

manufacturers instructions. Unless stated otherwise, 12% (w/v) acrylamide/bis-acrylamide (37.5:1), 375 mM Tris-HCl pH 8.7, 0.1% (w/v) SDS, 0.05% (w/v) $(NH_4)_2SO_4$, 0.5 % (w/v) TEMED separating gels and 4% (w/v) acrylamide/bis-acrylamide, 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) $(NH_4)_2SO_4$, 1.5 % (w/v) TEMED stacking gels were prepared. Alternative gel concentrations were prepared using the modified mixtures listed in table 3.3.

gel percentage	distilled water (ml)	acrylamide / bis-acrylamide (ml)
7.5	4.93	2.40
10	4.13	3.20
12.5	3.33	4.00
15	2.53	4.80
17.5	1.73	5.60

Table 3.3. Volumes of water and acrylamide stock used to obtain different percentage acrylamide gels.

Protein solutions were mixed with an equal volume of 2 x loading buffer (125 mM Tris-HCl pH 6.8, 6% (w/v) SDS, 0.01% (v/v) β-mecaptoethanol, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue). Lyophilised proteins were resuspended in 1 x loading buffer. In some cases the recommended heat treatment was omitted except were specified in individual experiments [Lammeli 1970]. The SDS stable oligomeric structure and enzyme activity was retained by the oxalate oxidase proteins if they were not heat treated prior to analysis by SDS-PAGE. Samples were heated to 100 °C for 2 min in the presence of loading buffer to completely denature the protein specimens. Electrophoresis was performed in 1 x running buffer (0.3% (w/v) Tris-HCl pH 8.3, 1.44% (w/v) glycine, 0.1% (w/v) SDS) at 50 V through the stacking gel and 150 V through the separating gel (constant voltage) using a Bio-Rad power pack 300.

Protein samples were also analysed and size fractioned by SDS-electrophoresis using the Bio-Rad Protean II vertical electrophoresis cell according to the method of Laemmli [1970]. The gels and protein samples were prepared as described above. These gels were run for about 18 hr at 16 mA (constant current).

Native-PAGE was carried out as described above the only exception was that SDS was not added to any of the gel, loading or running buffers.

3.14. Protein detection in polyacrylamide gels.

Proteins in the µg range were visualised in polyacrylamide gels by staining with 0.1% (w/v) Coomassie brilliant blue R250 CI 42660 in 16% (v/v) glacial acetic acid, 42% (v/v) methanol followed by destaining with 10% (v/v) glacial acetic acid, 40% (v/v) methanol [Sambrook *et al.* 1989].

Silver staining was 10-50 fold more sensitive than Coomassie blue staining allowing the visualisation of proteins in the ng range. A silver kit (Bio-Rad, Hemel Hempsted,

Herfordshire, U.K.) was used according to the manufacturers instructions for protein staining in acrylamide gels. Stained gels where photographed using a Nikon 801S camera and Technical Pan film.

3.15. Glycoprotein detection by periodic acid silver staining.

The method of Dubray and Bezard [1982] was used for the detection of glycoproteins in SDS-polyacrylamide gels. Following electrophoresis the gel was incubated overnight in 25% (v/v) isopropyl alcohol and 10 % (v/v) acetic acid at 25 °C. The gel was then incubated in 0.2 % (v/v) periodic acid at 4 °C for 60 min with gentle agitation (50 rpm) and then washed in several changes of pre-cooled water at 4 °C for 2 hr with gentle agitation (50 rpm). The water was removed and replaced with freshly prepared ammoniacal silver solution for 60 min at 25 °C with gentle agitation (50 rpm). The ammoniacal silver solution was prepared by adding 1.4 ml of ammonia solution (35% (w/v)) to 21 ml of 0.36% (w/v) NaOH. 4 ml of 19.4% $AgNO_3$ were then added drop by drop with vigorous stirring (magnetic stirrer) and the solution was made up to 100 ml with distilled water. The gel was then washed in distilled water for 2 min and transferred to a freshly prepared solution containing 0.05% (w/v) citric acid, 0.019% (v/v) formaldehyde and 10% (v/v) methanol. The gel was developed at room temperature until the bands of interest were visible (about 2 min). The reaction was stopped by placing the gel in commercial photographic fixative for 5 min. The gel was finally washed in several changes of distilled water for 30 min and then photographed using a Nikon 801S camera and Technical Pan film.

3.16. Concanavalin A sepharose chromatography.

Oxalate oxidase from total protein extracts and INF was isolated on a ConA-sepharose column according to the method of Dumas *et al.* [1993]. A 1 ml ConA-sepharose column was prepared in a 5 ml syringe and equilibrated with 10 ml of ConA-column buffer (100 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). Protein extracts were applied to the column, which was washed with 20 ml of column buffer until no further protein was eluted. The bound protein was eluted from the column with column buffer containing 5 mM α-mannopyranoside, collected as 200 µl fractions and assayed for oxalate oxidase activity, as described in section 3.11.4. Fractions exhibiting enzyme activity were pooled and stored frozen at -20 °C.

3.17. Electroblotting of proteins onto nitrocellulose membranes.

The method described by Burnette [1981] was modified to enable the transfer of SDS-PAGE fractioned proteins onto a nitrocellulose membrane using a Kem-en-Tec semi-dry electroblotting system (Whatman Ltd, Kent, U.K.). The apparatus was used according to the manufacturer instructions. The 'blotting sandwich' was assembled on the anodic graphite plate and consisted of nine Whatman no. 1 filter papers and the nitrocellulose membrane (0.45 µm) soaked in anode/cathode buffer (48 mM Tris-HCl, 39 mM glycine, 1.3 mM SDS, 20% (w/v) methanol). The SDS-PAGE gel was carefully laid on top of the nitrocellulose membrane. Air

bubbles were removed from beneath the gel to ensure complete contact with the membrane. A further 9 pieces of Whatman no. 1 filter paper soaked in anode/cathode buffer were placed on top of the gel. The 'blotting sandwich' was then covered with the cathodic graphite plate and connected to the power supply and operated at 0.8 mA/cm^2 for 1 hr at room temperature.

3.18. Dot blotting of proteins onto nitrocellulose membranes.

A variation of the electroblotting method was used to detect oxalate oxidase in a large number of samples. This involved the dot blotting of protein extracts onto a nitrocellulose membrane. This procedure was carried out using a Hybri-dot blotter (Bethesda Research Laboratories Ltd, Cambridge U.K.) attached to a Hybaid VAT/T blot processing pump (Hybaid Ltd, Middlesex, U.K.). The equipment was assembled according to the manufacturers instructions. Protein samples were diluted in the individual wells of a microtitre plate to a volume of 150 µl with water. The protein samples were applied onto a water rinsed nitrocellulose membrane supported by a layer of water saturated Whatman no. 1 filter paper, under a vacuum of 1×10^4 Pa (Nm^{-2}). Each well of the dot blotter was rinsed with 200 µl of distilled water once all of the samples were applied. The nitrocellulose filter was air dried and stored between two sheets of Whatman no. 1 filter paper in a sealed plastic bag at -20 °C.

3.19. Detection of membrane-immobilised proteins using labelled antibodies.

The nitrocellulose membrane containing blotted protein samples (from dot blots and electroblots) were incubated overnight with 20 ml of 5% (w/v) Marvel (dried skimmed milk powder)-TBS (100 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) at 4 °C. The purpose of blocking the nitrocellulose filters was to prevent the non-specific binding of antibodies. The filter was then incubated with primary antibodies in 10 ml of antiserum buffer (5% (w/v) Marvel-TBS, 0.1% (v/v) Tween 20) for 2 hr at 25 °C. Antibodies used in this study are listed in table 3.4. together with the dilutions used.

Antibodies	Dilution
anti- barley root oxalate oxidase	1:10,000
anti-pea ribulose bis-phosphate carboxylase (anti-RUBISCO)	1:1,000
anti-*Sinapis alba* germin-like protein (anti-*sa*glp)	1:250

Table 3.4. Primary antibody dilutions.

Filter were washed for 60 min with 4 changes of 20 ml of antiserum buffer. The procedure for blotting with secondary antibodies varied according to the method or kit used for the antibody detection, as described below.

3.19.1. Iodinated antibody detection.

Filter were incubated with 185 kBq of ^{125}iodine-conjugated donkey anti-rabbit antibodies in 10 ml of antiserum buffer for 2 hr at 25 °C. The filters were washed for 45 min

with 3 changes of 40 ml of antiserum buffer, followed by a single wash with 40 ml of TBS for 15 min at 25 °C. The filters were then exposed to Fuji RX medical x-ray film at -80 °C for between 1 and 14 days depending on the intensity of protein bands required.

3.19.2. Alkaline phosphatase colorimetric detection.

Filters were incubated with a 1:5,000 dilution of alkaline phosphatase-conjugated donkey anti-rabbit antibodies (Sigma, Poole, Dorset, U.K.) in 10 ml of antiserum buffer for 2 hr at 25 °C. The filters were washed for 45 min with 3 changes of 40 ml of antiserum buffer followed by a single wash with 40 ml of TBS for 15 min at 25 °C and then incubated in freshly prepared nitroblue tetrazolium (NBT) buffer. NBT buffer was prepared by adding 66 µl of 50 mg/ml NBT (stock in 70% (v/v) dimethyl formamide) and 33 µl of 50 mg/ml 5-bromo,4-chloro,3-indoyl phosphate (stock in distilled water) to 10 ml of buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The reaction was stopped after sufficient colour development (about 1 - 2 min) by washing the filters in several changes of distilled water. The filters were air dried and stored at room temperature.

3.20. Electroblotting for N-terminal protein sequencing.

SDS-PAGE was performed as described in section 3.14. with one modification. This involved pre-running the assembled gel with 200 µM thioglycolic acid in the upper reservoir (1 x running buffer) for 30 min at 50 V, before loading the protein samples. The thioglycolic acid acted as a scavenger of free amine groups in the polymerised gel which may cause N-terminal protein blocking. The SDS-PAGE separated proteins were then blotted onto a ProBlott™ membrane according to the manufacturers instructions (Applied Biosystems, California, USA). The polyacrylamide gel was soaked in electroblotting buffer (10 mM CAPS buffer pH 11 in 10% (v/v) methanol) for 5 min. The ProBlott™ was rinsed in 100% (v/v) methanol for a few seconds and then in electroblotting buffer for 2 min. The electroblotting was performed as described in section 3.18. Following electroblotting the membrane was rinsed in distilled water, soaked for a few seconds in 100% (v/v) methanol and then stained for 1 min with 0.1% (w/v) Coomassie blue R250 CI 42660, 40% (v/v) methanol, 1% (v/v) acetic acid. Excess stain was removed with 50% (v/v) methanol and the membrane was finally washed with distilled water. The stained protein bands of interest were excised from the ProBlott™ membrane for N-terminal sequencing.

3.21. N-terminal protein sequencing.

Excised membrane-immobilised proteins were loaded into a Blott™ cartridge on an Applied Biosystems 477A Pulsed Liquid Protein Sequencer (California, U.S.A.). Sequencing was carried out according to the standard automated protocol for PVDF (polyvinylidene difluoride)-blotted samples by John Gilroy (Department of Biological Sciences, University of Durham, U.K.).

3.22. Protein cross-linking with dimethyl suberimidate.

Protein oligomers were cross-linked with the bifunctional reagent dimethyl suberimidate (DMS) according to the method of Davies and Stark [1987]. DMS was used to produce amidine cross-links between the monomers of oligomeric proteins. Electrophoresis in the presence of SDS gives rise to a set of protein oligomers with molecular weights equal to integral multiples of the monomer molecular weight. The reaction mixture containing 5 µl protein (2 µg), 1 µl (0.8 mg / ml) DMS and 4 µl (400 mM) triethanolamine, was briefly vortexed, briefly spun in an MSE Microcentaur centrifuge and incubated at room temperature for at least 6 hr. The samples were then incubated with 1% (v/v) SDS, 1% (v/v) β-mecaptoethanol at 37 °C for 2 hr and finally mixed with 5 µl of 50% (v/v) glycerol containing 0.05% (w/v) bromophenol blue. Where stated the samples were heated 100 °C for 2 min. The proteins were separated by SDS-PAGE under standard conditions (section 3.14.).

3.23. Deglycosylation of protein with tunicamycin.

Tunicamycin is an inhibitor of N-acetylglucosamine transferases which blocks the formation of N-glycosidic linkages by inhibiting the first step in the lipid saccharide pathway i.e. the transfer of N-acetylglucosamine-1-phosphate to dolichol mono-phosphate. It is a nucleoside antibiotic composed of a uracil, a fatty acid and two glycosidically linked sugars (an N-acetylglucosamine and tunicamine an unusual 11-carbon aminodeoxydialdose). [Elbein 1987]. This was used to prevent the glycosylation of oxalate oxidase which enabled the degree of gylcosylation to be determined following analysis of the proteins by SDS-PAGE.

Barley and transgenic 3S1 oilseed rape seeds were surface sterilised in 10% (v/v) sodium hypochlorite for 10 min followed by 3 washes in sterile distilled water for 30 min. The seeds were briefly dried on paper towels and evenly spread over the surface of 2 layers of Whatman no. 1 filter paper in sterile tissue culture vessels. Isolated, surface sterilised barley and wheat embryos similarly were distributed over 2 layers of filter paper in sterile petri dishes. All the seeds and embryos were grown in the presence of 10 µg/ml chloramphenicol and half of the seeds and embryos were also grown in the presence of 10 µg/ml tunicamycin. The embryos were grown at 22 °C in darkness for 3 days. The seeds were grown at 22 °C in darkness until germination had begun (about day 3) and then they were transferred to a 16 hr light regime. The barley and oilseed rape seedlings were grown for 10 days under these conditions. Moisture levels were maintained every 24 hr by supplementing the embryos and seedlings with the appropriate antibiotic containing solutions. Total protein extracts were prepared from these plant materials in extraction buffer as described in section 3.3.

3.24. Pepsin digestion of proteins.

This procedure was used to digest virtually all of the contaminating proteins from crude total protein extracts to give a preparation of almost pure oxalate oxidase. Pepsin digestion of protein extracts was performed using a modified version of the method described by Grzelczak and Lane [1984]. The standard reaction mixture contained 2 µl protein extract, 10 µl 50 mM HCl and 2 µl pepsin (5 mg / ml). The reaction mixture had an overall pH of about

1.8. The mixture was briefly vortexed for 5 sec, briefly centrifuged in an MSE Microcentaur centrifuge and incubated in a water bath at 37 °C for 2 hr. The reaction was stopped by adding 4 µl of 3 M Tris-HCl pH 8.8. The samples were centrifuged at 18,000 x g for 10 min at 4 °C using a Beckman GS-1SR centrifuge in a F3602 rotor, the supernatant was removed and stored frozen at -20 °C.

3.25. Purification of oxalate oxidase for molecular weight determinations.

3.25.1. Protein extracts.

INF from leaves of the three transgenic plant lines (3S1 oilseed rape, C26 and SGS5 tobacco) was isolated as described in section 3.6.1. Total protein extracts of barley roots, barley embryos and wheat embryos were prepared according to section 3.3.

3.25.2. Ammonium sulphate precipitation.

INF samples (1 ml) were saturated to a level of 70% (relative saturation) ammonium sulphate for 2 hr at 0 °C with gentle agitation (150 rpm). Samples of total protein extracts (1 ml) were fractionated with 50% (relative saturation) and 70% (relative saturation) ammonium sulphate each for 2 hr at 0 °C with gentle agitation (150 rpm). The precipitated proteins were recovered by centrifugation at 18,000 g for 30 min at 4 °C using a Beckman GS-1SR centrifuge in a F3602 rotor. The supernatants were removed and the pellets from the 70% (relative saturation) ammonium sulphate fractions were resuspended in 25 µl of distilled water.

3.25.3. Superose 12 analytical gel filtration chromatography.

2.75 µl of 10 x superose 12 column buffer (1 M Na_2HPO_4, 2 M NaCl pH 7.0) was added to each of the protein samples, as prepared in section 3.25.2. The samples were centrifuged (30 min, 4°C, 18,000 g) and the resulting supernatants (27.5 µl) were separately loaded onto a 2.5 ml superose 12 column (Pharmacia Smart System, Southampton, U.K.) equilibrated with column buffer (100 mM Na_2HPO_4, 200 mM NaCl pH 7.0), at a flow rate of 40 µl/min. The eluant was collected in 50 µl fractions. 5 µl of fractions 1 to 22 were assayed for oxalate oxidase activity using the microtitre plate assay method (section 3.11.4.). Fractions which exhibited the highest levels of enzyme activity were pooled and store frozen at -20 °C. The column was calibrated with a mixture of proteins of known molecular weights.

3.26. Large-scale protein extraction from INF and total protein extracts.

Large-scale purifications of oxalate oxidases from transgenic and cereal plant materials were performed to provide sufficient enzyme to perform the various structural and kinetic studies. At each stage of the purification procedure a Bradford assay (section 3.10.) and a microtitre plate enzyme assay (section 3.11.4.) were performed on several dilutions of each of the protein samples. Dilutions of the protein samples were also immobilised onto nitrocellulose membranes, as described in section 3.18. Oxalate oxidase was detected by enzyme activity (section 3.11.1.)

3.26.1 Protein extraction.

INF from leaves of the three transgenic plant lines (3S1 oilseed rape, C26 and SGS5 tobacco) was isolated as described in section 3.6.1. Total protein extracts of barley root and embryo and wheat embryo were prepared according to section 3.3.

3.26.2. Ammonium sulphate precipitation.

All protein samples were fractionated with 50% (relative saturation) and 70% (relative saturation) ammonium sulphate each for at least 2 hr at 0 °C with gentle stirring. The precipitated proteins were recovered by centrifugation at 18,000 g for 30 min at 4 °C using a Beckman GS-1SR centrifuge in a F0850 rotor. The supernatants were removed and the pellets from the 50 % (relative saturation) and 70% (relative saturation) ammonium sulphate fractions were resuspended in 3-10 ml of distilled water. The volume of water was dependant on the size of the pellet to be resuspended.

3.26.3. G100 sephadex preparative gel filtration chromatography.

Pharmacia (St. Albans, Hertfordshire, U.K.) G100 sephadex was swollen and 300 ml was prepared according to the manufacturers instructions. A column (diameter 25 mm) was set up and run at 4 °C. The column was equilibrated with G100 buffer (200 mM NaCl, 50 mM $KHPO_4$ pH 7.5) and run at a flow rate of 2 ml / min and 5 ml fractions were collected. Aliquots of the fractions were assayed for oxalate oxidase using the microtitre plate enzyme assay as described in section 3.11.4.

Fractions exhibiting the highest levels of oxalate oxidase activity within the exclusion range of the column (i.e. proteins with a molecular weight greater than 100 kDa) were pooled and dialysed for 16 hr against several changes of DE52 equilibration buffer (50 mM $KHPO_4$ pH 7.5) at 4 °C. The column was washed and stored in G100 buffer containing 0.02% (w/v) sodium azide when not in use.

3.26.4. DE52 cellulose ion exchange chromatography.

Whatman (Maidstone, Kent, U.K.) DE52 cellulose was prepared according to the manufacturers instructions. A 200 ml column with a diameter of 25 mm was set up and run at 4 °C using a Pharmacia Biotech Gradifrac system (Southampton, U.K.). The column was equilibrated with DE52 equilibration buffer (50 mM $KHPO_4$ pH 7.5). The dialysed protein sample was centrifuged at 18,000 g for 10 min at 4 °C using a Beckman GS-1SR centrifuge in a F0850 rotor. The resulting supernatant was loaded onto the ion exchange column and run at a flow rate of 2 ml / min and 10 ml fractions were collected. Once all the unbound protein were washed from the column an NaCl gradient was applied. The production of the gradient was controlled by the Pharmacia Biotech Gradifrac system, which controlled the mixing of the equilibration buffer and the elution buffer (500 mM NaCl, 50 mM $KHPO_4$ pH 7.5). The total gradient comprised of 200 ml from 0-100% of elution buffer. Aliquots of the fractions were assayed for oxalate oxidase using the microtitre plate enzyme assay, as described in section 3.11.4. Fractions exhibiting the highest levels of oxalate oxidase activity were pooled and

dialysed for 16 hr against several changes of distilled water at 4 °C. The column was washed and stored in equilibration buffer containing 0.02% (w/v) sodium azide when not in use.

3.26.5. Freeze drying.

Dialysed protein solutions were centrifuged at 18,000 g for 10 min at 4 °C using a Beckman GS-1SR centrifuge in a F0850 rotor. The supernatant was then lyophilised as described in section 3.9.

3.26.6. Pepsin digestion.

The purified wheat embryo and barley embryo oxalate oxidase preparations still contained significant levels of contaminating proteins and so a scaled up pepsin digestion (100 x) was performed on these protein samples as described in section 3.24. in order to remove these proteins. The digested protein samples were passed down a G100 sephadex column as described in section 3.26.3., assayed, and the oxalate oxidase containing fractions were pooled, dialysed and lyophilised as described in section 3.26.5.

3.27. MALDI-ToF mass spectrometry.

MALDI-ToF (matrix assisted laser desorption ionisation-time of flight) mass spectrometry was carried out in collaboration with Amelia Jackson (KRATOS Analytical, Manchester, U.K.) using a Kompact Research MALDI 2 with a 77 cm flight tube with 'pulsed extraction, which increases resolution and mass accuracy. The lyophilsed protein samples (section 3.26.) were dissolved (1 mg / ml) in 0.1% (v/v) trifluoroacetic acid. The matrix used was sinapinic acid (3,5 dimethyl-4 hydroxycinnanmic acid) at 10 mg / ml in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. 0.5 ml of the protein sample was mixed with 0.5 ml of matrix, which was then added to the sample well on the sample slide. The sample was then crystallised by drying and inserted into the instrument. The y-axis on the plots represents percentage signal intensity. 100% signal represents the mV reading included in the figure legend.

3.28. Circular dichroism spectrometry.

Circular dichroism spectrometry was carried out in collaboration with Peter Wilde and Fiona Husband (Institute of Food Research, Norwich, U.K.) using a Jasco J710 spectropolarimeter (Jasco corporation, Tokyo, Japan). The sample chamber was purged with nitrogen to reduce absorption of CD signal by atmospheric oxygen. The lyophilised protein samples (section 3.26.) were resuspended and diluted in 10 mM sodium phosphate buffer pH 7.0 and loaded into quartz cuvettes with various pathlengths (0.1-10 mm cells) at various concentrations (0.01-1 mg/ml). CD spectra were measured over wavelengths of between 180-260 nm. The raw data was converted to Delta (Δ) Epsilon ($cm^2.mmole^{-1}$) with the equation 3.39×10^{-3} / ((protein concentration (mg / ml)) x pathlength (cm)).

The spectra were analysed using the selcon method [Sreerama and Woody 1993], fitting to 3 structural parameters, α-helix, β-sheet and aperiodic, using a series of standard spectra of known protein secondary structures.

3.29. Differential scanning calorimetry.

Differential scanning calorimetry was performed in collaboration with Tim Mann and Paul Gabbott at Perkin Elmer Thermal Analysis (Buckinghamshire, U.K.), using a Pyris 1 differential scanning calorimeter. The lyophilised protein samples (section 3.26.) were resuspended in Milli Q water in a stainless steel large volume pan. Temperature increase occurred at a rate of 10 °C / min, over a temperature range of 50-150 °C.

3.30. Densitometry.

Quantification of oxalate oxidase enzyme concentrations were determined using a Bio-Rad Gel Doc 1000 system with a GS690 imaging densitometer and the Bio-Rad molecular analyst 2D-gel data processing software programme. Autoradiographs of western dot blots of known concentrations of commercially available barley seedling oxalate oxidase (Sigma, Poole, Dorset, U.K.) and purified barley root oxalate oxidase were used to prepare standard curves of different enzyme concentrations. Standard error calculations were included on each of the graphs presented in this thesis, to show the degree of variation between the replicate data. Error bars are not visible on some of the graphs due to the scale used on the vertical axis, to enable direct comparison of different sets of data.

3.31. Genomic DNA isolation.

Genomic DNA was isolated from non-transgenic and transgenic 3S1 oilseed rape leaves using a CTAB isolation method [Boris Stanchev, personal communication]. 10 g of leaf material was frozen in liquid air, homogenised in a mortar and mixed with 50 ml of buffer B (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, pre-heated to 65 °C) and incubated at 65 °C for 15 min. The homogenate was centrifuged at 1,200 g for 10 min at room temperature using an MSE Centaur 2 centrifuge and an equal volume of chloroform:isoamyl alcohol (24:1) was added to the supernatant. The aqueous phase was removed to a fresh tube and the chloroform:isoamyl alcohol extraction repeated. To the aqueous phase was added an equal volume of buffer C (50 mM Tris.HCl pH 8.0, 10 mM EDTA, 1% (w/v) CTAB) which was incubated at room temperature for 30 min. The suspension was then centrifuged at 9,000 g for 30 min at room temperature using a Beckman GS-1SR centrifuge in a F0850 rotor. The DNA pellet was dissolved in 2 ml of 1 M CsCl containing 100 µg/ml DNAse-free RNAse and incubated for 2 hr at room temperature. The DNA was then precipitated with 1 volume of 13% (w/v) polyethylene glycol 8,000, washed twice with 70% (v/v) ethanol and air dried. The pellet was resuspended in 25-200 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.4), depending on the quantity of isolated DNA and incubated at 4 °C for 48 hr to aid resuspension and then stored frozen at -20 °C.

3.32. Restriction endonuclease digestion of DNA.

Restriction endonuclease reactions were carried out using the buffers and temperatures recommended by the manufacturers. Restriction digests of plasmid DNA were incubated at 37 °C for 2 hr. Restriction digests of genomic DNA were incubated at 37 °C for at least 16 hr. The restriction endonucleases were inactivated by incubating the reaction mixture at 70 °C for 15 min.

3.33. Oligonucleotide synthesis.

Oligonucleotides were synthesised using an Applied Biosystems 380 DNA synthesizer using the manufacturers reagents. The synthetic DNA was deblocked overnight by incubating at 55 °C. 100 µl of the oligonucleotide solution was aliquoted into a 1.5 ml microcentrifuge tube to which was added 1 ml of 100% (v/v) butanol at room temperature. The tube was briefly vortexed and centrifuged at 14,000 g for 5 min using an MSE Microcentaur centrifuge. The supernatant was removed, the pellet air dried and resuspended in 12.5 µl of sterile MilliQ water. The optical density of the oligonucleotide sample was measured at 260 nm (the conversion factor was OD_{260} x dilution x molar extinction coefficient (25) = µg / ml DNA), the sample was diluted with MilliQ water to give the working concentrations (100 µg / ml) and stored frozen at -20 °C.

name	oligonucleotide sequence
C1	5'-GAYTTYTGYGTIGTIGAYAAYACIGGIGA-3'
C2	5'-GTTGTCCAACAAGGAACCTTGCTTGTAGGG-3'
C3	5'-ATTGTCCAACAAGGAACCTTGCTTGTAGGG-3'

Table 3.5. PCR Oligonucleotide sequences.
I, inosine; Y, T/C.

3.34. Polymerase chain reaction.

PCR was used to amplify germin-like gene sequences from non-transgenic oilseed rape genomic DNA samples. All the reaction components were placed into 0.5 ml thin walled microcentrifuge tubes in the order described below, 'flicked' to mix and briefly centifuged in an MSE microfuge. A typical amplification reaction consisted of:

genomic DNA (1 µg)	10 µl
dNTP stock (1.25 mM)	16 µl
oligonucleotide primer 1 (100 µg / ml)	10 µl
oligonucleotide primer 2 (100 µg / ml)	10 µl
sterile distilled water	37 µl
10 x PCR buffer (200 mM Tris. HCl pH 8.4, 500 mM KCl)	13 µl
50 mM $MgCl_2$	3 µl
Taq polymerase (5 U / µl)	1 µl

The amplification reaction was carried out using a Techne PCH-3 thermal cycler (Cambridge, U.K.) with the following reaction conditions.

(1) 3 cycles of PCR amplification	Temp (°C)	Time (sec)
denaturing	94	30
annealing	35	30
extending	72	60
(2) 35 cycles of PCR amplification	Temp (°C)	Time (sec)
denaturing	94	30
annealing	55	30
extending	72	60
(3) 1 cycle of PCR amplification	Temp (°C)	Time (sec)
extending	73	360
storage	4	∝

Table 3.6. PCR amplification conditions.

Aliquots (10 µl) of the completed PCR reactions were run on low melting point agarose gels as described in section 3.35. The remaining reaction mixture was stored frozen at -20 °C.

3.35. DNA separation on agarose gels.

DNA was size fractioned by electrophoresis as described by Sambrook *et al.* [1989]. 0.7% (w/v) agarose gels were cast in 1 x TAE buffer (40 mM Tris-acetate pH 7.7, 1 mM EDTA, 0.5 µg/ml ethidium bromide). 4 µl of loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol) was added to 20 µl of DNA samples diluted in sterile MilliQ water prior to loading. The gels were run in 1 x TAE buffer at 90 v (constant voltage) and the separated DNA fragments were visualised at 300 nm on a ultra violet transilluminator. A Bio-Rad Gel Doc 1000 image analyser system was used to produce a thermal video printout of the gel.

3.36. Recovery of DNA restriction fragments from low melting point agarose.

DNA restriction fragments were isolated using a QIAquick gel extraction kit according to the manufacturers instructions. The recovered DNA restriction fragments were stored frozen at -20 °C.

3.37. Random primer DNA labelling.

DNA restriction fragments were isolated from low melting point agarose gels using a Geneclean kit (Bio 101 Inc., California, U.S.A.) according to the manufacturers instructions. The restriction fragments were labelled with [α-^{32}P] dCTP (1.85 x 10^9 Bq) using a Prime-It II random primer labelling kit (Stratagene, Cambridge, U.K.) according to the manufacturers instructions.

Labelled DNA was separated from unincorporated nucleotides using a 10 ml Sephadex G50 column (equilibrated with 300 mM NaCl, 0.1% (w/v) SDS, 50 mM Tris-HCl pH 9.5, 10 mM EDTA). Specific activities of 10^8-10^9 cpm/µg were routinely achieved.

3.38. Southern blotting.

Agarose gels of genomic DNA restriction endonuclease digests and PCR products were briefly washed with distilled water, incubated twice in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 10 min and blotted onto a Hybond N nylon membrane in 1 x denaturation solution as described by Sambrook *et al.* [1989]. The filters were washed twice in neutralisation solution (1.5 M NaCl, 0.5 M Tris.HCl pH 7.2, 0.001 M EDTA) for 10 min and for 5 min in 6 x SSC (20 x SSC stock; 175.3 g/L NaCl, 88.2 g/L Na citrate pH 7.0) before being fixed by heating at 80 °C for 2 hr.

The filter was pre-hybridised in 5 x SSC containing 0.5% (w/v) Marvel milk powder and 0.1% (w/v) SDS at 65 °C for 2 hr. The ^{32}P-labelled probe prepared according to section 3.37. was added to the membrane and hybridised overnight at 65 °C. The excess probe was collected and stored frozen at -20 °C for re-use. The filter was washed twice in 2 x SSC, 0.1% (w/v) SDS and twice in 1 x SSC and 0.1% (w/v) SDS each for 15 min at 65 °C. The filter was then exposed to Fuji RX medical x-ray film at -80 °C for between 1 and 14 days depending on the intensity of the DNA bands required.

3.39. Dephosphorylation and ligation.

PCR amplified fragments were cloned into digested pUC18 plasmid vector DNA which was dephosphorylated using shrimp alkaline phosphatase (Boehringer Mannheim, East Sussex, U.K.) according to the manufacturers instructions. Ligations were performed using T4 DNA ligase (Boehringer Mannheim, East Sussex, U.K.) according to the manufacturers instructions.

3.40. Transformation, growth and selection of competent *E. coli* cells.

The maintenance, growth and storage of *E. coli* strain DH5α was carried out as described by Sambrook *et al.* [1989]. Competent DH5α *E. coli* cells were grown in Luria broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl pH 7.0) overnight at 37 °C. 200 µl of cells were grown to the exponential phase (optical density of 0.5 at 600 nm) in 10 ml Luria broth at 37 °C. The suspension was centrifuged at 1,200 g for 5 min at room temperature in an MSE Centaur 2 centrifuge and the pellet resuspended in 1 ml of TSS buffer (Luria broth, 10% (w/v) polyethylene glycol 6,000, 30 mM $MgCl_2$ and 5% (v/v) dimethyl sulphoxide pH 6.5 at 0 °C) [Sambrook *et al.* 1989]. 20 µl of plasmid DNA was incubated on ice with 200 µl of cell suspension for 60 min. The cells were heat-shocked (42 °C for 2 min), immediately cooled on ice, mixed with 800 µl of Luria broth and incubated at 37 °C for 45 min.

The cell suspension was briefly centrifuged at 1,200 g at room temperature using an MSE Centaur 2 centrifuge and 900 µl of the supernatant was removed. The cells were resuspended in the remaining 100 µl of buffer and plated out onto 20 ml of L-agar (Luria broth

containing 1.5% (w/v) bactoagar) containing 250 mg / L ampicillin, 0.3 mg / L IPTG, 66 mg / L x-gal and incubated at 37 °C overnight. Single white transformed colonies were removed with sterile tooth picks and grown in 10 ml of 20 µg/ml ampicillin containing Luria broth. The cells were stored frozen in 15% (v/v) glycerol at -80 °C according to the method of Sambrook *et al.* [1989].

3.41. Plasmid DNA isolation.

Plasmid DNA was isolated using a Wizard™ mini-prep plasmid DNA isolation kit (Promega, Southampton, U.K.) according to the manufacturers instructions. The isolated DNA was stored frozen at -20 °C.

3.42. DNA sequencing.

Sequencing was performed by Julia Bartley (Department of Biological Sciences, University of Durham, U.K.) using the dideoxy terminator chemistry method [Sanger *et al.* 1977] using fluorescent dye-linked M13 forward and reverse primers. The reaction was performed using a Perkin Elmer thermocycler 1 (Warrington, U.K.) using AmpliTaq®DNA polymerase, FS (Perkin Elmer, Warrington, U.K.).

4. RESULTS AND DISCUSSION.

Oxalate oxidase expression in transgenic oilseed rape and tobacco.

Experiments were carried out on transgenic 3S1 oilseed rape and C26 tobacco expressing the extensin-oxalate oxidase chimeric gene (*N. plumbaginifolia* extensin signal peptide and barley root oxalate oxidase mature open reading frame) and transgenic SGS5 tobacco expressing the wheat embryo oxalate oxidase (gf-2.8) gene (wheat embryo oxalate oxidase signal peptide and mature open reading frame). Thompson *et al.* [1995] carried out segregation analysis on the 3S1 oilseed rape plants taking them through three generations to produce homozygous plants containing a single transgenic locus. Basic oxalate oxidase enzyme assays were performed to confirm the expression of the extensin-oxalate oxidase gene in the transgenic plants. The experiments described in this chapter further expanded upon the experiments performed by Thompson *et al.* [1995] and they included the two transgenic (T1 heterozygous) tobacco lines (C26 and SGS5). Experiments were carried out to confirm expression of the oxalate oxidase genes, to monitor expression during growth and development and to determine expression levels in different organs of the transgenic plants. Leaves from non-transgenic and transgenic oilseed rape and tobacco plants were also treated with oxalic acid to see if the expression of active oxalate oxidase in the transgenic plants provided protection against the effect of oxalic acid (the primary phytotoxin produced by the fungal pathogen *S. sclerotinia*) compared with non-transgenic plants. The results of these experiments are described in this chapter.

Plants grown from seeds of the three transgenic lines grew to produce healthy plants which matured, flowered and set seed under the conditions described in section 3.1. When grown under high intensity light conditions transgenic 3S1 oilseed rape grew at the same rate and formed mature plants indistinguishable from non-transgenic (control) plants, in terms of size, rate of maturation and onset of flowering (transgenic and non-transgenic seeds were of the cultivar Westar). The two transgenic (C26 and SGS5) and the non-transgenic tobacco plants exhibited slight morphological differences (i.e. plant and leaf size and shape). This was due to the different tobacco cultivars (samson and xanthi) which were studied rather than as a result of the introduction and expression of the transgenes. Leaves of mature plants were the experimental material of choice, unless stated otherwise.

4.1. Verification of the presence of the extensin-oxalate oxidase chimeric gene in transgenic 3S1 oilseed rape.

DNA isolated using the CTAB method as described in section 3.31. provided a high yield of good quality genomic DNA from oilseed rape leaves. Fig. 4.1. lane C illustrates the restriction endonuclease analysis and isolation of the KpnI extensin-oxalate oxidase chimeric gene (KpnI-EXTOX) and the linearised cloning vector pNEXTOX (lane C, KpnI-pNEXTOX). Fig. 4.1. lane B shows the unrestricted pNEXTOX cloning vector in its circularised form. Fig. 4.2. confirmed the presence of the extensin-oxalate oxidase chimeric gene in transgenic 3S1 oilseed rape plants as detected using the ^{32}P-KpnI extensin-oxalate oxidase labelled probe

Figure 4.1. Analysis of pNEXTOX plasmid DNA isolated from transformed DH5α *E. coli* cells.

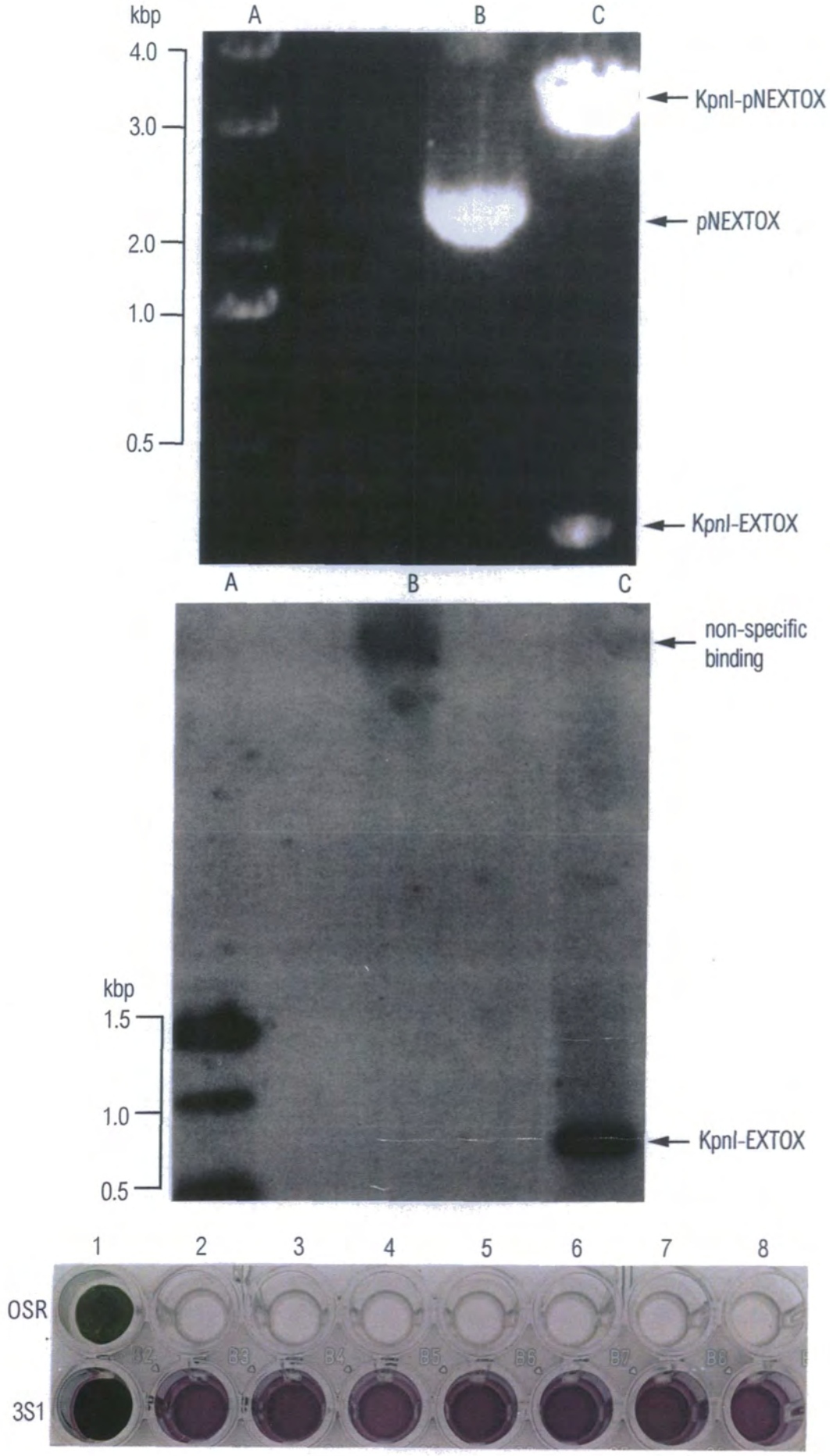
Ethidium bromide stained 0.7% (w/v) agarose gel of A, 1 kb lambda marker DNA; B, undigested pNEXTOX plasmid DNA; C, KpnI digest of pNEXTOX plasmid DNA. 10 µl of restriction digest samples were loaded into each well of the agarose gel. The arrows indicate the undigested plasmid DNA (pNEXTOX), KpnI digested plasmid DNA (KpnI-pNEXTOX) and the 771 bp extensin-oxalate oxidase chimeric gene (KpnI-EXTOX).

Figure 4.2. Detection of the extensin-oxalate oxidase chimeric gene in oilseed rape genomic DNA.

Autoradiograph of a Southern blot probed with the ^{32}P-labelled extensin-oxalate oxidase KpnI restriction fragment. A, 1 kb lambda marker DNA; B, KpnI restriction digest of non-transgenic oilseed rape leaf genomic DNA; C, KpnI restriction digest of 3S1 transgenic oilseed rape leaf genomic DNA. 10 µl of restriction digest samples were loaded into each well of a 0.7% (w/v) agarose gel. The DNA was transferred onto a nylon membrane and Southern blotted as described in section 3.38. The filter was washed to a final stringency of 1 x SSC and 0.1% (w/v) SDS at 65 °C for 15 min and exposed to x-ray film at -80 °C for 10 hr. The arrows indicate non-specific binding of the ^{32}P-labelled probe to high molecular weight DNA and the extensin-oxalate oxidase chimeric gene (KpnI-EXTOX).

Figure 4.3. Leaf disk oxalate oxidase enzyme assay.

Eight 4.5 mm leaf disks of non-transgenic (OSR) and transgenic oilseed rape (3S1) were assayed for oxalate oxidase activity using the microtitre plate leaf disk enzyme assay, as described in section 3.12. The wells of lane 1 still contain the intact leaf disks.


kbp
A
B
C
4.0
3.0
2.0
1.0
0.5
KpnI-pNEXTOX
pNEXTOX
KpnI-EXTOX
A
B
C
non-specific
binding
kbp
1.5
1.0
0.5
KpnI-EXTOX
1
2
3
4
5
6
7
8
OSR
3S1

(Fig. 4.1. lane C KpnI-EXTOX). Lane C contained the 771 bp extensin-oxalate oxidase chimeric gene, which was excised from 3S1 oilseed rape genomic DNA by restriction endonuclease digestion at the two KpnI sites that flank the extensin-oxalate oxidase sequence (See Fig. 2.2.). The oxalate oxidase gene sequence was absent from non-transgenic oilseed rape genomic DNA (digested with KpnI), however the KpnI probe appeared to hybridise to gene sequences in the DNA sample (lane B). This was originally thought to have resulted from non-specific binding, but it was later demonstrated that the probe probably hybridised to gene sequences encoding germin-like proteins (i.e. genes with significant levels of DNA sequence homology to oxalate oxidase, See chapter 10 for further details).

4.2. Confirming the expression of active oxalate oxidase in transgenic oilseed rape and tobacco.

The Southern blot shown in Fig 4.2. confirmed the presence of the chimeric gene in transgenic 3S1 oilseed rape. Isolating good quality DNA from tobacco using the CTAB method was more problematic and so assaying for enzyme activity was also used as a means of confirming gene expression and synthesis of active oxalate oxidase in the transgenic plants. The leaf disk enzyme assay method was developed to enable the rapid processing of large numbers of samples. Freshly excised leaf disks (taken from leaves at the same stage of growth) of mature plants were treated as described in section 3.12. The disks had a diameter of about 4.5 mm which fitted neatly into the bottom of the microtitre plate well without being folded or damaged (Fig. 4.3. lane 1). The disks were isolated from leaves using a stationary hole punch; cork borers were found to cause too much damage to the excised leaf disks. The use of the hole punch also ensured that the disks were always isolated from approximately 8 mm from the edge of the leaf. If the disks became folded or crushed during isolation they were discarded. It was important that excised leaf disks did not contain any large leaf veins, which tended to result in elevated activity, presumably as a result of the presence of oxalate oxidase in the vascular tissue, which may have been accessible to the assay substrate. Only the circumference of the leaf disk was exposed to the substrate to ensure that the method was reasonably quantitative between different samples. The microtitre plate was rapid shaken during the incubation period (900 rpm) to ensure that the edges of the disks were constantly in contact with the oxalic acid (substrate) solution. It was possible to obtain a rough determination of the levels of oxalate oxidase activity in the different transgenic lines using the leaf disk assay. Fig. 4.3. shows an example of the leaf disk enzyme assay for non-transgenic and transgenic 3S1 oilseed rape. Non-transgenic oilseed rape and tobacco plants were shown to lack any detectable endogenous oxalate oxidase activity when assayed on several separate occasions, even with increased assay incubation times (up to 3 hr). Therefore any detectable oxalate oxidase activity in the transgenic plants was deemed to have resulted from the expression of the introduced oxalate oxidase transgene.

The transgenic 3S1 oilseed rape was segregated and homozygous for the transgene and all the plants grown during the study expressed oxalate oxidase at very similar levels (3.13-3.63 µmol H_2O_2 / min / leaf disk). The 3S1 oilseed rape was shown to contain a single

transgenic locus (as determined from segregation analysis, carried out by Thompson *et al.* [1995] and the seeds supplied were of the T3 generation. The two transgenic tobacco lines (C26 and SGS5) used during this study were not segregated (i.e. they were heterozygous for the oxalate oxidase transgene) and therefore the levels of expression between different plants of these two transgenic lines varied considerable, presumably as a result of variation in the copy number of the oxalate oxidase transgenes in the different plants. The seeds supplied were of the T1 generation. C26 and SGS5 tobacco expressed higher levels of oxalate oxidase (up to about 4.13 and 4.56 μmol H_2O_2 / min / leaf disk, respectively) in mature flowering plants compared with 3S1 oilseed rape. Time constraints prevented the segregation of the T1 generations of the two transgenic tobacco lines used in this work, although it would have been preferable to have studied homozygous tobacco plants known to express similar and high levels of the transgenic proteins.

Leaf disk enzyme assays were used to enable the selection of the highest oxalate oxidase expressing C26 and SGS5 tobacco plants from each set of T1 seeds sown. Selection was not performed on the 3S1 oilseed rape as all the plants sown had similar expression levels. The leaf disk assay also overcame the problem of loss of enzyme activity observed in total protein extracts where levels of activity were often <0.1 μmol H_2O_2 / min / mg leaf dry weight. The loss of activity may have resulted from the release of inhibitors into the extract or as a result of the presence of NaCl in the extraction buffer. Suzuki and Meeuse [1965] had reported the inhibition of moss oxalate oxidase by chloride ions. The effect of NaCl on oxalate oxidase activity is examined in chapter 9. Only tobacco plants with high levels of oxalate oxidase expression were selected for use in future experiments.

4.3. Determining the expression levels and accumulation of oxalate oxidase throughout growth and development in transgenic oilseed rape and tobacco.

Leaf disk enzyme assays carried out on 4 week old transgenic tobacco plants initially demonstrated an apparent lack of any detectable oxalate oxidase expression in C26 tobacco compared with SGS5 tobacco, where high but variable levels of activity were detected (results not shown). Therefore a simple experiment was carried out to monitor the levels of oxalate oxidase activity in the non-transgenic (as negative controls) and the three transgenic plant lines (3S1, C26 and SGS5). Two weeks after germination the oilseed rape and tobacco seedlings were transplanted to 8 cm (diameter) pots in John Innes no. 1 compost and grown under conditions described previously in section 3.1., to promote rapid growth. Tobacco and oilseed rape were found to 'bolt', rapidly mature and flower if the root systems of the plants were confined under these growth conditions.

Fig. 4.4. illustrates enzyme activity detected in the transgenic plants monitored over a twelve week period from the point when the plants began active growth after transplantation (4 weeks after germination) through to flowering (12 weeks after germination). No oxalate oxidase activity was detected in any of the non-transgenic oilseed rape or tobacco plants during the course of the experiment. Four weeks after germination, no oxalate oxidase activity was detected in the 3S1 oilseed rape or C26 tobacco (both expressing the extensin-oxalate

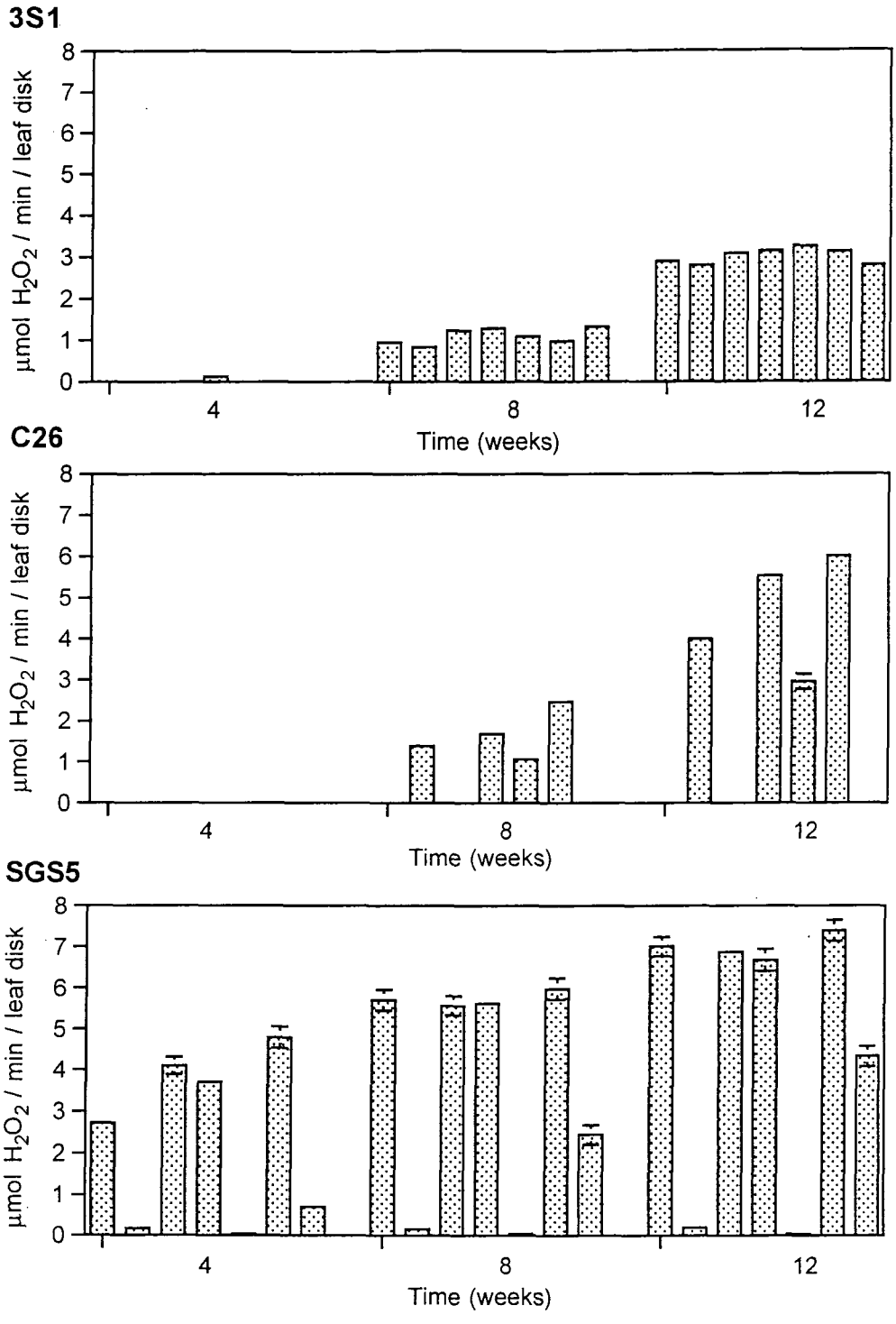


Figure 4.4. Oxalate oxidase expression throughout growth and development in the transgenic plants.
Leaf disk enzyme assays carried out on leaf disks isolated from 7 individual transgenic oilseed rape and tobacco plants, as described in section 3.12. Three leaf disks were isolated from leaves at the same nodal position on each of the selected plants. 3S1 oilseed rape, C26 tobacco and SGS5 tobacco. 4, 8 and 12 weeks after germination. Error bars show mean ± standard errors.

oxidase gene construct) (Fig. 4.4. 3S1 and C26), Activity was detected in one of the 3S1 plants (about 0.120 µmol H_2O_2 / min/ leaf disk), but this was barely above background levels. In contrast, four week old SGS5 tobacco plants (containing the wheat oxalate oxidase gene construct) exhibited much higher activity, for example one of the plants exhibited activity of 4.8 µmol H_2O_2 / min / leaf disk (Fig. 4.4. SGS5). However, the activity was quite variable due to the use of plants germinated from unsegregated seeds of the T1 generation. Eight weeks after germination the oilseed rape and tobacco plants were growing rapidly and oxalate oxidase was detected in all three of the transgenic plant lines (Fig. 4.4. 3S1, C26, and SGS5). Levels of activity in 3S1 oilseed rape were similar between the seven plants tested (0.82 - 1.32 µmol H_2O_2 / min / leaf disk). Only four of the seven C26 plants contained detectable levels of oxalate oxidase (1.07 - 2.46 µmol H_2O_2 / min / leaf disk). Although the C26 'positive' plants exhibited considerably higher activity on average than the 3S1 plants which were expressing the same chimeric gene. The levels of oxalate oxidase activity in the SGS5 tobacco plants had on average (for the seven plants) increased by 36% compared with the levels of enzyme activity detected in the same plants at week four. By week twelve activity in the 3S1 plants (Fig. 4.4. 3S1) had increased by 64% and the levels of activity in the seven C26 (Fig. 4.4. C26) and SGS5 (Fig 4.4. SGS5) tobacco plants increased by 64% and 22% compared with oxalate oxidase activity in same plants at week eight, respectively. If the tobacco plants with activities of < 0.2 µmol H_2O_2 / min / leaf disk were removed from the calculations then the flowering 3S1, C26 and SGS5 plants had activities in the region of 3.00, 4.62 and 6.49 µmol H_2O_2 / min / leaf disk, respectively. The highest activity was detected in SGS5 tobacco, which was 54% and 39% higher than 3S1 oilseed rape and C26 tobacco, respectively.

Not only were the SGS5 transgenic tobacco plants exhibiting the highest levels of oxalate oxidase they also expressed detectable levels of the enzyme at an earlier stage of growth compared with the transgenic 3S1 oilseed rape and C26 tobacco plants (Fig. 4.4.). The SGS5 transgenic tobacco plants were expressing an oxalate oxidase enzyme with superior kinetic properties compared with the oxalate oxidases expressed in 3S1 oilseed rape and tobacco, which may explain the higher levels of activity observed in this transgenic tobacco line (see chapter 9 for enzyme kinetics). Oxalate oxidase activity in the transgenic plants expressing detectable levels of the enzyme increased in the leaves over time, which may have resulted from the increased expression of the transgenes in the mature leaves. Stefanov *et al.* [1994] reported increased expression of the β-glucuronidase gene (GUS) in mature leaves of oilseed rape containing a CaMV 35S-GUS construct. The younger leaves in the upper parts of the transformed plants exhibited lower GUS expression [Stefanov *et al.* 1994]. The increase in activity observed in the leaves over time may have resulted from accumulation of oxalate oxidase in the mature leaves of the transgenic plants. Immunoassays were carried out on leaf extracts of the three transgenic lines to determine if the levels of enzyme activity observed were related to the levels of enzyme present in the transgenic leaves.

4.4. Immunological detection of oxalate oxidase levels in transgenic oilseed rape and tobacco.

Figure 4.5. illustrates the levels of oxalate oxidase protein detected in leaves from mature flowering plants of the three transgenic lines (3S1, C26 and SGS5). The C26 and SGS5 tobacco plants were selected on the basis of high oxalate oxidase activity as determined from leaf disk assays. No detectable levels of oxalate oxidase (i.e. above background levels on the autoradiograph) were observed in non-transgenic oilseed rape or tobacco plants, as determined using dot blots of total leaf protein extracts and immunological assays with anti-oxalate oxidase antibodies, as described in sections 3.18. and 3.19.1. The levels of oxalate oxidase detected were related to the levels of total protein recovered per gram of leaf dry weight (calculated using Bradford assays). In 3S1 oilseed rape, oxalate oxidase constituted about 0.84% of the total protein compared with the two transgenic tobacco lines (C26 and SGS5) where oxalate oxidase constituted about 2.2 and 2.7% of the total protein, respectively. Detergents were not used in the preparation of the total extracts as these interfere with the Bradford assay. Levels of oxalate oxidase in SGS5 tobacco were over 3 fold higher compared with levels in 3S1 oilseed rape. Fig. 4.4. had shown that the level of enzyme activity in mature SGS5 tobacco leaves (week 12) was over twice that observed in 3S1 oilseed rape, presumably resulting from the presence of higher levels of the transgenic enzyme in SGS5 tobacco leaves.

4.5. The distribution of oxalate oxidase expression and accumulation in different organs of transgenic oilseed rape and tobacco.

The distribution of oxalate oxidase transgene expression (controlled by the CaMV 35S promoter) and accumulation of oxalate oxidase in different organs of the transgenic plants was examined in fresh homogenates of 3S1 oilseed rape, C26 and SGS5 tobacco (Fig. 4.6.). Total protein extracts were prepared from roots, stems, leaves and petals harvested from mature flowering plants using extraction buffer containing 400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5, 1 % (w/v) SDS. SDS was added to the extraction buffer as the levels of oxalate oxidase in the extracts were to be determined from autoradiographs of immunoassays and not enzyme activity assays. Stems and leaves were isolated from the same nodal regions of the different plants, to ensure the organs were at comparable stages of development. The petals of fully opened flowers were isolated and young roots were taken from the edges of the root mass. Estimates of the dry weights of all of the organ materials were determined separately by freeze drying, as described in section 3.4. Protein determination was not performed on the samples because of the use of SDS in the extraction buffer, which interferes with the Bradford assay. The organ total protein extracts (from roots, stems, leaves and petals) and serial dilutions of purified barley root oxalate oxidase were applied onto a nitrocellulose membrane using the dot blotting technique described in section 3.18., followed by immunological assays with anti-oxalate oxidase antibodies for the detection of oxalate oxidase. The density of the dots on the autoradiographs were calculated using a Bio-Rad Gel Doc 1000 system with a GS690 imaging densitometer and molecular analyst 2D-gel data processing software, as

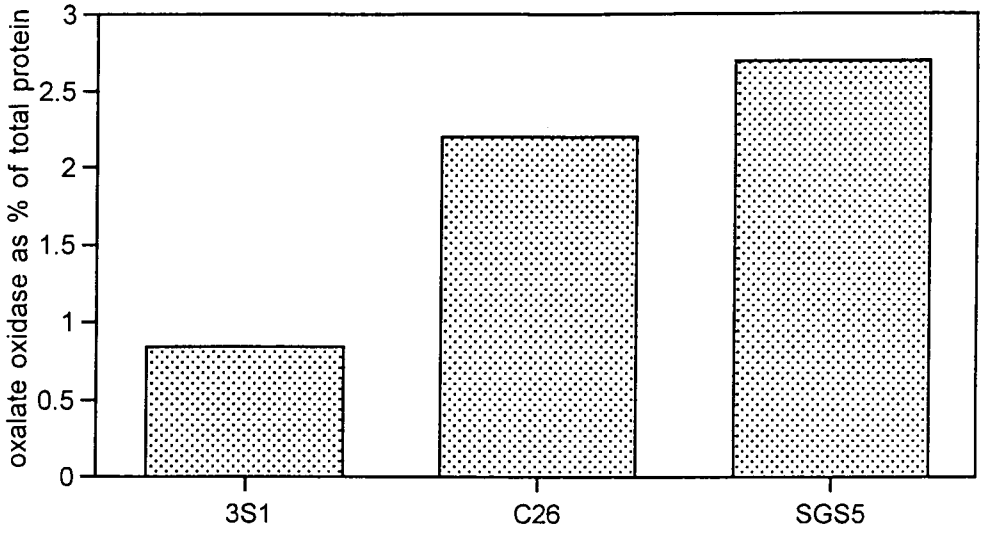


Figure 4.5. Immunoassays of oxalate oxidase expression in mature transgenic leaves.
Total protein extracts were prepared from leaf material isolated from mature, flowering plants of 3S1 oilseed rape, C26 and SGS5 tobacco using extraction buffer (400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5). Three replicates of each extract were dot blotted onto a nitrocellulose membrane. Anti-oxalate oxidase antiserum was used as the primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. Oxalate oxidase protein concentration was related to the total protein concentration per g leaf dry weight.

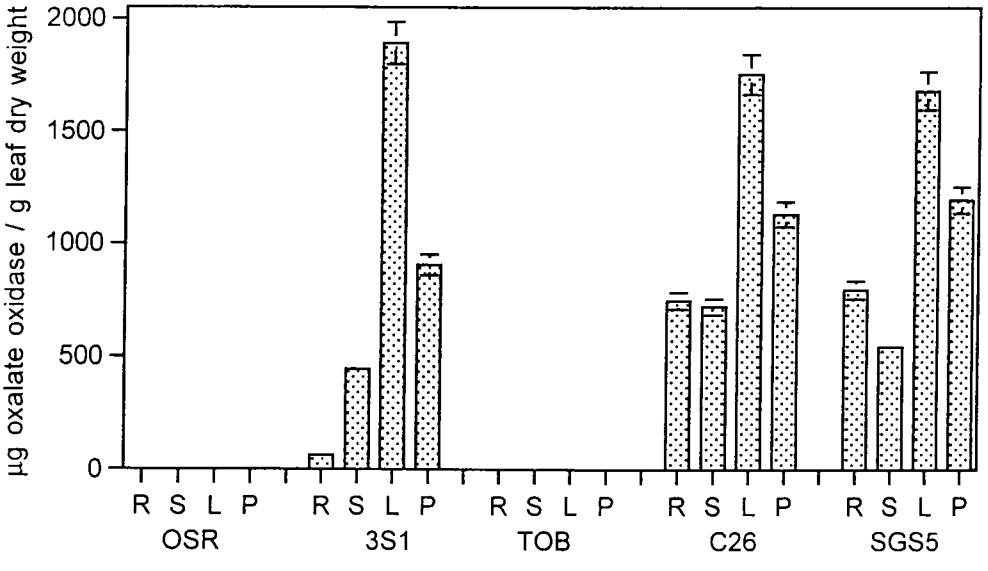


Figure 4.6. Distribution of oxalate oxidase expression in different transgenic plant organs as determined by immunoassays.
Oxalate oxidase concentrations in roots (R), stems (S), leaves (L), and petals (P) were determined from dot blots (3 replicates) of total protein extracts of non-transgenic oilseed rape (OSR) and tobacco (TOB) and transgenic 3S1, C26 and SGS5 mature plants. Extracts were prepared using extraction buffer (400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5, 1% (w/v) SDS). Anti-oxalate oxidase antiserum was used as the primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antbodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. Densitometry of the autoradigraphs was carried out as described in section 3.30. Error bars show mean ± standard errors.

described in section 3.30. The densitometry data was then converted using a spreadsheet to µg of oxalate oxidase from standard curve densitometry data generated from known concentrations of barley root oxalate oxidase dilutions. The dot density values were then converted to µg of oxalate oxidase / g dry weight of plant material to allow for a more quantitative comparison of protein distribution between the four main plant organs and the two plant species studied (oilseed rape and tobacco).

No detectable levels of oxalate oxidase were observed in any of the non-transgenic oilseed rape or tobacco extracts (Fig. 4.6. OSR and TOB, respectively). Therefore any oxalate oxidase detected in the transgenic plant organs was assumed to have resulted from the expression of the oxalate oxidase transgenes. All three of the transgenic plant lines exhibited highest levels of oxalate oxidase expression in the leaves (1681-1892 µg / g dry weight of plant material). C26 and SGS5 tobacco petals expressed similar levels of oxalate oxidase which represented about 65% of the expression levels measured in the leaves. The level of expression in 3S1 oilseed rape petals was about 48% of that observed in 3S1 leaves. Expression in C26 tobacco roots and stems were very similar (about 700 µg / g dry weight representing 42% of leaf expression). The difference in expression between SGS5 tobacco roots and stems was more significant (about 47% and 32% of leaf expression, respectively). The most significant difference in oxalate oxidase organ expression was seen in 3S1 oilseed rape stems (24% of leaf expression) and roots which expressed less than 4% of the oxalate oxidase detected in leaves. The CaMV 35S promoter was constitutive in the sense that the oxalate oxidase genes were expressed in all organs tested albeit at different levels. These results were in agreement with Stefanov *et al.* [1994] were considerable variation was observed in the expression of GUS under the control of the CaMV 35S promoter between organs and tissues at various developmental stages in transgenic oilseed rape. The significance of oxalate oxidase expression levels in the various organs of the transgenic plants with regard to improved fungal pathogen resistance will be discussed in chapter 11.

4.6. The effect of incubating non-transgenic and transgenic leaves of oilseed rape and tobacco in oxalic acid.

This experiment was performed to determine if the active oxalate oxidase expressed and accumulated in the transgenic plants provided improved protection against the symptoms of exposure to oxalic acid, compared with the non-transgenic plants. Oxalic acid is the initial phytotoxin produced by *S. sclerotiorum* during infection. It was hoped that the expression of oxalate oxidase in the transgenic plants would reduce the damaging effect of exposure to oxalic acid. Fig. 4.7. and 4.8. demonstrate the effect of incubating non-transgenic and transgenic excised leaves in distilled water and increasing concentrations of oxalic acid. The experimental procedure performed by Thompson *et al.* [1995] was repeated to include higher oxalic acid concentrations and the transgenic tobacco plant lines (C26 and SGS5). Excised leaves from non-transgenic and transgenic oilseed rape (3S1) and tobacco (C26 and SGS5) plants were incubated in oxalic acid solutions.

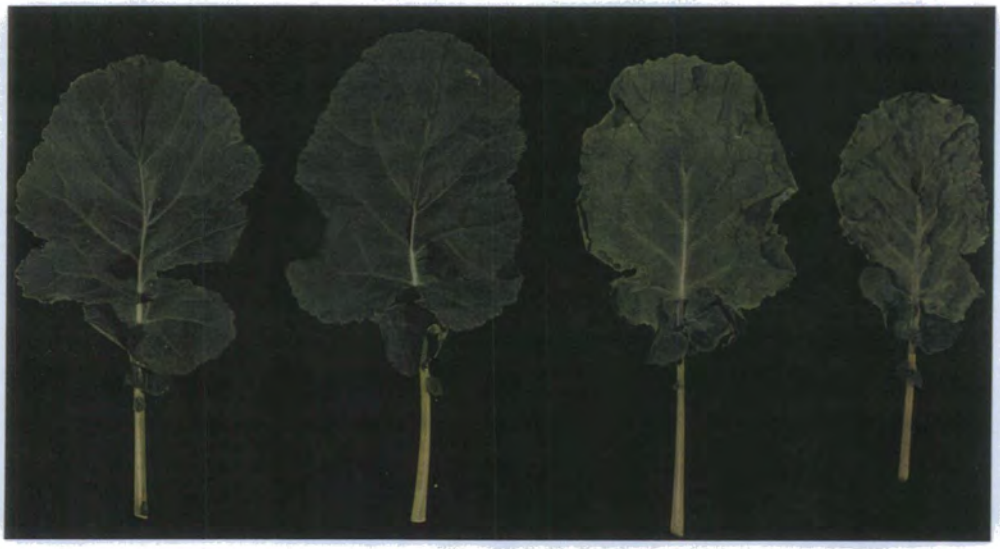
Figure 4.7. Effect of oxalic acid on excised oilseed rape leaves.

Identical intact leaves were excised from mature non-transgenic oilseed rape and transgenic 3S1 oilseed rape and incubated at 22 °C for 24 hr in distilled water at pH 4.0 (0) or in 25, 50 or 100 mM oxalic acid at pH 4.0.

Non-transgenic

Oxalic acid (mM)

0 25 50 100



3S1

Oxalic acid (mM)

0 25 50 100

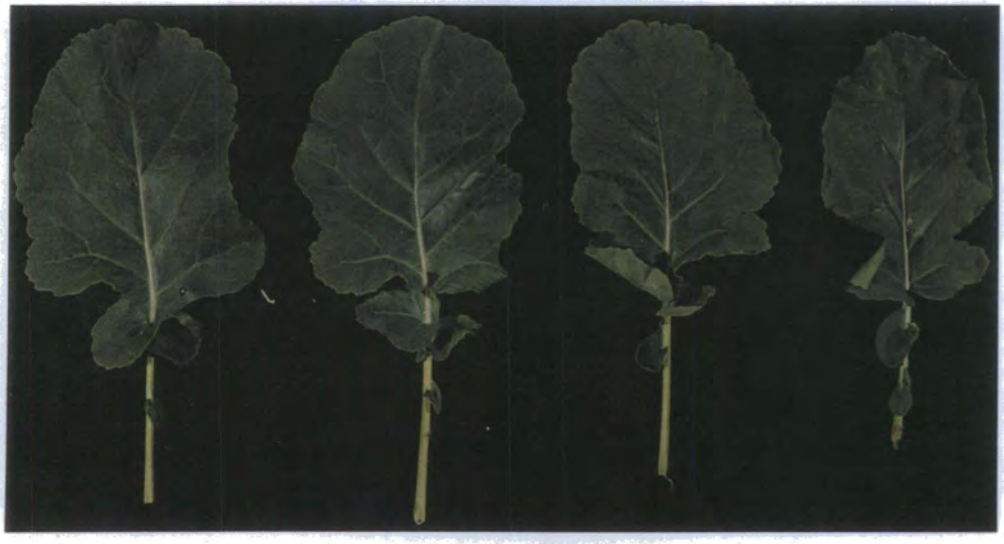


Figure 4.8. Effect of oxalic acid on excised tobacco leaves.

Identical intact leaves were excised from mature non-transgenic tobacco; C26 transgenic tobacco, and SGS5 transgenic tobacco plants and incubated at 22 °C for 24 hr in distilled water at pH 4.0 (0) or in 25, 50 or 100 mM oxalic acid at pH 4.0.

Non-transgenic

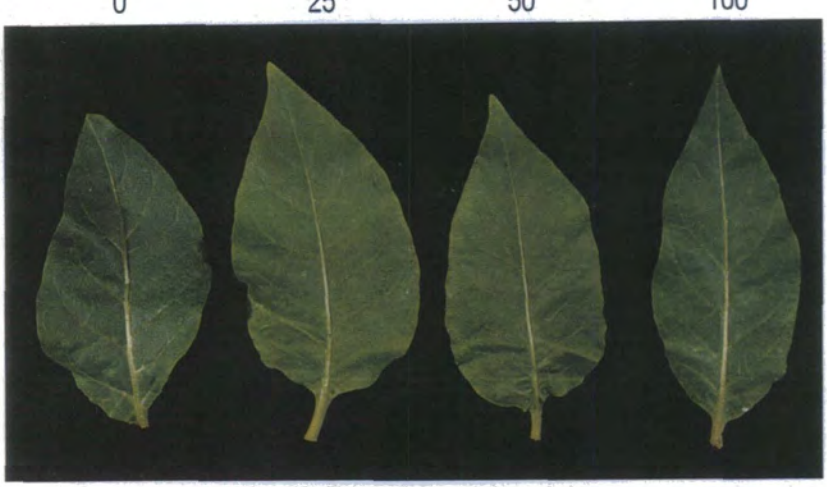
Oxalic acid (mM)

0 25 50 100

C26

Oxalic acid (mM)

0 25 50 100



SGS5

Oxalic acid (mM)

0 25 50 100

Photosynthetically active leaves were isolated from transgenic and non-transgenic plants. The leaves were of a similar age and isolated from the same nodal position of the mature plants. Tobacco leaves were selected from plants which were previously identified as expressing similar and high levels of oxalate oxidase, using leaf disk enzyme assays. The stems were cut under water and the petioles of the excised leaves were placed into water (pH 4) as a control or 25, 50, or 100 mM oxalic acid pH 4 and incubated at room temperature for 24 hr, before being examined for signs of dehydration, wilting and necrosis. The degree of tissue damage was measured using a grid of 5 mm^2 squares, if more than half of a square contained damaged material then it was counted and the percentage of wilted and/or necrotic tissue was calculated.

When incubated in distilled water adjusted to pH 4.0 the non-transgenic (Fig. 4.7. and 4.8. non-transgenic 0 mM) and transgenic (Fig. 4.7. 3S1 0 mM, and Fig. 4.8. C26 0 mM and SGS5 0 mM) oilseed rape and tobacco leaves were unaffected by the treatment and appeared healthy, with no signs of wilting or necrosis. In 25 mM oxalic acid the non-transgenic oilseed rape leaf showed signs of significant dehydration and necrosis of the veins around the edge of the leaf and approximately 30% wilting around the leaf edges (Fig. 4.7. non-transgenic 25 mM). In 50 mM oxalic acid the entire leaf had wilted and approximately 80% of the leaf veins were dehydrated and had turned brown (Fig. 4.7. non-transgenic 50 mM). Complete desiccation of the leaf occurred in 100 mM oxalic acid and the leaf disintegrated under light pressure (Fig 4.7. 100 mM). When oxalic acid enters the leaf it collates calcium ions in the middle lamella which results in embolism in the xylem. Air then enters the xylem and the leaves wilt. Wilting may also be due to the formation of oxalate crystals which blocked water movement in the xylem as a result of oxalic acid uptake into the leaves. The severity of symptoms observed were related to the concentration of oxalic acid to which the leaves were exposed. The 3S1 transgenic oilseed rape leaf expressing high levels of oxalate oxidase was unaffected by 25 mM oxalic acid (Fig 4.7. 3S1 25 mM) but showed signs of approximately 20% wilting around the leaf edges, but no dehydration of the leaf veins in 50 mM oxalic acid (Fig. 4.7. 3S1 50 mM). The leaf incubated in 100 mM oxalic acid exhibited approximately 50% wilting and dehydration but no necrosis of the veins around the leaf edges (Fig. 4.7. 3S1 100 mM).

The non-transgenic tobacco appeared to be unaffected when incubated in 25 and 50 mM oxalic acid (Fig. 4.8. non-transgenic 25 and 50 mM). Even in 100 mM oxalic acid (Fig. 4.8. non-transgenic 100 mM) there were only signs of very slight necrosis of the small leaf veins around the edges of the leaf. Oxalic acid treatment of C26 tobacco (Fig. 4.8. C26 25, 50 and 100 mM) and SGS5 tobacco (Fig. 4.8. SGS5 25, 50 and 100 mM) had no affect on the leaf tissue even at the highest concentration tested (100 mM, Fig 4.8. C26 and SGS5 100 mM). This assay was repeated on three separate occasions (with 3 replicates per treatment) and gave the same results each time. In fact the transgenic tobacco leaves (C26 and SGS5) showed no signs of dehydration or wilting when the leaves were incubated for 3 days (under standard growth conditions, section 3.1) in 25, 50 or 100 mM oxalic acid.

This experiment demonstrated that it was not the low pH which led to the dehydration and wilting observed but the presence specifically of oxalic acid, since none of the leaves incubated in distilled water (pH 4.0) were affected by the low pH treatment (Fig. 4.7. and 4.8. non-transgenic 0 mM). Expression of the transgene in 3S1 oilseed rape appeared to significantly improve the plants ability to withstand exposure to oxalic acid, confirming the results of Thompson *et al.* [1995]. The oxalic acid treatment did not have any detectable affect on tobacco (non-transgenic or transgenic), which implied that tobacco was already equipped with mechanisms capable of coping with oxalic acid. Throughout this study no endogenous oxalate oxidase activity was ever detected in tobacco. Tobacco may contain oxalate decarboxylase capable of converting oxalic acid to CO_2 and formate although there are no recent reports of the discovery of this enzyme in tobacco. There may be other mechanisms in tobacco capable of degrading oxalic acid. For example, Giovanelli and Tobin [1964] suggested that oxalic acid underwent decarboxylation via a thiokinase reaction to form CO_2 and formate in peas. Another possibility was that the oxalic acid reacted with Ca^{2+} to form insoluble calcium oxalate crystals in specialised crystal idioblast cells found in tobacco. The insoluble form of oxalic acid was not toxic to the plant. There are no reports of crystal idioblasts in oilseed rape and these results illustrated the plants (non-transgenic) inability to withstand oxalic acid treatment. The relevance of this experiment to fungal pathogen resistance will be discussed in chapter 11.

5. RESULTS AND DISCUSSION.

Oxalate oxidase localisation in transgenic oilseed rape and tobacco.

Lane *et al.* [1986, 1992] used subcellular fractionation and immunological techniques to demonstrate the association of oxalate oxidase with the cell walls of germinated wheat embryos. Approximately 40% of total wheat embryo oxalate oxidase was shown to be associated with the apoplast in the wall bound form. Two oxalate oxidase isoforms were identified in wheat embryos, the G' isoform (oligomer M_r about 120 kDa) was found to be released by washing germinated embryos in an aqueous buffer compared with G (oligomer Mr about 136 kDa) which was the dominant species in total protein extracts [Lane *et al.* 1986; McCubbins *et al.* 1987]. The two oxalate oxidase isoforms (G and G') have the same apoprotein but are differentially glycosylated [Lane *et al.* 1986, 1987; Jaikaran *et al.* 1990]. The ability to isolate oxalate oxidase simply by washing germinated wheat embryos in an aqueous buffer suggested that some of the protein was located in the extracellular spaces or even on the surfaces of the germinated embryos. Berna and Bernier [1997] discovered a similar pattern of oxalate oxidase localisation in transgenic tobacco plants expressing the intact wheat oxalate oxidase gf-2.8 gene under the control of the native wheat gf-2.8 promoter instead of the CaMV 35S promoter used in their SGS5 transgenic tobacco line.

Experiments were carried out to determine the efficiency of various buffers for isolating oxalate oxidase from total leaf homogenates and from leaf extracellular spaces. The vacuum infiltration procedure used for isolating extracellular proteins with minimum intracellular contamination was used as a method for quantifying the levels of oxalate oxidase associated with the extracellular matrix and to facilitate purification. The results of these experiments are described in this chapter.

5.1. The influence of buffer composition on oxalate oxidase isolation from total leaf homogenate of transgenic 3S1 oilseed rape.

Differential total extraction was initially used as a means of determining the chemical components which would achieve optimal yields of total oxalate oxidase if included in the extraction buffer. A 20 g sample of homogenised transgenic 3S1 oilseed rape leaf material was sequentially extracted in the chemical buffers listed in table 3.1. Distilled water washes were included between each of the chemical buffer extractions to remove all of the previous chemical from the leaf material, so that any variation in levels of oxalate oxidase isolated would result from the presence of a single chemical component in the buffer. The different chemicals tested were selected because of their ability to release proteins which interacted with different extracellular matrix components. $CaCl_2$ releases positively charged proteins from negatively charged pectins in the cell walls. EDTA led to the release of chelated and metal ion containing proteins. DTT disrupts proteins which are disulphide bonded to insoluble matrix components. Glycoproteins are released as a result of sodium borate treatment which disrupts complementing between proteins and carbohydrates. The sodium and chloride ions in NaCl competed with protein-protein and protein-matrix interacts resulting in the release of charged

proteins. SDS is a detergent which disrupts protein-lipid complexes, causing the dissociation and release of susceptible proteins.

Fig. 5.1. illustrates the efficiency of each of the chemical buffers tested at releasing oxalate oxidase from the homogenised leaf material. A proportion of oxalate oxidase (in excess of 50% of leaf total oxalate oxidase) was effectively isolated using double distilled water (Fig. 5.1. MQ 1-4). The levels of oxalate oxidase isolated with each subsequent water extraction (MQ 1-4) resulted in the release of progressively lower levels of oxalate oxidase (28.6%, 23.1, 10.0 and 3.7% of total oxalate oxidase, respectively), until no detectable oxalate oxidase was isolated by the fifth water extraction (Fig. 5.1. MQ 5). EDTA (0.1 M) and DTT (0.002 M) were not effective at releasing any detectable levels of oxalate oxidase from the homogenised leaf material. Sodium borate (0.2 M) resulted in the release of about 5.1% of total oxalate oxidase and $CaCl_2$ (200 mM) released about 1% of total oxalate oxidase. NaCl (1 M) was by far the most effective buffer used for the isolation of oxalate oxidase from the leaf material (26.9% of total oxalate oxidase). Only about 1.5% of total oxalate oxidase was released by extracting the leaf homogenate in SDS buffer (0.035 M) (1% (w/v)), which suggested that the majority of oxalate oxidase was released from the leaf material by water and the subsequent NaCl extraction. The high molarity NaCl buffer presumably caused substantial interference of ionic bonds between oxalate oxidase and cellular components, resulting in the release of high levels of the bound protein. The results of this experiment provided information on the extraction of total oxalate oxidase from cell artefacts (i.e. cell walls and membranes), which suggested that the transgenic 3S1 oilseed rape oxalate oxidase protein existed in both free and bound forms which were released most effectively by water or NaCl, respectively. The other chemical buffers tested did not lead to the release of significant levels of total oxalate oxidase from the transgenic 3S1 oilseed rape leaf homogenate. As a result of this experiment NaCl was included in the extraction / infiltration buffers used in all further experiments.

5.2. The use of vacuum infiltration for studying the extracellular location of proteins in plants.

Vacuum infiltration was used for the isolation of intercellular fluid (INF) from oilseed rape and tobacco leaves, which allowed the selective analysis of proteins associated with the extracellular matrix. Initial attempts using the method described by Hammond-Kosack [1992] resulted in the isolation of low yields of oilseed rape leaf INF (5 - 10 µl/2 cm^2 leaf piece). Therefore the method was adapted as described in section 3.6.1. to achieve a more uniform water-logging of the leaves in an attempt to retrieve higher yields of INF. Oilseed rape leaf surfaces repelled aqueous solutions and the leaf pieces tended to associate with the air-buffer interface. To overcome this problem the leaf material was held below the surface of the buffer with wire mesh and a 1 kg weight. However, during evacuation of the leaf pieces air drawn from the extracellular spaces remained associated as small air bubbles with the leaf surfaces. Upon release of the vacuum this air was drawn back into the leaf material resulting in minimal water-logging. Vigorous shaking of the vacuum jar was introduced to dislodge the air bubbles

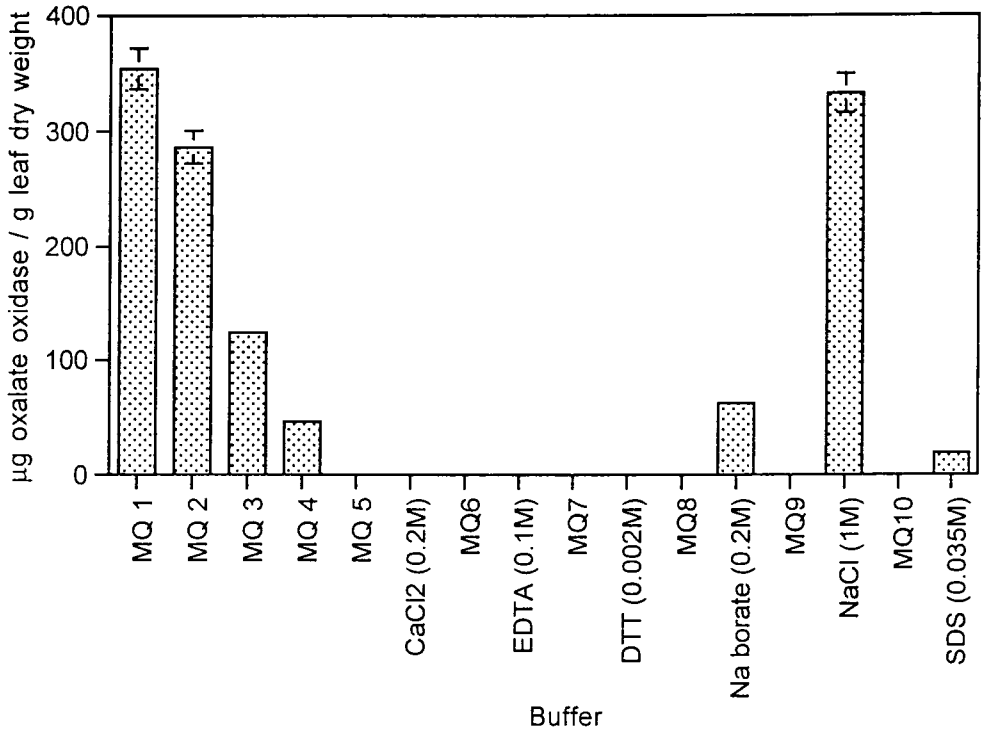


Figure 5.1. Differential extraction of transgenic 3S1 oilseed rape leaf and the detection of oxalate oxidase by immunoassays.

20 g of transgenic 3S1 oilseed rape leaf material was ground in liquid air and sequentially extracted in a series of chemical buffers (concentrations in parentheses) and MilliQ (MQ) quality sterile distilled water (as described in section 3.5.). 3 replicates of each extract (5 µl) were dot blotted onto a nitrocellulose membrane. Anti-oxalate oxidase antiserum was used as the primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. The dot blots were autoradiographed and the film quantifed as described in section 3.30. Error bars show mean ± standard errors.

from the leaf surfaces; this treatment did not appear to have any damaging effects on the leaf material. 30 sec evacuations were found to be quite sufficient to remove all the air from the extracellular spaces rather than the suggested 10 min [Hammond-Kossack 1992]. To achieve complete water-logging of the oilseed rape material the leaf pieces were repeatedly evacuated up to five times. Tobacco leaf pieces were much easier to infiltrate, often only requiring a single 30 sec evacuation to achieve complete water-logging of the extracellular spaces, as the leaf surfaces did not attract the air drawn out from the extracellular spaces.

The centrifugal forces used to retrieve INF were also increased from 865 g to 2,500 g for tobacco and 10,000 g for oilseed rape, resulting in higher yields. When oilseed rape leaf pieces were spun at 10,000 g a clear fluid was recovered with no visible signs of intracellular contamination, i.e. small green pellets in the base of the microfuge tubes, resulting from the release of chloroplasts / chlorophyll into the INF. Husted and Schjoerring [1995] reported that it was possible to isolate uncontaminated (cytosol-free) oilseed rape INF at forces of up to 12,000 g, because of the mechanical strength of the leaf material. The tobacco leaf pieces were spun at a lower speed of 2,500 g, because the material was more easily damaged compared with oilseed rape. However, the yields of INF were significantly higher from tobacco leaf compared with oilseed rape (50-150 µl and 25-50 µl, respectively). The INF isolated from tobacco leaf material had a slight russet colour. An average of 320 µl (oilseed rape) and 550 µl (tobacco) of INF were isolated per gram fresh weight of leaf material using the modified vacuum infiltration method. For oilseed rape this represented a significant increase in INF yield of up to 40% compared with Hammond-Kossack's [1992] method. The INF isolated from the extracellular spaces of oilseed rape and tobacco leaves had pH's of about 5.6-5.8, following infiltration with distilled water, which was comparable with the pH of oilseed rape INF reported by Husted and Schojoerring [1995] (pH 5.8). The pH's of the INF were measured immediately after the centrifugation step.

The yield of extracellular proteins isolated in the INF was too low to be visualised on Coomassie blue stained SDS-PAGE gels and so the proteins were concentrated. This was achieved using one of several methods. Ammonium sulphate precipitation was initially used to concentrate the extracellular proteins in INF. The INF was saturated to 100% (relative saturation) $(NH_4)_2SO_4$ for at least 2 hr at 0 °C, followed by centrifugation at 14,000 g for 30 min at 4 °C. The main problem encountered using this method was the inability to fully resuspend some of the precipitated proteins. Therefore the protein profiles obtained on SDS-PAGE gels may not be a true representation of all of the extracellular proteins isolated in the original INF. Acetone precipitation was found to be an unacceptable method for obtaining a concentrated sample of extracellular proteins particularly if proteins of interest had enzyme activity, as this was often lost during the precipitation process. As with ammonium sulphate precipitation the proteins were sometimes very difficult to resuspend in aqueous buffers or 1 x SDS-PAGE loading buffer. Freeze drying was found to be the most effective method of concentrating the proteins isolated in the INF, following dialysis to remove the low molecular weight materials in the infiltrate (including NaCl in the infiltration buffer). The lyophilised proteins were easily resuspended in water or 1 x SDS-PAGE loading buffer, often in volumes

100 fold lower than that of the original INF. Freeze drying and resuspending the extracellular proteins in such small volumes of buffer meant that the proteins were usually separated well on SDS-PAGE gels and a significant number of proteins were clearly visible following Coomassie blue staining (about 20-30 protein bands). These experiments were carried out to optimise the vacuum infiltration procedure which was subsequently used to confirm the extracellular nature of the oxalate oxidase proteins expressed in the transgenic oilseed rape and tobacco plants. Two methods were then used to confirm that the proteins isolated in INF were selectively isolated from the extracellular spaces free from intracellular contamination.

5.3. The detection of intracellular contamination in leaf INF following isolation by vacuum infiltration.

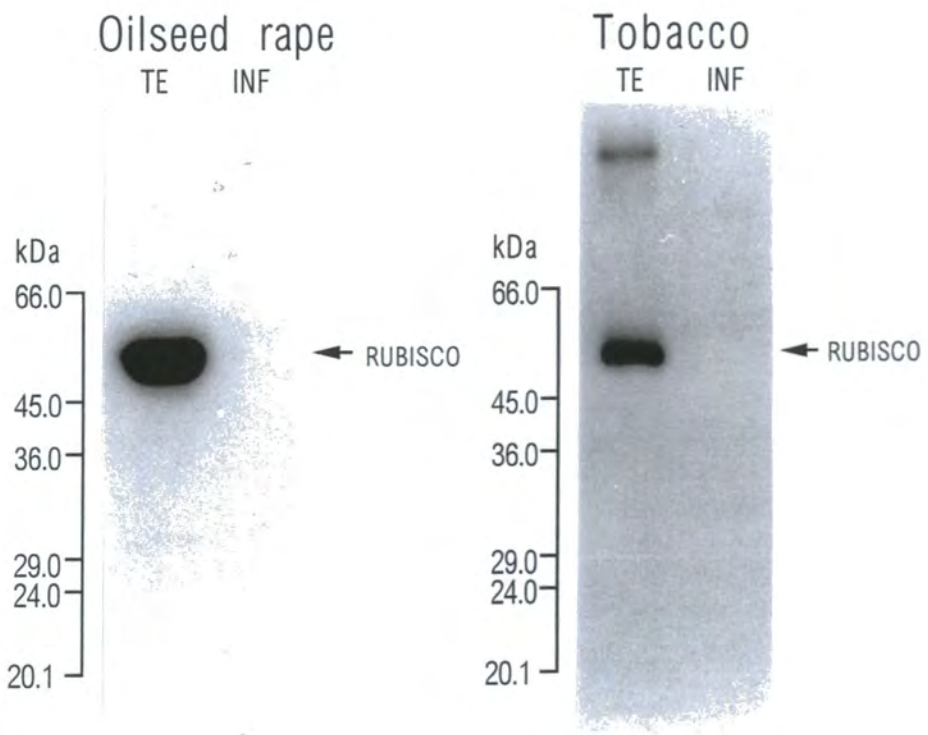
Occasionally a few of the INF samples had a green coloration which was a primary indicator of intracellular contamination, such samples were discarded. This contamination probably resulted from disruption of the cell wall or plasma membrane, possibly as a result of the vacuum applied to the leaf material or the centrifugal force being too high during recovery of the INF. The leaf pieces were handled with care to prevent any ripping or squashing when inserting the material into the microfuge tubes, any leaf pieces which were visibly damaged were always discarded.

The absence of intracellular contaminants from the isolated INF was initially confirmed by immunodetection of chloroplastic material using anti-RUBISCO antibodies, by comparing RUBISCO levels in INF with leaf total protein extracts. RUBISCO was used as an indicator of the leakage of chloroplastic proteins or whole / fragmented chloroplasts into the extracellular spaces. RUBISCO is the most abundant intracellular plant protein constituting about 25% of leaf total proteins, with a molecular weight of about 45-50 kDa. The concentration of RUBISCO in the leaf made it a suitable candidate for monitoring intracellular contamination of INF, since slight damage of the leaf would probably result in the presence of this protein in the INF and its subsequent detection on western blots. The results from Fig 5.2. showed the absence of detectable levels of RUBISCO in the INF samples of non-transgenic oilseed rape and tobacco. RUBISCO was clearly detected by immunodetection in the leaf total protein extracts. However, this procedure was time consuming and so a more rapid and simple enzyme assay technique was used to monitor cytosolic contamination. A method for detecting cytosolic contamination was also more desirable as damage to the integrity of the cell wall / membrane was more likely to have resulted in the leakage of cytosolic compared with chloroplastic contaminants (i.e. RUBISCO) into the INF.

Malate dehydrogenase (MDH) is an abundant cytosolic enzyme which catalyses the oxidation of malate to oxaloacetate using NAD^+ as the oxidising agent. MDH enzyme assays were used to detect intracellular contamination in isolated INF relative to leaf total protein extracts. Levels of MDH activity in INF compared with leaf total protein extract were generally in the region of 1%, which was an acceptable level of contamination [Ting 1968; Husted and Schjoerring 1995]. The modified MDH assay described in section 3.7. was heavily weighted towards the detection of any potential MDH contamination in the INF, because of the addition

Figure 5.2. Analysis of INF for intracellular contamination.

Immunoblots of 12% (w/v) SDS-PAGE gels of INF and total protein extracts (TE) prepared from non-transgenic oilseed rape (OSR) and tobacco (TOB) leaves. The extracellular proteins in 1 ml of INF were concentrated by freeze drying and the lyophilised proteins were resuspended in 10 µl of distilled water. 20 µg of resuspended extracellular proteins and 50 µg of leaf total protein extracts prepared as described in section 3.3. were loaded onto the gel. The gels were electroblotted onto nitrocellulose membranes as described in section 3.17. The immunoblots were probed with anti-RUBISCO antiserum (1:1,000 dilution) as the primary antibodies and ^{125}I-conjugated donkey anti-rabbit antibodies (185 kBq) as secondary antibodies for the detection of RUBISCO. Blots were exposed to x-ray film for 2 weeks at -80 °C. The arrows indicate RUBISCO.


Oilseed rape
TE
INF
kDa
66.0
45.0
36.0
29.0
24.0
20.1
RUBISCO
Tobacco
TE
INF
kDa
66.0
45.0
36.0
29.0
24.0
20.1
RUBISCO

of a larger sample volume of infiltrate compared with the method described by Husted and Schjoerring [1995]. Small leaf veins were always removed from the leaf material to be infiltrated, as reports indicate that their presence in the infiltrated material result in significantly increased MDH activity [Husted and Schojoerring 1995]. Husted and Schojoerring [1995] also demonstrated that MDH activity in oilseed rape INF was independent of centrifugal force in the range 1,000 to 12,000 g and always remained below 1% of that in leaf total protein extracts. Tobacco leaves had lower mechanical strength compared with oilseed rape, which was reflected in the lower centrifugal force used to obtain the INF, in order to prevent increased cellular damage and contamination. Monitoring MDH activity in the INF was shown to be a much more effective and rapid method for monitoring cytosolic contamination compared with screening with anti-RUBISCO antibodies, although both are valid methods for monitoring intracellular contamination of INF.

5.4. Isolation of extracellular oxalate oxidase from transgenic oilseed rape and tobacco leaves.

Vacuum infiltration was used to confirm the extracellular nature of the transgenic oxalate oxidases. Initial experiments were carried out using water as the infiltration medium, which allowed the selective isolation of the transgenic 3S1 oxalate oxidase from oilseed rape leaf extracellular spaces. Fig. 5.3. shows the results of an experiment involving the repeated infiltration of 3S1 oilseed rape leaf pieces (as described in section 3.6.2.), which was carried out to determine how effective the vacuum infiltration technique was for the isolation of oxalate oxidise from leaf extracellular spaces. The levels of oxalate oxidase isolated following each of the four infiltrations were monitored by measuring enzyme activity. The second infiltration led to the recovery of approximately 14.8% (0.044 µmol H_2O_2 / min / g leaf dry weight, Fig 5.3. A and B, INF 2) of the extracellular oxalate oxidase compared with the initial infiltration (76.5 %, 0.228 µmol H_2O_2 / min / g leaf dry weight, Fig 5.3. A and B, INF 1). Approximately 3.4% of extracellular oxalate oxidase was isolated following a fourth infiltration of the leaf pieces (0.011 µmol H_2O_2 / min / g leaf dry weight, Fig 5.3. A and B, INF 4). This experiment illustrated that the majority of soluble extracellular oxalate oxidase was released from water-logged leaves following three repeated infiltrations.

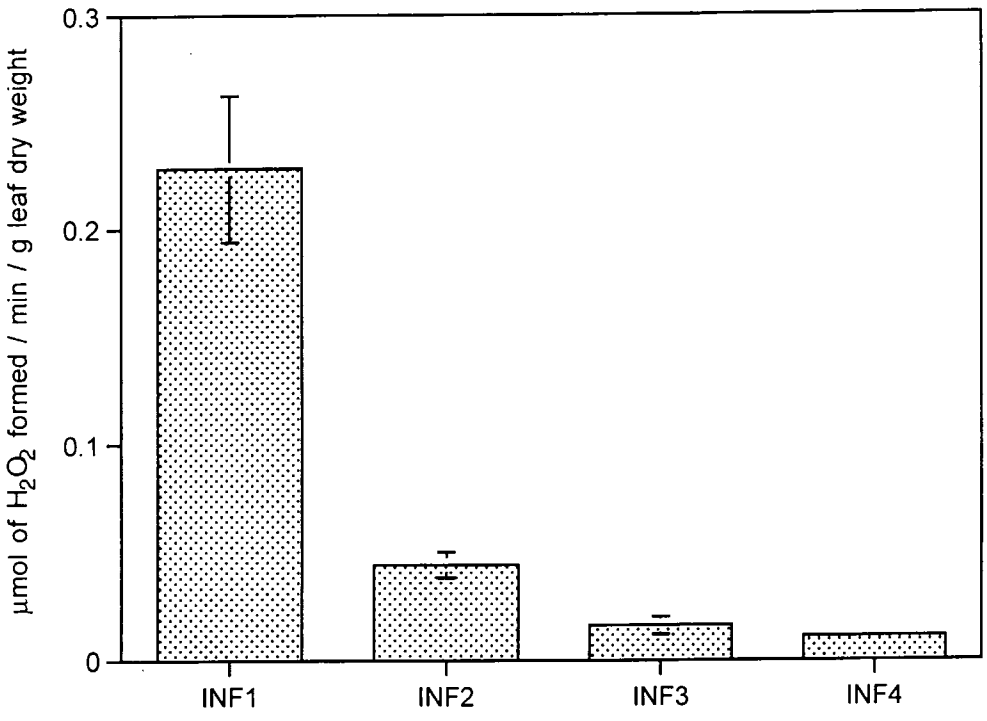
A further vacuum infiltration experiment was carried out with mature transgenic 3S1 oilseed rape leaf pieces to determine the efficiency of oxalate oxidase release from leaf extracellular spaces with different chemical buffers. The efficiency of the vacuum infiltration procedure for isolating extracellular proteins was not only dependant on the plant species, organs, vacuum and centrifugal forces used but also on the composition of the infiltration buffer. Proteins undergo different interactions in the extracellular spaces, for example some proteins may be unassociated or weakly bound and easily isolated by infiltration with distilled water alone, whereas others may undergo more substantial interactions (involving ionic bonds, such as between basic proteins and pectic substances), which require the presence of salts to disrupt these interactions before the proteins can be retrieved in the INF by vacuum infiltration. This experiment used the same extraction buffers used in the differential total protein

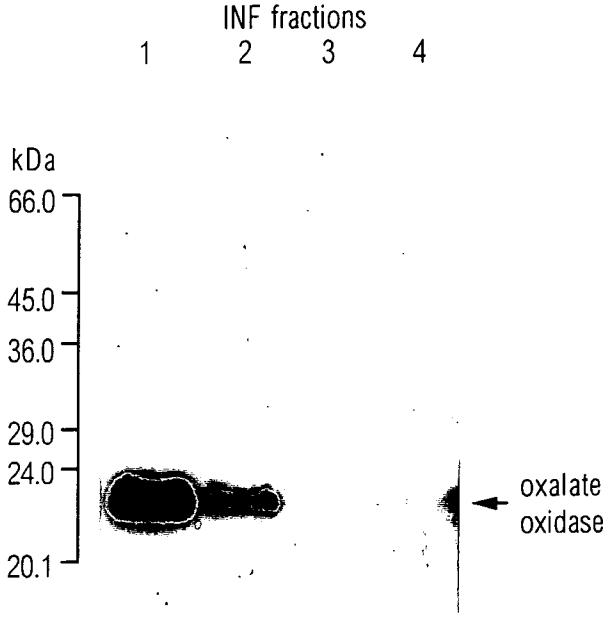
Figure 5.3. Release of extracellular oxalate oxidase by repeated vacuum infiltration of transgenic 3S1 oilseed rape leaves.

Twelve 2 cm^2 pieces of transgenic 3S1 oilseed rape leaves were sequentially infiltrated four times (INF 1-4) with distilled water.

A, 5 µl of each INF sample were assayed for oxalate oxidase enzyme activity using the cuvette enzyme assay method with the Unicam UV2 spectrophotometer, as described in section 3.11.2. The assays were incubated at 37 °C for 60 min. Error bars represent mean ± standard errors.

B, immunoblot of a 12% (w/v) SDS-PAGE gel of INF 1-4. The extracellular proteins in 1 ml of INF were concentrated by freeze drying and the lyophilised proteins were resuspended in 100 µl of distilled water. 10 µl of resuspended extracellular proteins were loaded onto the gel. The gel was electroblotted onto a nitrocellulose membrane, as described in section 3.17. Anti-oxalate oxidase antiserum (1:10,000 dilution) was used as the primary antibodies and ^{125}I-conjugated donkey anti-rabbit antibodies (185 kBq) as secondary antibodies for the detection of oxalate oxidase. Blots were exposed to x-ray film for 2 weeks at -80 °C. The position of the oxalate oxidase protein is indicated with an arrow.


0.3
0.2
0.1
0
µmol of H_2O_2 formed / min / g leaf dry weight
INF1
INF2
INF3
INF4


INF fractions
1
2
3
4
kDa
66.0
45.0
36.0
29.0
24.0
20.1
oxalate
oxidase

extraction method (as described in section 3.6.3.). The only difference was the lower concentration of the NaCl (100-400 mM)), which was reduced from 1 M as this caused the leaf pieces to go flaccid (as a result of osmosis and water loss from the cells), making them difficult to handle and more prone to damage during the vacuum infiltration procedure. Preventing damage to the leaf pieces was essential to reduce the risk of intracellular contamination of the INF. The purpose of performing this experiment was to determine which chemicals were most effective at isolating oxalate oxidase from leaf extracellular spaces. The results of this experiment were important to define which buffer composition was most effective for use in all of the subsequent vacuum infiltration experiments, to obtain a preparation of INF enriched with oxalate oxidase. Fig. 5.4. shows the effect of each of the infiltration buffers on the volumes of INF isolated from equivalent dry weights of leaf material. The majority of the chemical buffers (including water) resulted in the isolation of 0.749 - 0.958 ml / g leaf dry weight. The increased volumes of INF recovered as a result of elevated NaCl concentrations in the infiltration buffer (0.1 - 0.4 M) was probably due to the movement of water out of the cells (by osmosis) and into the extracellular spaces. The increase in the volumes of INF did not resulted from cellular disruption, since the levels of MDH activity in the isolated INFs were not significantly different as a result of using infiltration buffers containing different chemical components and at different concentrations (Fig. 5.5.). The majority of MDH activity in the different infiltrates was below 1% (relative to total extracted MDH), confirming that the protein extracts were selectively isolated from the leaf extracellular spaces. Fig. 5.6. shows the levels of proteins isolated from the extracellular spaces with buffers containing different chemical components. In terms of total extracellular protein isolated, water was the least effective medium; relatively low levels of proteins were also recovered with DTT (0.005 M), EDTA (0.1 M) and sodium borate (0.1 and 0.2 M). Increasing concentrations of NaCl (0.1, 0.2 and 0.4 M) led to the isolation of large amounts of extracellular proteins, probably resulting from increased disruption of ionic bonds between proteins and the extracellular matrix. $CaCl_2$ (0.2 M) was also particularly effective at removing high levels of extracellular proteins for the same reason as NaCl. However, as Fig. 5.7. illustrates $CaCl_2$ was not very effective at isolating extracellular oxalate oxidase. The only infiltration buffers which were effective at isolating extracellular oxalate oxidase contained NaCl. Increasing the concentration of NaCl (0.1 - 0.4 M) led to the isolation of increased levels of oxalate oxidase from leaf extracellular spaces. Fig. 5.8. illustrates the levels of oxalate oxidase isolated from the extracellular spaces with each infiltration buffer in relation to the levels of total extracellular proteins. Interestingly, the use of water alone enabled the isolation of a more highly enriched oxalate oxidase fraction, compared with the infiltration buffers containing DTT, $CaCl_2$, EDTA and sodium borate (Fig 5.8.). However, the highest levels of oxalate oxidase were isolated using 0.1 - 0.4 M NaCl. In the light of these results infiltration buffer containing 0.2 M NaCl and 50 mM Na_2HPO_4, pH 7.5 was used for all subsequent vacuum infiltration experiments where high levels of extracellular oxalate oxidase were to be isolated. Infiltration of leaves with 0.4 M NaCl led to the isolation of marginally higher levels of extracellular oxalate oxidase compared with 0.2 M NaCl, however 0.4 M NaCl made the leaf pieces more flaccid and difficult to handle

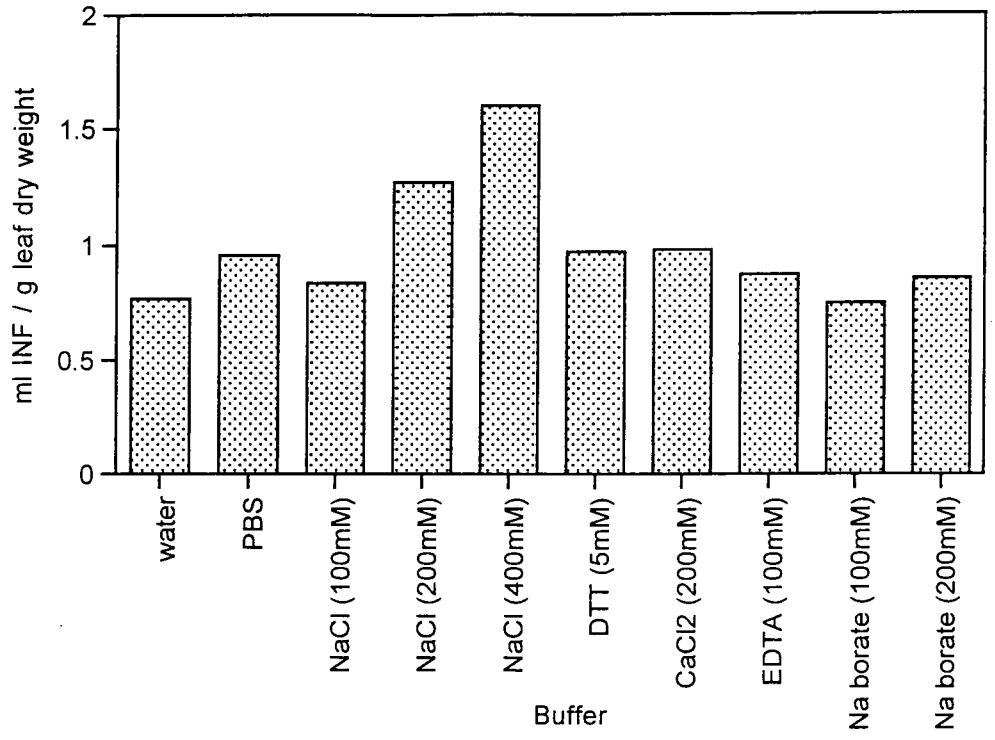


Figure 5.4. Effect of infiltration buffer on the volumes of isolated INF.
Volumes of INF isolated from 3S1 oilseed rape leaves material following vacuum infiltration with different infiltration buffers (concentrations in parentheses).

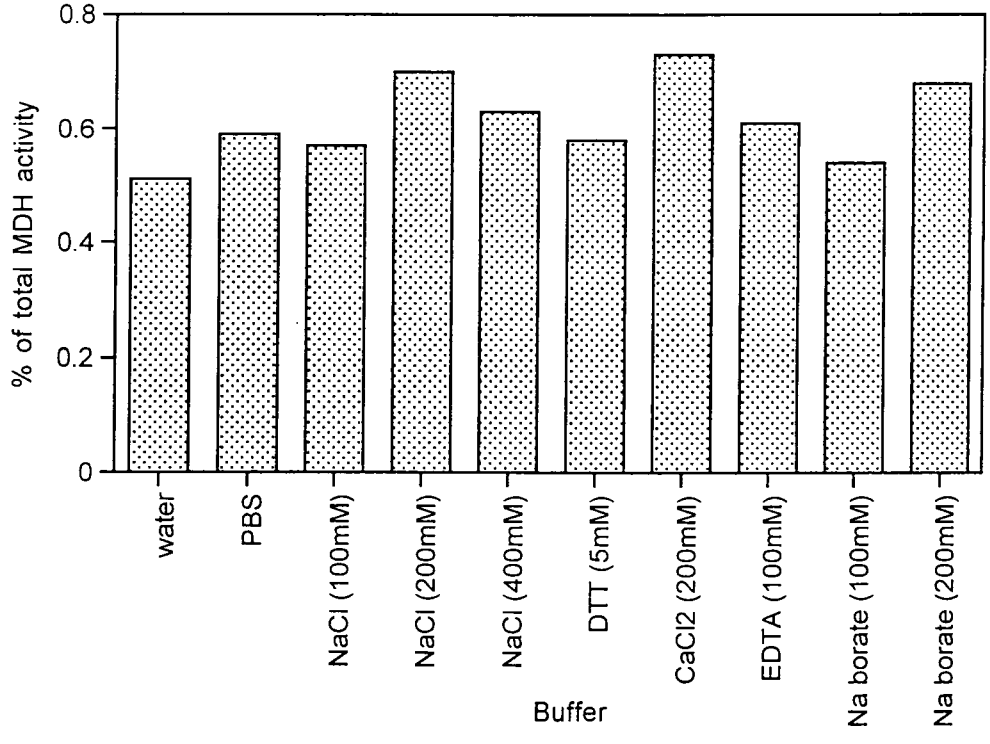


Figure 5.5. MDH activity in leaf INF compared with total protein extracts.
MDH activity in 50 µl of 3S1 leaf INF was compared with MDH activity in 10 µl of leaf total protein extract. The total protein extracts were prepared as described in section 3.3. using the different infiltration buffers (concentrations in parentheses) used for the isolation of INF (as described in section 3.6.3).

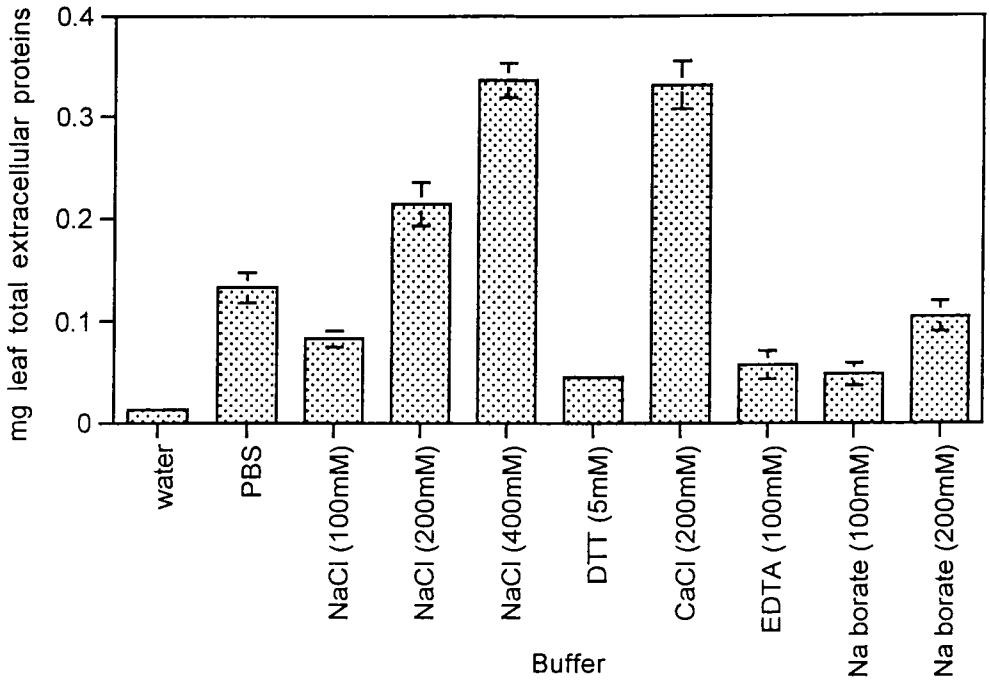


Figure 5.6. Effect of infiltration buffer on the isolation of leaf total extracellular proteins.
Concentrations of extracellular proteins in the INF isolated from 3S1 oilseed rape leaves following vacuum infiltration with different infiltration buffers (concentrations in parentheses). Error bars show mean ± standard errors.

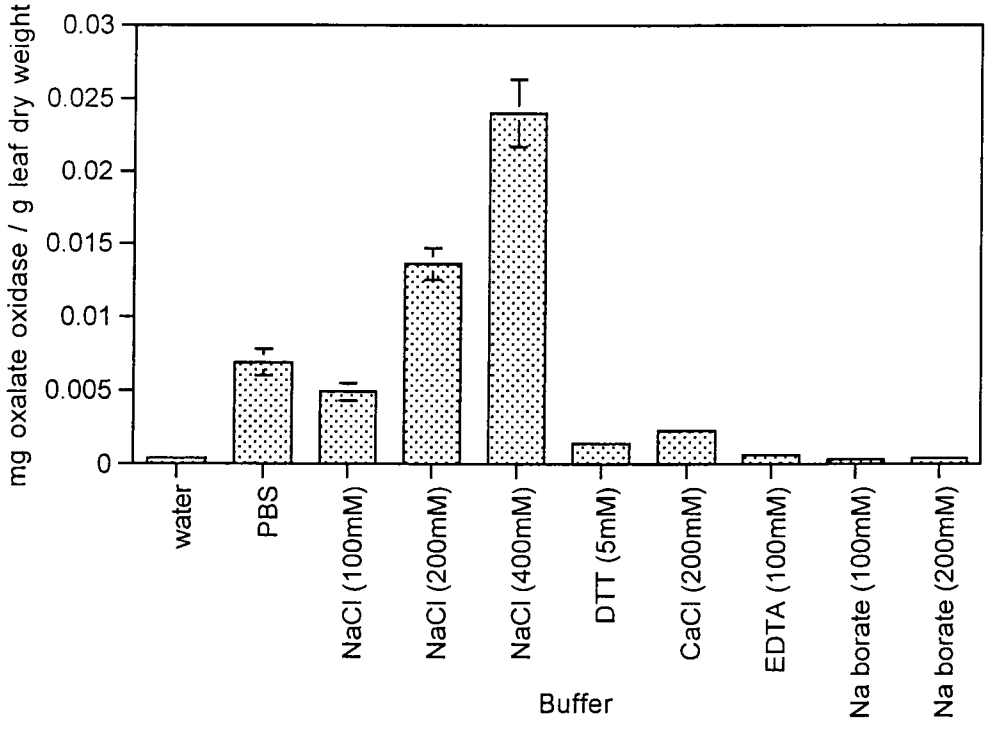


Figure 5.7. Effect of infiltration buffer on the isolation of oxalate oxidase from leaf extracellular spaces.
Concentrations of extracellular oxalate oxidase in the INF isolated from 3S1 oilseed rape leaves following vacuum infiltration with different infiltration buffers (concentrations in parentheses). Error bars show mean ± standard errors.

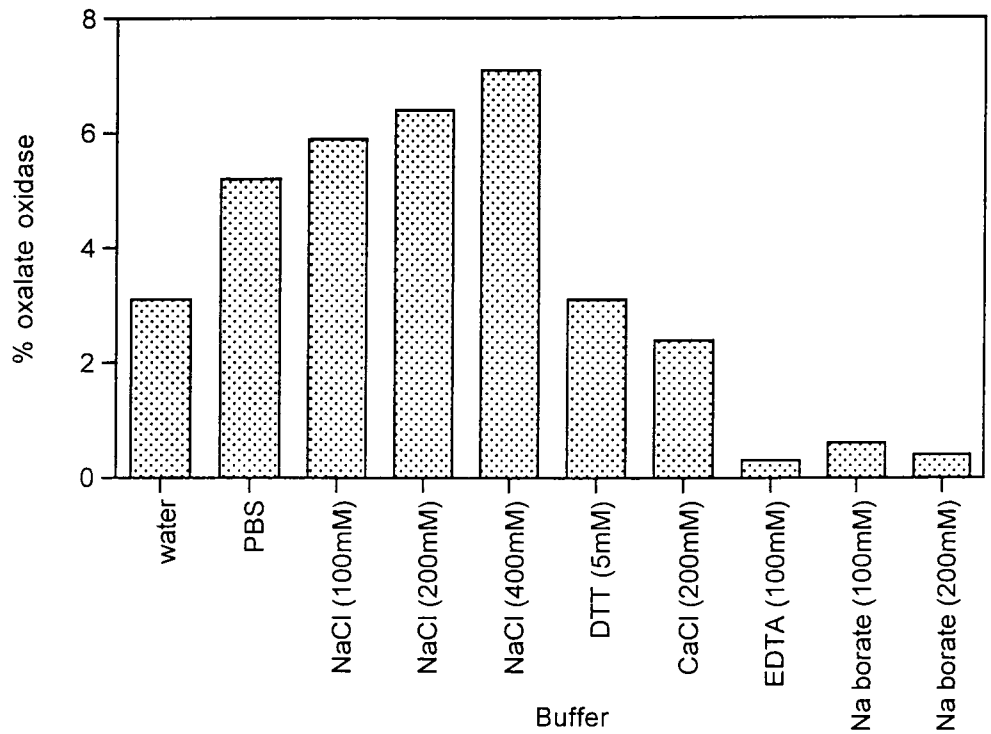


Figure 5.8. Efficiency of oxalate oxidase isolation from leaf extracellular spaces.

Extracellular oxalate oxidase concentration expressed as a percentage of total extracellular protein concentration (using a Bradford assay as described in section 3.10.) in the INF isolated from 3S1 oilseed rape leaves following vacuum infiltration with different infiltration buffers (concentrations in parentheses).

without causing damage and so 0.2 M NaCl was used in infiltration buffer for almost all subsequent vacuum infiltration experiment.

Fig. 5.9. shows Coomassie blue stained SDS-PAGE gels illustrating the protein profiles of transgenic 3S1 oilseed rape, C26 and SGS5 tobacco INF and leaf total protein extracts. As can be seen the transgenic oxalate oxidase proteins (3S1, C26 and SGS5 indicated by the arrows) formed a significant proportion of the total extracellular proteins isolated in the leaf INF when using the modified vacuum infiltration method. The extracellular protein profiles of the two transgenic tobacco lines where considerably different, which may have reflected the differential expression and accumulation of extracellular proteins in these two different tobacco cultivars (samson and Xanthi).

Confirmation of the extracellular localisation of a proportion of the transgenic oxalate oxidases was obtained by confirming the absence of RUBISCO and a lack of MDH activity in the INF isolated from transgenic 3S1, C26 and SGS5 leaves. Fig. 5.10. A, illustrates the presence of oxalate oxidase in non-heat treated leaf INF and total protein extracts of the three transgenic plant lines (3S1, C26 and SGS5). No oxalate oxidase was detected in the non-transgenic oilseed rape (OSR) or tobacco (TOB) leaf INF or in the corresponding total protein extracts. However, the anti-oxalate oxidase antibodies cross-reacted faintly with a protein band in the INF and total protein extract of non-transgenic tobacco, with a molecular weight of about 40 kDa (the relevance of this germin like-protein will be discussed in chapter 10). Fig. 5.10. B, shows the presence of RUBISCO in leaf total protein extracts of the three transgenic plant lines and its absence from the leaf INF, thus indicating an absence of intracellular contamination. The autoradiographs were over-exposed in order to detect any RUBISCO in the isolated INF. MDH activities in the INF was generally less than 1% of the activity in leaf total protein extracts. The absence of RUBISCO and measured MDH activity of less than 1% in the leaf INF confirmed that a proportion of the transgenic oxalate oxidases (3S1, C26 and SGS5) were isolated from the extracellular spaces using this vacuum infiltration procedure.

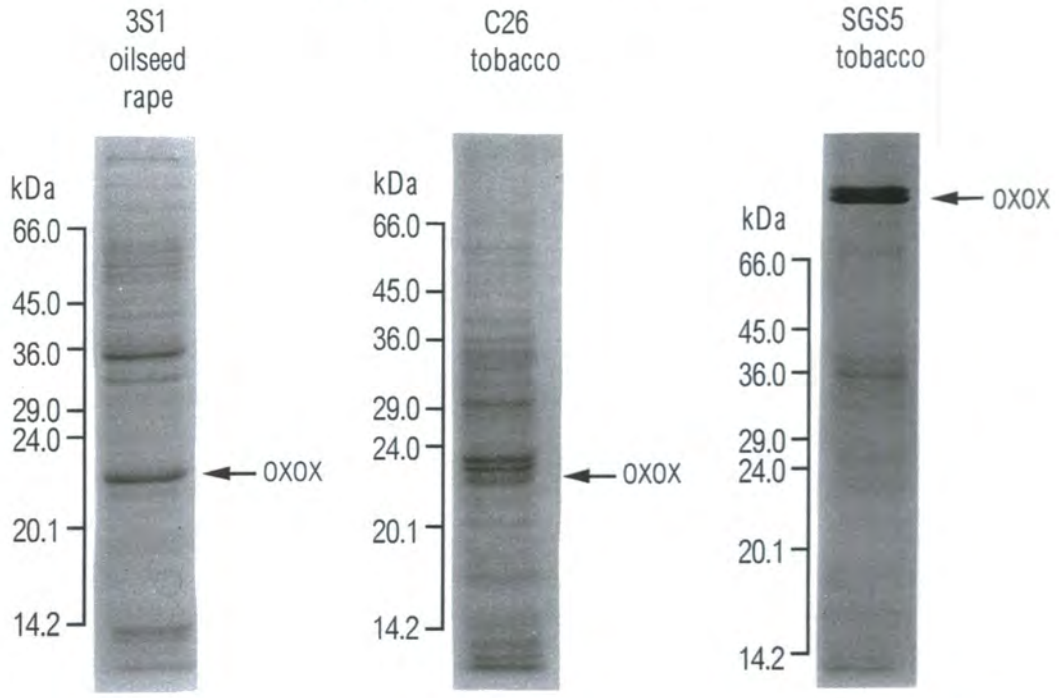
5.5. Quantifying the extracellular accumulation of oxalate oxidase in transgenic oilseed rape and tobacco leaves.

A vacuum infiltration experiment was designed to estimate the levels of oxalate oxidase released by the repeated infiltration of transgenic 3S1 oilseed rape, C26 and SGS5 tobacco leaves with infiltration buffer containing 400 mM NaCl and 50 mM Na_2HPO_4 pH 7.5. Detergents were excluded from the infiltration buffer so that total protein estimations could be performed on the leaf total protein extracts and INF samples. Vacuum infiltration was performed as described in section 3.6.2. and each leaf piece was infiltrated 3 times. Previous experiments had already demonstrated that little additional extracellular oxalate oxidase was isolated in a fourth infiltration (Fig. 5.3.). Total protein extracts were prepared from samples of the non-infiltrated leaf material using the same saline extraction buffer. All the data generated was related back to the dry weight of the non-infiltrated leaf material (mg protein / g dry weight), as determined according to section 3.4.

Figure 5.9. SDS-PAGE analysis of proteins extracted from transgenic oilseed rape and tobacco leaves.

12% (w/v) SDS-PAGE analyses of INF and total protein extracts from transgenic oilseed rape (3S1) and tobacco (C26 and SGS5) leaves, using extraction buffer containing 400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5. The extracellular proteins in 1 ml of INF were concentrated by freeze drying and resuspended in 10 µl of distilled water. Approximately 30 µg of resuspended extracellular proteins and 80 µg of leaf total protein extracts were loaded onto the gels without heat treatment. Gels were stained with Coomassie blue. The positions of the transgenic oxalate oxidases are indicated by arrows (oxox).

Leaf Intercellular fluids



Leaf total protein extracts

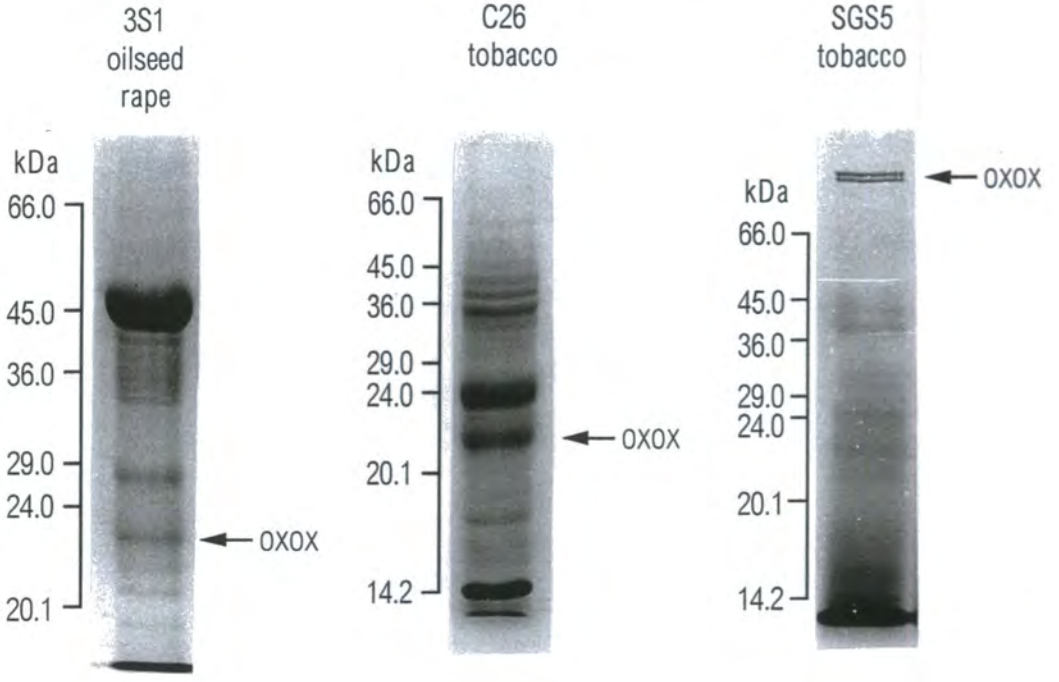
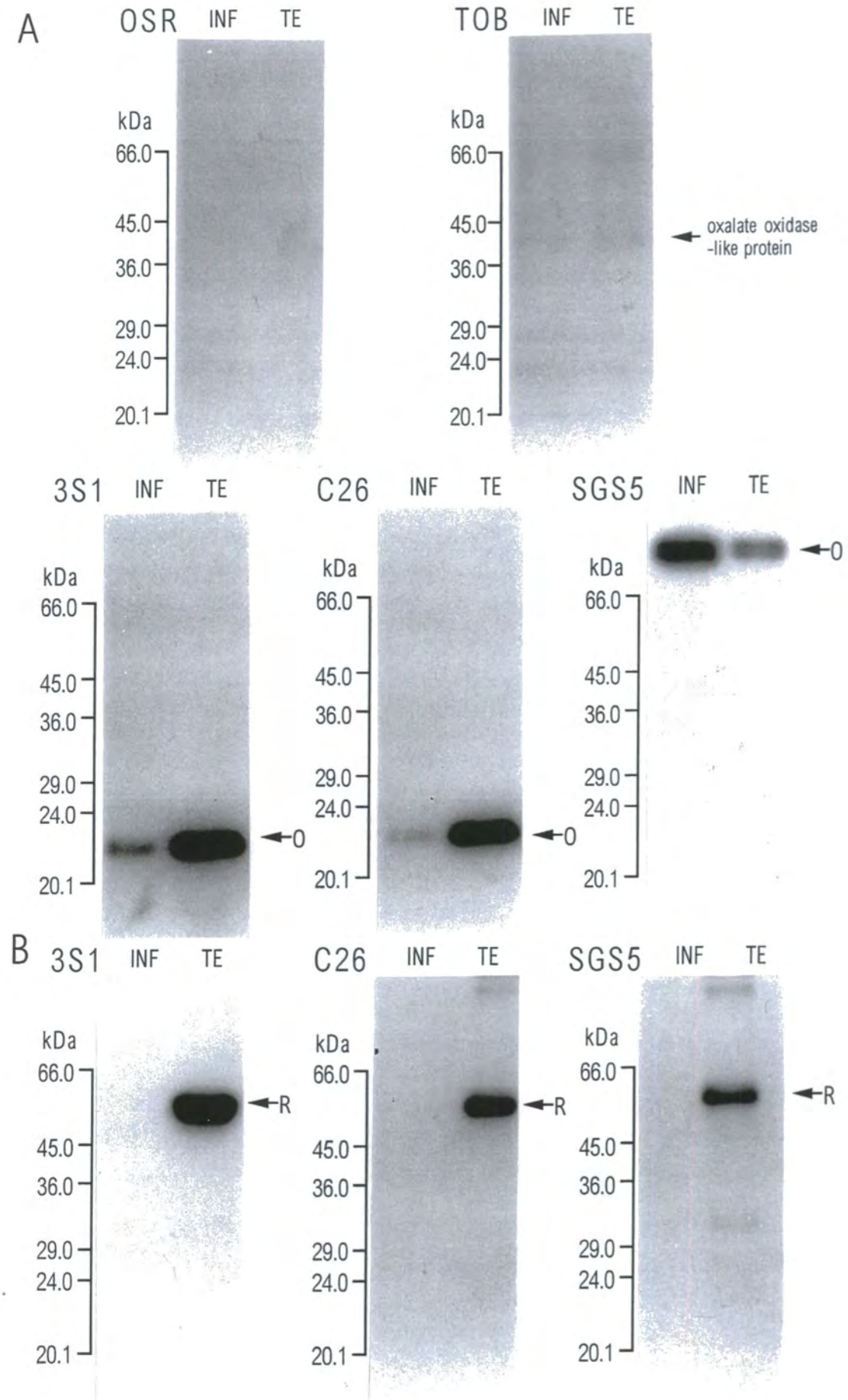


Figure 5.10. Immuno-detection of oxalate oxidase and RUBISCO in leaf INF and total protein extracts of oilseed rape and tobacco.

12% (w/v) SDS-PAGE analyses of INF and total protein extracts (TE) prepared from non-transgenic oilseed rape (OSR) and tobacco (TOB) and transgenic oilseed rape (3S1) and tobacco (C26 and SGS5) leaves. The proteins in 1 ml of INF were concentrated by freeze drying and resuspended in 100 µl of distilled water. Approximately 20 µg of resuspended extracellular proteins and 50 µg of leaf total protein extracts (in 1 x loading buffer) were loaded onto the gels without heat treatment. The gels were electroblotted onto nitrocellulose membranes as described in section 3.17. The immunoblots were reacted with either,

A, anti-oxalate oxidase antiserum as the primary antibodies (1:10,000 dilution).

B, anti-RUBISCO antiserum as the primary antibodies (1:1,000 dilution) and then ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase and RUBISCO, respectively. The blots were exposed to x-ray film for 2 week at -80 °C. The arrows indicate oxalate oxidase (O) and RUBISCO (R).


A
OSR
INF
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29.0
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20.1
TOB
INF
TE
kDa
66.0
45.0
36.0
29.0
24.0
20.1
oxalate oxidase
-like protein
3S1
INF
TE
kDa
66.0
45.0
36.0
29.0
24.0
20.1
O
C26
INF
TE
kDa
66.0
45.0
36.0
29.0
24.0
20.1
O
SGS5
INF
TE
kDa
66.0
45.0
36.0
29.0
24.0
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B
3S1
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29.0
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20.1
R
C26
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TE
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29.0
24.0
20.1
R
SGS5
INF
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kDa
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29.0
24.0
20.1
R

INF of 3S1 oilseed rape was clear while INF of C26 and SGS5 tobacco had a slight russet colour, which was quite normal for INF isolated from some plant species [Hammond-Kossack 1992]. The volumes of INF recovered from oilseed rape (3S1 INF, 1.43 ml / g leaf dry weight) were considerably lower than the levels isolated from tobacco (C26 INF, 2.23 ml / g leaf dry weight and SGS5 INF, 2.25 ml / g leaf dry weight), even though a much higher centrifugal force was used to isolate the INF from oilseed rape compared with tobacco (10,000 g and 2,500 g, respectively). Fig. 5.11. shows the results of MDH activity assays carried out on the INF and leaf total protein samples. For each of the three transgenic lines the levels of MDH activity increased with each subsequent infiltration, which presumably resulted from increased cellular disruption and cytosolic leakage into the extracellular spaces. However, the levels of MDH activity for all of the INF samples were approximately 1-1.5% and so the proteins were assumed to have originated largely from the extracellular spaces.

Leaf total protein extracts prepared from 3S1 oilseed rape leaves contained about 45.1 mg protein / g leaf dry weight (Fig. 5.12. A, 3S1 TE) compared with C26 and SGS5 tobacco which contained about 15.9 and 13.0 mg protein / g dry weight, respectively (Fig. 5.12. B, C26 TE and SGS5 TE). The two tobacco lines contained similar levels of protein on a dry weight basis. Fig. 5.12. B shows the level of extracellular proteins isolated in the 3 infiltrations expressed as a percentage of leaf total proteins. The levels of extracellular proteins in each of the subsequent INF samples declined, presumably resulting from the release of less proteins with each subsequent infiltration. The concentration of total extracellular proteins isolated from the 3 infiltrations was lower in 3S1 oilseed rape (0.687 mg / g leaf dry weight) compared with C26 (1.271 mg / g leaf dry weight) and SGS5 tobacco (1.046 mg / g leaf dry weight). This may have been due to the more effective infiltration of the tobacco leaves compared with the oilseed rape leaves resulting in higher yields of tobacco extracellular proteins in the INF.

Fig. 5.13. illustrates the levels of oxalate oxidase isolated in leaf total protein extracts (TE) of 3S1 oilseed rape (0.378 mg / g leaf dry weight), C26 tobacco (0.350 mg / g leaf dry weight) and SGS5 tobacco (0.353 mg / g leaf dry weight). The three transgenic plant lines contained very similar levels of total oxalate oxidase per gram of leaf dry weight. Fig. 5.13. also shows the levels of oxalate oxidase isolated from the INF of the three repeated vacuum infiltrations for 3S1 oilseed rape, C26 tobacco and SGS5 tobacco. The highest level of extracellular oxalate oxidase was isolated from INF 1, with only minute quantities being retrieved from INF 3. This suggested that the majority of extracellular oxalate oxidase which could be isolated using the repeated vacuum infiltration method with buffer containing 400 mM NaCl was retrieved from the extracellular spaces with three infiltrations. It was unlikely that a fourth infiltration would have removed any further oxalate oxidase from the extracellular spaces and MDH activity would have been above acceptable levels in the INF. The total levels of oxalate oxidase isolated from the leaf extracellular spaces were approximately 1.5 fold higher in C26 tobacco compared with 3S1 oilseed rape or SGS5 tobacco (Fig. 5.13. C26 TINF, 3S1 TINF and SGS5 TINF, respectively).

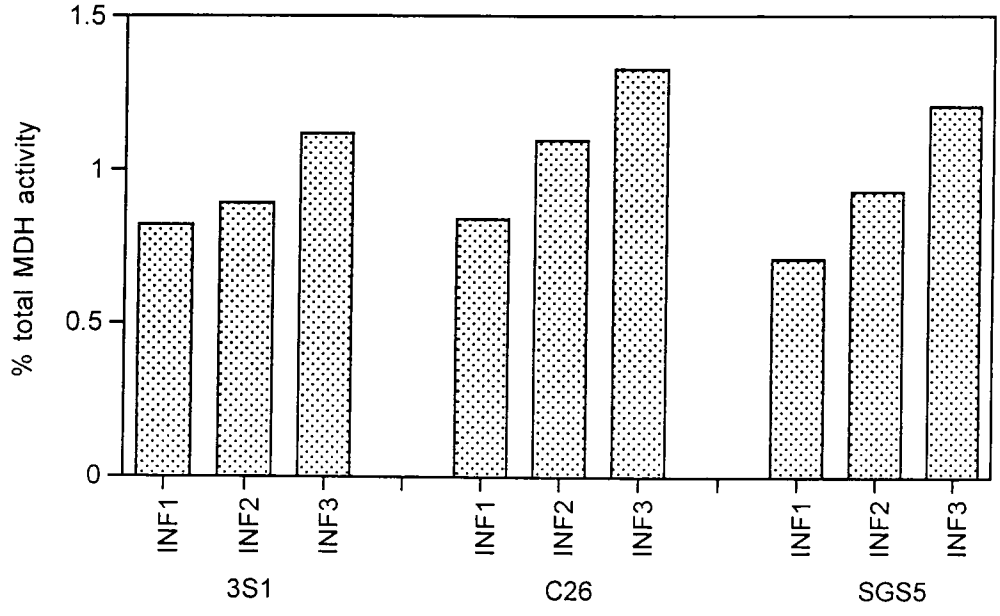


Figure 5.11. MDH activity in leaf INF compared with levels in total protein extracts.

MDH activity in 50 µl of leaf INF (1 - 3) was compared with MDH activity in 10 µl of leaf total protein extracts, as described in section 3.7. The total protein extracts were prepared as described in section 3.3. using infiltration buffer (400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5). 3S1 transgenic oilseed rape, C26 and SGS5 transgenic tobacco.

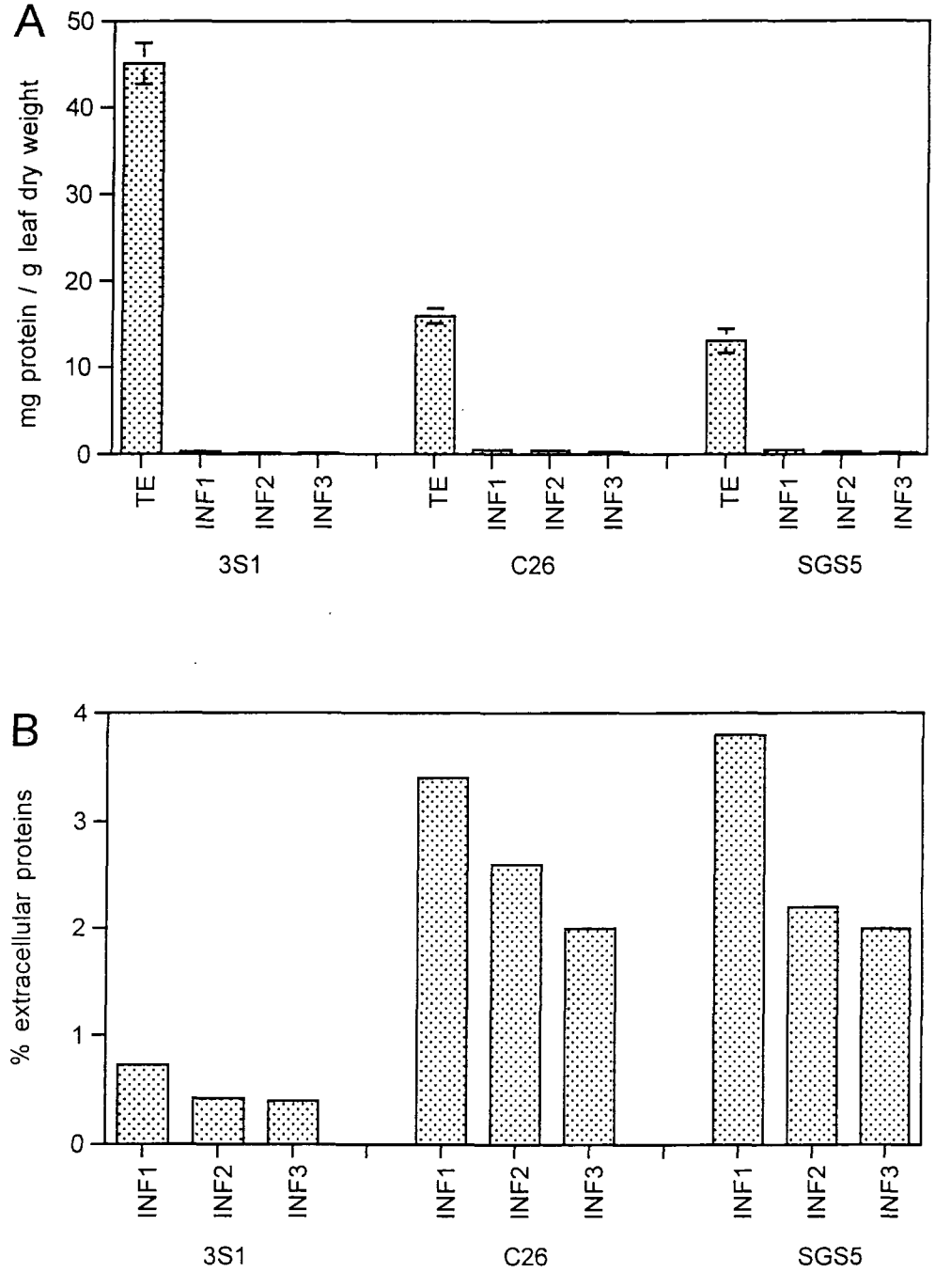


Figure 5.12. Effect of repeated vacuum infiltration on the isolation of total extracellular proteins.

A, concentrations of total proteins in leaf total extracts (TE) and in the INF from sequential vacuum infiltrations (INF 1-3); **B**, extracellular proteins in the INF from sequential vacuum infiltrations (INF 1-3) expressed as percentages of leaf total proteins. INF and total protein extracts were prepared with infiltration buffer (400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5). 3S1 transgenic oilseed rape, C26 and SGS5 transgenic tobacco. Error bars show mean ± standard errors.

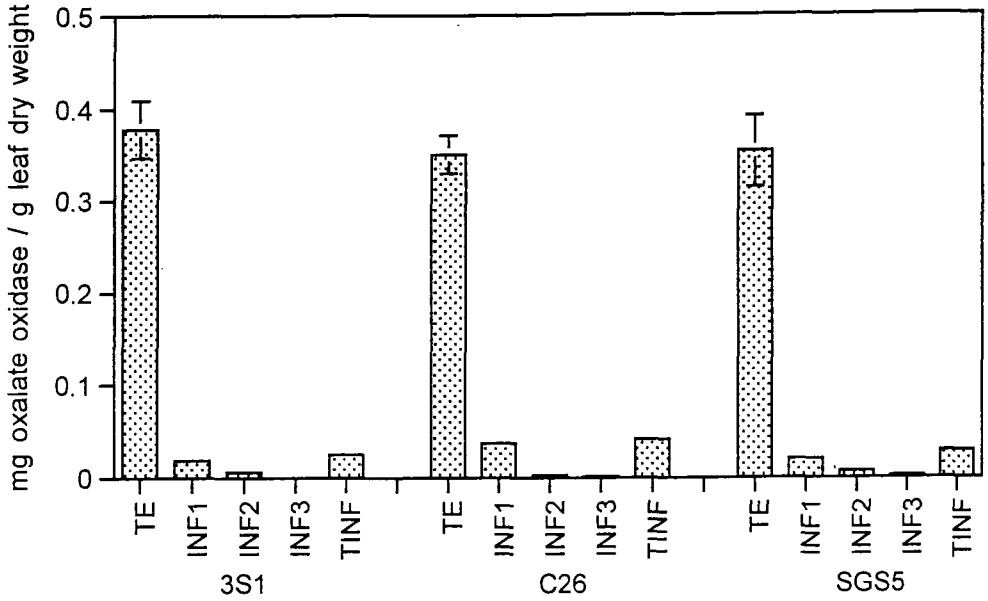


Figure 5.13. Effect of repeated infiltration on the isolation of oxalate oxidase from leaf extracellular spaces.
Levels of oxalate oxidase isolated from leaf total protein extracts (TE), extracellular oxalate oxidase isolated in the INF from sequential vacuum infiltrations (INF 1-3) and the total concentration of extracellular oxalate oxidase (TINF). INF and total protein extracts were prepared with infiltration buffer (400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5). 3S1 transgenic oilseed rape, C26 and SGS5 transgenic tobacco. Error bars show mean ± standard errors.

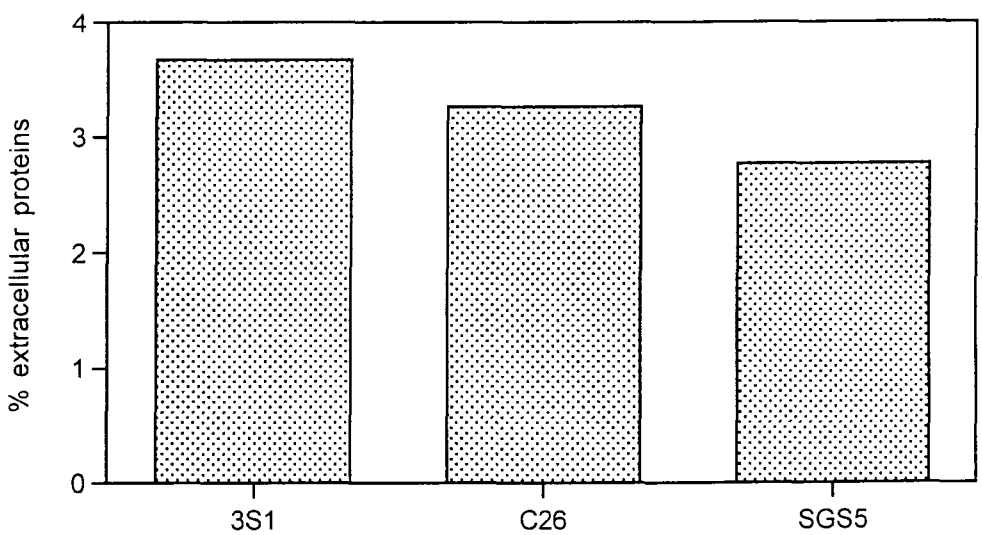


Figure 5.14. Extracellular localisation of oxalate oxidase.
Concentration of oxalate oxidase expressed as a percentage of total extracellular proteins in the INF from sequential vacuum infiltrations (INF 1-3). INF was prepared with infiltration buffer (400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5). 3S1 transgenic oilseed rape, C26 and SGS5 transgenic tobacco.

The results of the three vacuum infiltrations were combined so that the total levels of oxalate oxidase present in the leaf extracellular spaces of the three transgenic plant lines could be related to the levels of total extracellular proteins (Fig. 5.14.). Approximately 3.67% of the total proteins isolated from the extracellular spaces by repeated vacuum infiltration of 3S1 oilseed rape leaves was extracellular oxalate oxidase. C26 and SGS5 tobacco leaves contained lower levels of extracellular oxalate oxidase compared with total extracellular proteins (3.26% and 2.77%, respectively). However, the concentration of total extracellular proteins isolated from the three infiltrations of 3S1 leaves was significantly lower (30-50%) than the levels isolated from the tobacco leaf infiltrations (C26 and SGS5). Either oilseed rape contained less extracellular proteins which were isolated using this infiltration protocol or tobacco leaves were a more suitable material for this procedure resulting in the isolation of higher levels of extracellular proteins per g of leaf dry weight. These results show that 3S1 oilseed rape oxalate oxidase constituted a higher percentage of total extracellular proteins than C26 or SGS5 tobacco oxalate oxidases.

Fig. 5.15. illustrates the levels of total oxalate oxidase which were located in the extracellular spaces of the three transgenic plant lines. The levels of extracellular oxalate oxidases were 6.7%, 11.8% and 8.2% for 3S1, C26 and SGS5, respectively. The transgenic tobacco lines contained higher levels of extracellular oxalate oxidase compared with the transgenic oilseed rape. Tobacco secreted higher levels of transgenic oxalate oxidases to the extracellular spaces compared with oilseed rape, which may have resulted from differences in protein processing pathways in these two species. Lane *et al,* [1992] demonstrated that about 40% of oxalate oxidase was associated with the pellet fraction of a total homogenate of germinated wheat embryos, suggesting an association with cell walls. The exact location of the other 60% of the protein isolated in the soluble fraction has yet to be determined. Immuno-cytological techniques confirmed the association of oxalate oxidase with cell walls in freeze-cleaved germinated wheat embryos [Lane *et al.* 1992]. Berna and Bernier [1997] demonstrated that in transgenic tobacco expressing the intact gf-2.8 wheat embryo oxalate oxidase gene approximately 30% of the protein was released by washing the seedlings. Approximately 60% was associated with the soluble fraction as judged from enzymatic activity and the other 10% was associated with the cell wall fraction. Berna and Bernier [1997] calculated that approximately 30% of the transgenic oxalate oxidase was associated with the apoplast, which was recovered in INF following vacuum infiltration. The significance of the extracellular localisation of oxalate oxidase in the transgenic oilseed rape plants with regard to fungal pathogen resistance will be discussed in chapter 11.

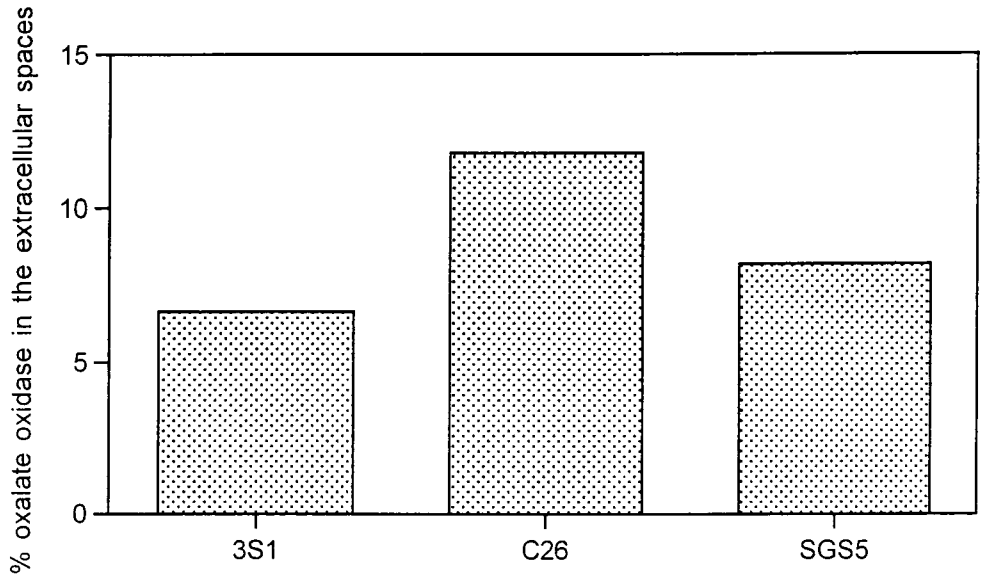


Figure 5.15. Distribution of oxalate oxidase in the leaf.
Extracellular oxalate oxidase expressed as a percentage of leaf total oxalate oxidase. INF and total extracts were prepared with infiltration buffer (400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5). 3S1 transgenic oilseed rape, C 26 and SGS5 transgenic tobacco.

6. RESULTS AND DISCUSSION.

Protein and DNA sequencing of native and transgenic oxalate oxidases.

The experiments described in this chapter relate to the N-terminal sequencing of the native cereal and transgenic oxalate oxidases. These experiments were performed to confirm that the transgenic oxalate oxidases had identical N-terminal sequences to the native proteins thus providing evidence that the signal peptides were correctly cleaved from the mature oxalate oxidase proteins in the transgenic plants. The extensin-oxalate oxidase chimeric gene expressed in transgenic 3S1 oilseed rape and C26 tobacco was sequenced to confirm that the gene sequence was identical to the native barley root oxalate oxidase gene.

6.1. N-terminal sequencing of proteins isolated from leaf INF.

Leaf INF provided an ideal starting material from which N-terminal sequencing data of extracellular proteins could be obtained in a relatively short period of time. INF was isolated free of intracellular contaminants as determined by MDH activity assays. Therefore any proteins isolated in the INF were known to have originated from the extracellular spaces. It was possible to obtain repeatable N-terminal sequence information for a number of extracellular proteins by separating the proteins from INF directly on SDS-PAGE gels after isolation by vacuum infiltration. In some cases it was necessary to concentrate the protein solution prior to gel electrophoresis, usually by freeze drying following overnight dialysis against water at 4 °C, as described in section 3.9. The separated polypeptide bands were then transferred onto a ProBlott™ membrane as described in section 3.20. The polypeptides of interest were carefully excised and the first 10 to 15 amino acid residues sequenced. The number and accuracy of amino acid residues obtained was dependant on the concentration and quality of polypeptide present on the membrane.

The use of this particular protocol assumed that (a) some if not all of the protein of interest was located in the extracellular spaces, (b) the protein of interest was present in the INF at sufficient levels to be easily observed on the ProBlott™ membrane in the presence of other extracellular proteins, and (c) the protein did not separate in close proximity to other proteins of similar molecular weight on the SDS-PAGE gel, which would have led to contamination and an unreliable result when sequencing. Approximately 100 pmoles of protein were required to obtain N-terminal sequence information over the first 10-15 amino acid residues.

6.2. N-terminal sequences of the native barley and wheat oxalate oxidases.

N-terminal sequencing from partially purified protein samples of 10 day old barley roots, 3 day germinated barley embryos and 3 day germinated wheat embryos was used to obtain oxalate oxidase protein sequence information. Crude total protein extracts prepared from these three plant materials were fractionated with ammonium sulphate (50 and 70% relative saturation). Proteins which precipitated in the presence of 70% (relative saturation) ammonium sulphate were retrieved following centrifugation, resuspended in water, dialysed

overnight against water at 4 °C and lyophilised according to the method described in section 3.9. This basic level of purification was sufficient to provide protein samples which when analysed on 15% (w/v) SDS-PAGE gels separated the oxalate oxidase pentameric isoforms free from other major protein contamination in the 110-120 kDa region of the gel. The sequence information shown in Fig. 6.1. confirmed that the G and G' isoforms of barley root, barley embryo and wheat embryo oxalate oxidases possessed identical N-terminal sequences. These results also confirmed that an additional oxalate oxidase identified in barley root extracts had an identical N-terminal sequence compared with the G and G' isoforms (see chapter 8 for more details of this protein). Lane *et al.* [1991] published the entire protein sequence of two wheat embryo oxalate oxidases translated from the open reading frames of isolated embryo oxalate oxidase genes (gf-2.8 and gf-3.8). They stated that the N-terminal residue of the protein translated from the wheat embryo gf-2.8 gene sequence was threonine and not serine. However, in the present study serine was shown to be the N-terminal residue for the six native oxalate oxidase isoforms sequenced (Fig. 6.1.). The wheat embryo oxalate oxidase G and G' isoforms were resequenced, which confirmed that serines were the N-terminal residues of the two isoforms. The N-terminal sequences of the barley root and embryo oxalate oxidases were identical to the sequence published by Lane *et al.* [1993] for barley seedling oxalate oxidase. The translated product from a second wheat embryo oxalate oxidase gene (gf-3.8) identified by Lane *et al.* [1991] had overall protein sequence identity to gf-2.8 of 93% but only 70% homology over the first 10 residues at the N-terminus. The N-terminal residue of the translated mature gf-3.8 protein was serine.

6.3. N-terminal sequences of the transgenic oilseed rape and tobacco oxalate oxidases.

N-terminal sequencing was performed on 3S1 oilseed rape, C26 and SGS5 tobacco oxalate oxidases isolated from INF of mature leaves which were concentrated by freeze drying, separated by SDS-PAGE and electroblotted onto a ProBlott™ membrane. INF used in this experiment was obtained from mature leaves of the three transgenic plant lines which were shown to contain high levels of active enzyme, as determined using the leaf disk assay method described in section 3.12. The oxalate oxidases isolated from 3S1, C26 and SGS5 transgenic plants were detectable as major bands in the INF when analysed on 15% (w/v) SDS-PAGE gels, without heat treatment (Fig. 5.9.). The single monomeric 3S1 oilseed rape and C26 tobacco oxalate oxidases (about 22-24 kDa) and the two oligomeric SGS5 tobacco oxalate oxidase isoforms (about 110-120 kDa) were easily excised from the ProBlott™ membrane without risk of contamination from other extracellular proteins. These experiments were performed to confirm that the oxalate oxidase were correctly processed in the three transgenic plant lines, to give proteins with N-terminal sequences identical to the native cereal proteins (i.e. SDPDPLQDFCV).

The results of the N-terminal sequencing of the transgenic oxalate oxidases are shown in Fig. 6.1. These results confirmed that the two SGS5 tobacco oxalate oxidase isoforms (G and G') were correctly processed in the transgenic plants giving rise to mature

Figure 6.1. N-terminal amino acid sequences of the native and transgenic oxalate oxidases.

N-terminal sequences of the native cereal oxalate oxidase isoforms were obtained from the partially purified proteins without heat treatment (70% (relative saturation) $(NH_4)_2SO_4$ fractions of total protein extracts). N-terminal sequences of the transgenic 3S1 oilseed rape, C26 and SGS5 tobacco oxalate oxidase isoforms were obtained from the proteins in samples of dialysed and lyophilised INF, without heat treatment. The protein samples were separated on 12% (w/v) SDS-PAGE gels and electroblotted onto ProBlott™ membrane as described in section 3.20. N-terminal sequences derived from the gene sequences of wheat germins isolated by Dratewka-Kos *et al.* [1989] (gf-2.8) and Lane *et al.* [1991] (gf-3.8) and barley seedling oxalate oxidase identified by Lane *et al.* [1993] are included in the alignment. Differences in the N-terminal amino acid sequences are in bold. The G and G' oxalate oxidase isoforms are denoted.

SDPDPLQDFCV	Barley root (G)
SDPDPLQDFCV	Barley root (G')
SDPDPLQDFCV	Barley root (X)
SDPSPLQDFCV	Barley embryo (G)
SDPDPLQDFCV	Barley embryo (G')
SDPDPLQDFCV	Wheat embryo (G)
SDPDPLQDFCV	Wheat embryo (G')
SETDPDPLQDFCV	3S1 oilseed rape
SETDPDPLQDFCV	C26 tobacco
SDPDPLQDFCV	SGS5 tobacco (G)
SDPDPLQDFCV	SGS5 tobacco (G')
TDPDPLQDFCV	Wheat germin (gf-2.8)
S**H**P**H**PLQDFCV	Wheat germin (gf-3.8)
SDPDPLQDFCV	Barley seedling

proteins with N-terminal sequences identical to those of the native wheat embryo oxalate oxidase isoforms (i.e. SDPDPLQDFCV).

These results implied that the native wheat embryo oxalate oxidase signal peptide present on the N-terminus of the nascent transgenic SGS5 oxalate oxidase polypeptide was recognised and correctly cleaved by signal peptidase (a membrane-associated multi sub-unit enzyme) in the rough ER of the tobacco plants.

The N-terminal sequences for the transgenic 3S1 and C26 oxalate oxidases were expected to be identical to barley root oxalate oxidase (i.e. SDSDPLQCFCV) because the gene which codes for this particular oxalate oxidase was expressed in these two transgenic lines. However, the N-terminal sequences for the 3S1 and C26 oxalate oxidases contained an additional 2 residues and a threonine instead of the native N-terminal serine residue (i.e. **SET**DPDPLQDFCV) (Fig. 6.1.). Therefore these two transgenic lines were not expressing proteins which were identical to the mature barley root oxalate oxidase. The serine (S) and glutamic acid (E) residues on the N-terminal sequences of the 3S1 and C26 proteins appeared to have originated from the C-terminal sequences of the extensin signal peptide. This may have resulted from the incorrect cleavage of the extensin signal peptide from the mature oxalate oxidase protein during processing of the nascent polypeptide by signal peptidase in the rough ER of the transgenic oilseed rape and tobacco plants. The consequences of the incomplete cleavage of the extensin signal peptide from the mature oxalate oxidase proteins expressed in transgenic 3S1 oilseed rape and C26 tobacco will be discussed in chapter 11.

6.4. Sequencing of the extensin-oxalate oxidase chimeric gene.

Following the discovery that the oxalate oxidases expressed in transgenic 3S1 oilseed rape and C26 tobacco plants did not have N-terminal sequences identical to the native barley root protein the decision was taken to clone and sequence the extensin-oxalate oxidase chimeric gene introduced and expressed in these transgenic plants. The reason for sequencing the chimeric gene was to determine if there were any alterations to the introduced DNA sequence that might have led to changes in the amino acids in the primary protein sequence at the N-terminus or elsewhere in the mature protein. Mutations or cloning artefacts in the DNA sequence could lead to changes in the amino acid sequence of the transgenic protein. Mutations in the primary protein sequence may have caused some of the unusual structural and kinetic properties exhibited by the 3S1 and C26 transgenic proteins. These properties will be discussed later in comparison with the native cereal proteins (see chapter 8 and 9).

6.5. Preparation of a subclone from pNEXTOX.

The extensin-oxalate oxidase chimeric gene was excised from pNEXTOX as a Kpn1 restriction fragment, 771 bp in length (Fig. 4.1.). The Kpn1 restriction fragment was ligated into pUC18, restriction digested with Kpn1 and dephosphorylated using shrimp alkaline phosphatase to give pEXTOX+. Plasmid pEXTOX+ was transformed into DH5α and

transformed clones were selected on x-gal / IPTG plates. The transformation efficiency with pEXTOX+ was greater than 90%, as judged by the number of white (transformed) to blue (untransformed) bacterial colonies. Plasmid pEXTOX+ was isolated from two independently transformed colonies, each of which were sequenced in both the forward and reverse directions, as described in the methods section 3.42.

6.6. DNA sequence analysis of the extensin-oxalate oxidase chimeric gene.

Figure 6.2. illustrates the sequence alignment of the native barley root oxalate oxidase DNA sequence (BROX) and the extensin-oxalate oxidase chimeric gene sequence, which was introduced into the transgenic plants. The N-terminal residue of mature barley root oxalate oxidase was serine, encoded by the codon TCC. Zeneca Seeds introduced a point mutation which changed the N-terminal residue of the mature protein from serine (TCC) to threonine (ACC), as a 'safe-guard' to ensure complete cleavage of the *N. plumbaginifolia* extensin signal peptide in the transgenic plants. During cotranslational processing of the primary polypeptide sequence the extensin signal peptide appeared to have successfully ensured that the growing polypeptide was directed to the rough ER fulfilling its main requirement in the transgenic plants. However, during the subsequent cleavage of the signal peptide by signal peptidase in the rough ER the protein was processed at an alternative site to yield an oxalate oxidases in the transgenic oilseed rape (3S1) and tobacco (C26) with the observed different N-terminal sequences (Fig. 6.1. 3S1 and C26) This resulted in the production of oxalate oxidases in the 3S1 and C26 transgenic plants with N-terminal sequences which were different from the native cereal oxalate oxidases (i.e. SETDPDPLQDFCV instead of SDPDPLQDFCV).

Six other nucleic acid base changes were also discovered in the extensin-oxalate oxidase chimeric gene which are highlighted in Fig 6.2. However, all of these mutations occurred in the third base of the codons and did not result in any change in the assignment of amino acid residues at those positions in the polypeptide sequence. Three of these point mutations occurred in the codon coding for glutamic acid, where GAG had mutated to GAA (both encoded glutamic acid). These point mutations presumably arose as a result of the use of Taq DNA polymerase in the PCR used to obtain the extensin oxalate oxidase chimeric gene, which was subsequently introduced into the transgenic 3S1 and C26 plants. Taq DNA polymerase has an error rate attributable to non-templated nucleotide addition of about 1-3% [Smith *et al.* 1995].

The consequences of the presence of the additional two amino acids (SE) and the threonine residue on the N-termini of the 3S1 and C26 transgenic oxalate oxidases became apparent in subsequent experiments on the oxalate oxidase proteins, particularly when comparing the structural and kinetic properties of the native cereal and SGS5 transgenic proteins with the 3S1 and C26 transgenic oxalate oxidases (see chapters 8 and 9).

Figure 6.2. Comparison of the barley root and the extensin-oxalate oxidase DNA sequence expressed in transgenic 3S1 oilseed rape and C26 tobacco.

An alignment of the barley root oxalate oxidase gene (BROX) and the extensin-oxalate oxidase chimeric gene expressed in transgenic 3S1 oilseed rape and C26 tobacco plants (EXTOX). The extensin signal peptide sequence is underlined and the amino acid sequence of the translation product of the extensin-oxalate oxidase chimeric gene is in italics. The bold characters represent difference identified between the two gene sequences and changes in the amino acid sequences. The numbers correspond to the bases in the mature protein coding region. *, stop codon (TAA).

```
EXTOX                    ATGGGAAAAATGGCTTCTCTATTTGCCACATTTTTAGTGGTTTTA
                          M  G  K  M  A  S  L  F  A  T  F  L  V  V  L

BROX                                  TCCGACCCAGACCCACTCCAGGACTTCTGTGTCGCG 36
EXTOX GTGTCACTTAGCTTAGCTTCTGAAACCGACCCAGACCCACTCCAGGACTTCTGCGTCGCG 36
       V  S  L  S  L  A  S  E  T  D  P  D  P  L  Q  D  F  C  V  A

BROX  GACCTCGATGGCAAGGCGGTCTCGGTGAACGGGCATACGTGTAAGCCCATGTCGGAGGCC 96
EXTOX GACCTCGATGGCAAGGCGGTCTCGGTGAACGGGCATACGTGTAAGCCCATGTCGGAGGCC 96
       D  L  D  G  K  A  V  S  V  N  G  H  T  C  K  P  M  S  E  A

BROX  GGCGACGACTTCCTCTTCTCGTCCAAGCTGACCAAGGCCGGCAACACGTCCACCCCGAAC 156
EXTOX GGCGACGACTTCCTCTTCTCGTCCAAGCTGACCAAGGCCGGCAACACGTCCACCCCGAAC 156
       G  D  D  F  L  F  S  S  K  L  T  K  A  G  N  T  S  T  P  N

BROX  GGGTCGGCCGTGACGGAGCTCGACGTGGCCGAATGGCCCGGTACGAACACGCTGGGCGTG 216
EXTOX GGCTCGGCCGTGACGGAGCTCGACGTGGCCGAGTGGCCCGGTACGAACACGCTGGGCGTG 216
       G  S  A  V  T  E  L  D  V  A  E  W  P  G  T  N  T  L  G  V

BROX  TCCATGAACCGTGTGGACTTCGCGCCGGGCGGCACCAACCCGCCGCACATCCACCCGCGT 276
EXTOX TCCATGAACCGTGTGGACTTCGCGCCGGGGGGCACCAACCCGCCGCACATCCACCCGCGT 276
       S  M  N  R  V  D  F  A  P  G  G  T  N  P  P  H  I  H  P  R

BROX  GCAACCGAAATCGGCATGGTGATGAAAGGTGAACTCCTCGTTGGAATCCTCGGCAGCCTT 336
EXTOX GCAACCGAGATCGGCATGGTGATGAAAGGTGAGCTCCTCGTTGGAATCCTCGGCAGCCTT 336
       A  T  E  I  G  M  V  M  K  G  E  L  L  V  G  I  L  G  S  L

BROX  GACTCCGGAAACAAGCTCTACTCCAGGGTGGTGCGTGCCGGAGAGACTTTCGTCATCCCG 396
EXTOX GACTCCGGAAACAAGCTCTACTCCAGGGTGGTGCGTGCCGGAGAGACTTTCGTCATCCCG 396
       D  S  G  N  K  L  Y  S  R  V  V  R  A  G  E  T  F  V  I  P

BROX  CGCGGCCTCATGCACTTCCAGTTCAACGTTGGTAAGACGGAAGCCTACATGGTTGTGTCC 456
EXTOX CGCGGCCTCATGCACTTCCAGTTCAACGTTGGTAAGACGGAAGCCTACATGGTTGTGTCC 456
       R  G  L  M  H  F  Q  F  N  V  G  K  T  E  A  Y  M  V  V  S

BROX  TTCAACAGCCAGAACCCTGGCATCGTCTTCGTGCCGCTCACACTCTTCGGCTCCGACCCT 516
EXTOX TTCAACAGCCAGAACCCTGGCATCGTCTTCGTGCCGCTCACACTCTTCGGCTCCGACCCT 516
       F  N  S  Q  N  P  G  I  V  F  V  P  L  T  L  F  G  S  D  P

BROX  CCCATCCCCACGCCCGTGCTCACCAAGGCTCTCCGGGTGGAGGCCGGAGTCGTGGAACTT 576
EXTOX CCCATCCCCACGCCCGTGCTCACCAAGGCTCTCCGGGTGGAGGCCGGAGTCGTGGAACTT 576
       P  I  P  T  P  V  L  T  K  A  L  R  V  E  A  G  V  V  E  L

BROX  CTCAAGTCCAAGTTCGCCGGTGGGTCTTAA 606
EXTOX CTCAAGTCCAAGTTCGCCGGTGGGTCTTAA 606
       L  K  S  K  F  A  G  G  S  *
```

7. RESULTS AND DISCUSSION.

Purification of native and transgenic oxalate oxidases.

In order for enzyme kinetic and detailed structural studies to be carried out on the native (barley root, barley embryo and wheat embryo) and transgenic (3S1, C26 and SGS5) oxalate oxidases, a protocol was developed to attempt purification to homogeneity of all 6 proteins under investigation. The transgenic plants were expressing genes originating from barley roots and wheat embryos, so these proteins were purified and included in the kinetic and structural studies as controls to the transgenic proteins. Barley embryo oxalate oxidase was purified for comparison with wheat embryo oxalate oxidase (i.e. did the same organs of these two different species produce oxalate oxidases with similar properties). The purification protocol was developed according to published methods used previously by other researchers to purify oxalate oxidase from various sources [Chiriboga 1966; Sugiura *et al.* 1979; Pietta *et al.* 1982; Lane *et al.* 1987; Koyama *et al.* 1988; Schmitt 1991; Satyapal and Pundir 1993]. This chapter will review the adaptations, advantages and drawbacks of the various purification steps used to purify the oxalate oxidases.

Crude total protein extracts were prepared from roots and embryos of barley and wheat as starting materials for the purification of the cereal oxalate oxidases. The embryos were initially frozen in the presence of dry ice ground in a blender to a powder and then homogenised in extraction buffer (400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5), as described in section 3.3. This buffer was previously shown to lead to the release of the majority of the oxalate oxidase protein from leaf material in the differential total protein extraction of transgenic 3S1 oilseed rape leaves (Fig. 5.2.). The powdered cereal material was homogenised in the presence of quartz sand to ensure that the majority of cells were disrupted, to optimise total protein release. All of the purification stages were performed at 4 °C or on ice to reduce the risk of proteolysis as a result of release of proteolytic enzymes into the total protein extract. The brief heat treatment step (3 min at 80 °C) to precipitate contaminating proteins as used by several groups [Chiroboga 1966; Sugiura *et al.* 1979; Pietta *et al.* 1982; Schmitt 1991] at this stage of barley oxalate oxidase purification was omitted. The purified proteins were eventually to be used in structural and kinetic experiments (including a study of temperature effects, see chapters 8 and 9); exposing the proteins to high temperatures at this stage may have affected the results of these experiments. The pepsin digestion step initially discovered by Grzelczak and Lane [1984] as a means of removing the majority of unwanted proteins from a crude total protein extract of wheat embryos to give a preparation composed almost exclusively of oxalate oxidase, was also omitted from the purification procedure. The effect of proteolysis on the structural properties of native and transgenic oxalate oxidases was to be examined later in this study (see chapter 8). Wheat oxalate oxidase was shown to be structurally stable and resistant to broad specificity proteases [Grzelczak and Lane 1984], however at that point in time (1984) no information was available regarding the affect of pepsin treatment on the enzyme activity of this protein.

INF (approximately 40-50 ml) isolated from leaves of the three transgenic plant lines (3S1, C26 and SGS5) were used as the initial extract for the purification of the transgenic oxalate oxidases. INF was used rather than total leaf protein extracts because the vacuum infiltration process itself offered a high degree of purification since it only resulted in the release and isolation of mainly leaf extracellular proteins, including oxalate oxidase. Although only about 10% of total oxalate oxidase was located in the leaves of the transgenic plants (Fig. 5.15.). The leaves of mature flowering plants were used as starting material for the purification procedure because they were known to contain the highest levels of oxalate oxidase, compared with leaves at any other stage of the plants life cycle (Fig. 4.4.). Single infiltrations were carried out on each leaf piece with infiltration buffer containing 400 mM NaCl, 50 mM Na_2HPO_4 pH 7.5 to ensure high oxalate oxidase release from the extracellular spaces. The heat treatment and pepsin digestion steps were omitted from this stage of the purification protocol, for the reasons described previously.

Bradford and oxalate oxidase enzyme assays were carried out at each purification step using the microtitre plate methods described in sections 3.10. and 3.11.4., respectively. Fig. 7.1. and 7.2. illustrate the results obtained from the microtitre plate reader when standard curves were generated from serial dilutions of BSA (Bradford assay) and hydrogen peroxide (enzyme assay), respectively. These graphs highlight the efficiency and reproducibility of the Bradford and enzyme assays performed using the microtitre plate methods and the Dynatech MT5000 microtitre plate reader. Samples taken from each purification step were also dot blotted onto nitrocellulose membranes to enable the rapid detection of oxalate oxidase enzyme activity, particularly in the different column fractions collected.

Ammonium sulphate fractionation (50 and 70% relative saturation) of the total protein extracts and INF were carried out for 3 hr at 0 °C with gentle stirring. The majority of oxalate oxidase enzyme activity was detected in the pellet of the 70% (relative saturation) fractions, almost no activity was detected in the pellet of the 50% (relative saturation) fractions, which were discarded. Pietta *et al.* [1982] designed a purification protocol which did not include an ammonium sulphate fractionation step as they found the fractionation step to result in the considerable loss of oxalate oxidase isolated from barley roots.

A simple G100 sephadex column was prepared to remove contaminating proteins with molecular weights of less than 100 kDa. The 70% (relative saturation) ammonium sulphate pellets were resuspended in G100 column buffer (see section 3.26.3.) with a volume sufficient to produce a non-viscous protein solution. The pellets from the INF samples were small and easily resuspended in 3 ml of column buffer. The pellets from the cereal total extracts were resuspended in larger volumes (7-10 ml), particularly the embryo samples because of the presence of high levels of proteins and carbohydrates, which made the samples very viscous and difficult to resuspend in smaller volumes of column buffer. The G100 column had a void volume of about 70 ml. Fractions 14-20 (collected as 5 ml fractions) were found to contain the highest levels of oxalate oxidase activity on dot blots, corresponding to proteins with molecular weights of greater than 100 kDa. These column fractions were pooled and dialysed against DE52 column buffer (section 3.26.4.), to remove the NaCl present in the G100 column buffer.

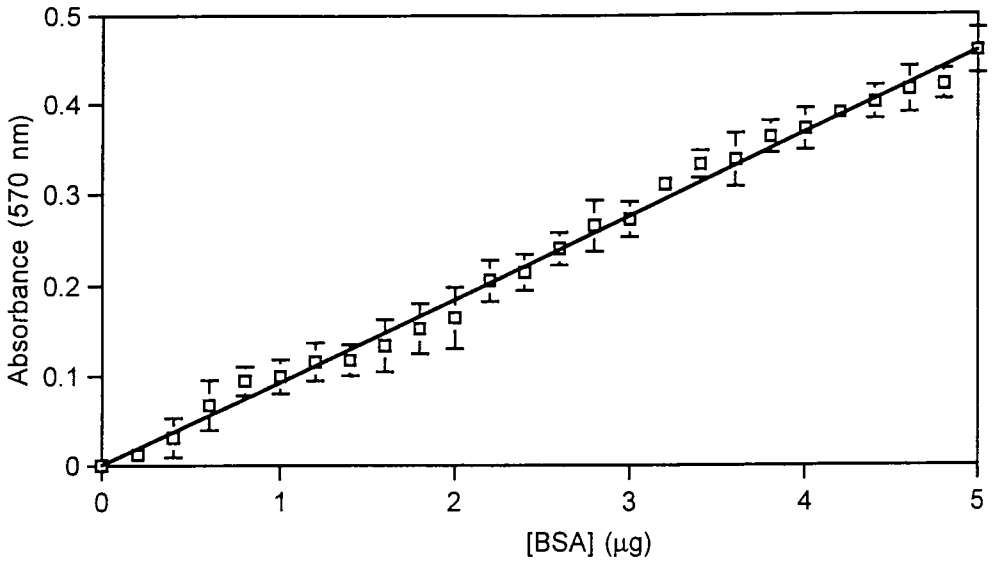


Figure 7.1. Protein determination standard curve.
A serial dilution of BSA (0.2-5 µg) was prepared in distilled water and a Bradford assay was performed as described in section 3.10. with 3 replicates for each dilution. The absorbance was measured on a Dynatech MR5000 microtitre plate reader at 570 nm. Error bars show mean ± standard errors.

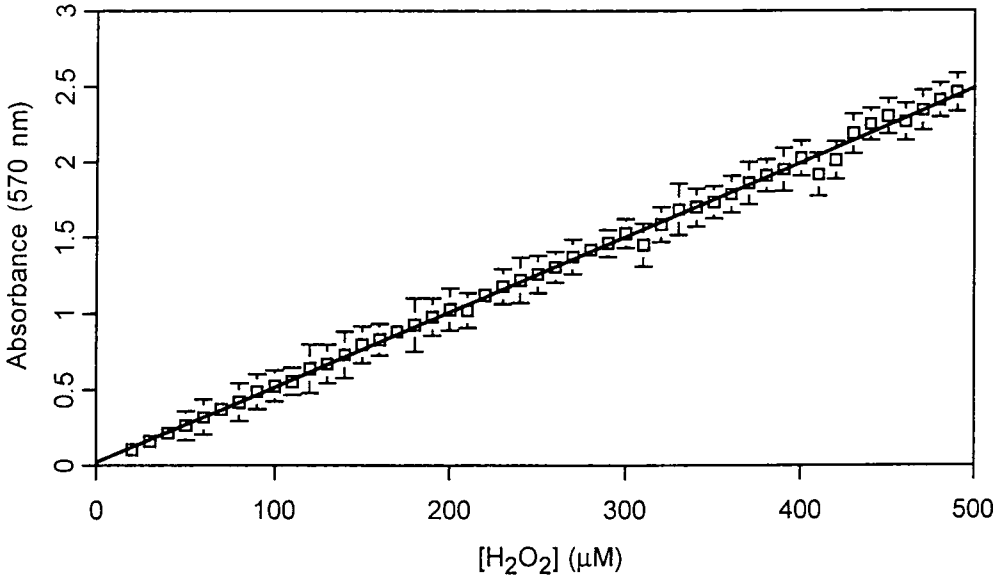


Figure 7.2. Hydrogen peroxide concentration standard curve.
A serial dilution of hydrogen peroxide was prepared in distilled water so that the final concentrations in the assay were in the range of 0-500 µM. Three replicates were prepared for each dilution and assayed using the oxalate oxidase enzyme assay described in section 3.11.4. The assays were measured at 570 nm on a Dynatech MR5000 microtitre plate reader. Error bars show the mean ± standard errors.

Some protein precipitation occurred during this dialysis step, but no oxalate oxidase activity was detected in the pellet.

A DE52 cellulose column was used to remove the majority of contaminating proteins still present in the G100 eluant, containing the oxalate oxidase protein. Schmitt [1991] showed that barley root oxalate oxidase had a pI of about 7.5, so it was expected that the oxalate oxidase proteins would not bind to the anion exchange column. Oxalate oxidase activity was detected in the DE52 unbound fractions (8-11, collected as 10 ml fractions) on enzyme assay stained dot blots. The majority of the contaminating proteins actually bound to the column and were eluted with the application of a gradient up to 0.5 M NaCl. Fig. 7.3. shows the traces obtained from the barley root (BR) and transgenic oilseed rape (3S1) G100 fractions dialysed and applied to the DE52 anion exchange column. The DE52 wash fractions exhibiting the highest enzyme activity (8-11) were combined, dialysed against water, lyophilised and stored at 4 °C.

SDS-PAGE analysis of the wheat and barley embryo preparations indicated that the DE52 wash fractions still contained very high levels of contaminating protein. Therefore these two samples were treated with pepsin in an attempt to remove the contaminating proteins (section 3.26.6.). Following pepsin digestion the hydrolysate was passed down a G100 column to remove the pepsin protein and any low molecular weight protein contamination resulting from the digestion procedure. The eluted fractions were collected and tested for enzyme activity on dot blots (high levels of activity were detected), the fractions exhibiting the highest oxalate oxidase activity were combined, dialysed against water, lyophilised and stored at 4 °C.

Fig. 7.4. shows a silver stained 12% (w/v) SDS-PAGE gel of commercial barley seedling oxalate oxidase obtained from Sigma (Poole, Dorset, U.K.). The bands representing monomeric and pentameric oxalate oxidase in the heat (+) and non-heat (-) treated samples were barely visible and are indicated by arrows. The enzyme supplied by Sigma was purified according to the method of Sugiura *et al.* [1979], which resulted in an enzyme preparation containing high levels of contamination, which can be clearly seen in Fig. 7.4.

Aliquots of the purified protein samples were analysed on 12% (w/v) SDS-PAGE gels and silver stained to detect the oxalate oxidase proteins and any contaminating proteins still present in the samples (Fig. 7.5.). For each of the six protein samples (barley roots, barley embryos, wheat embryos, 3S1, C26 and SGS5) the oxalate oxidases appeared to be purified to homogeneity, with little if any contamination, particularly when compared with the Sigma preparation (Fig. 7.4.). The oligomeric form of the protein in the non-heat treated samples (Fig. 7.5. BR-, BE-, WE-, SGS5-) appeared to stain more intensely than the monomers in the heat treated samples (Fig. 7.5. BR+, BE+, WE+, SGS5+). This procedure resulted in the successful purification of the differentially glycosylated G and G' oxalate oxidase isoforms. The pentamers were clearly visible in the barley root, barley embryo, wheat embryo and SGS5 tobacco non-heat treated samples (Fig. 7.5. BR-, BE-, WE-, SGS5-, respectively), as indicated by the arrows (G and G'). The G and G' isoforms were not clearly visible in the heat treated samples due to poor separation in the 20-25 kDa region of the 12% (w/v) SDS-PAGE gels (Fig 7.5. BR+, BE+, WE+, 3S1+, C26+, SGS5+). The barley embryo and wheat embryo oxalate oxidase proteins both appeared to be stable and retained their oligomeric structures in non-

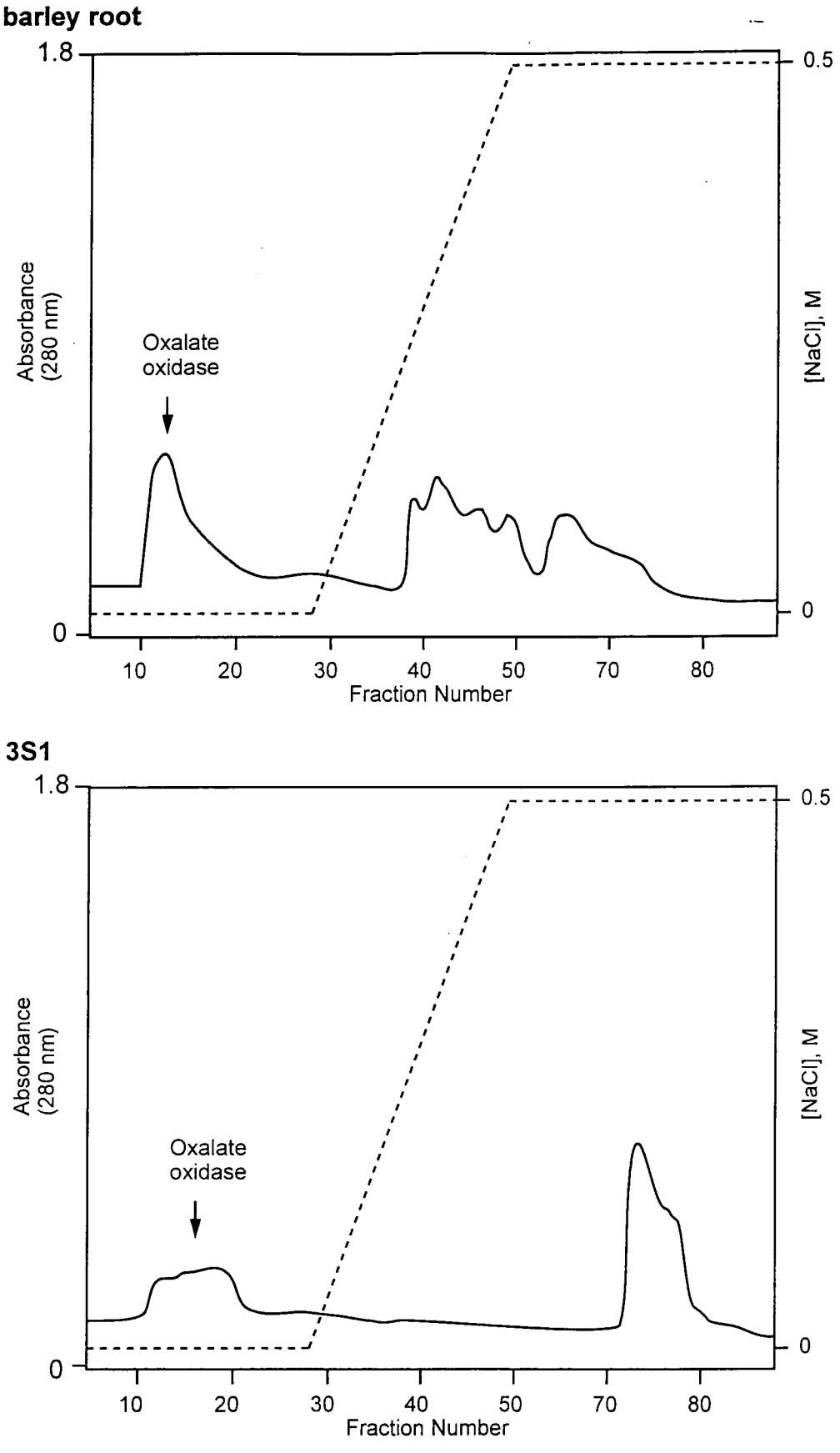
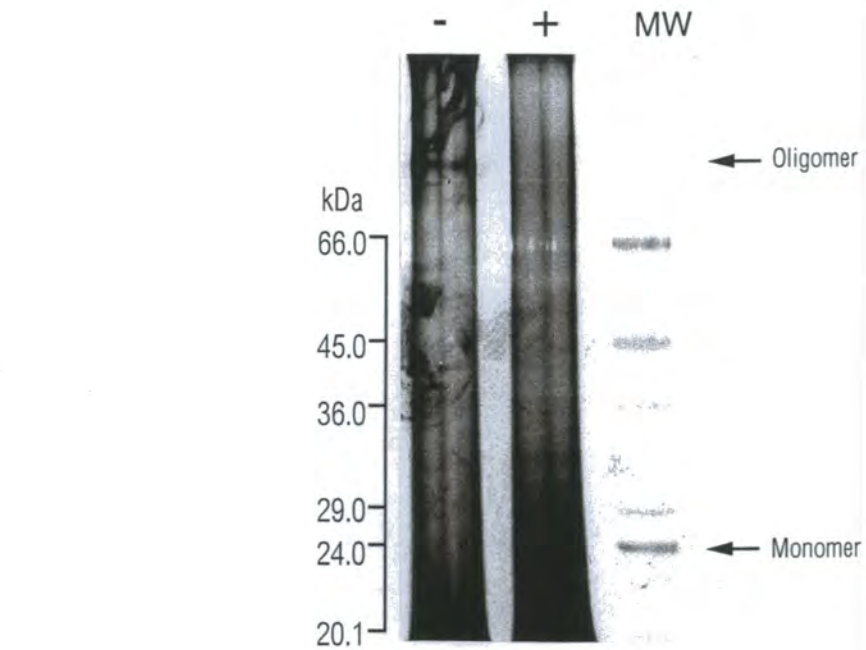
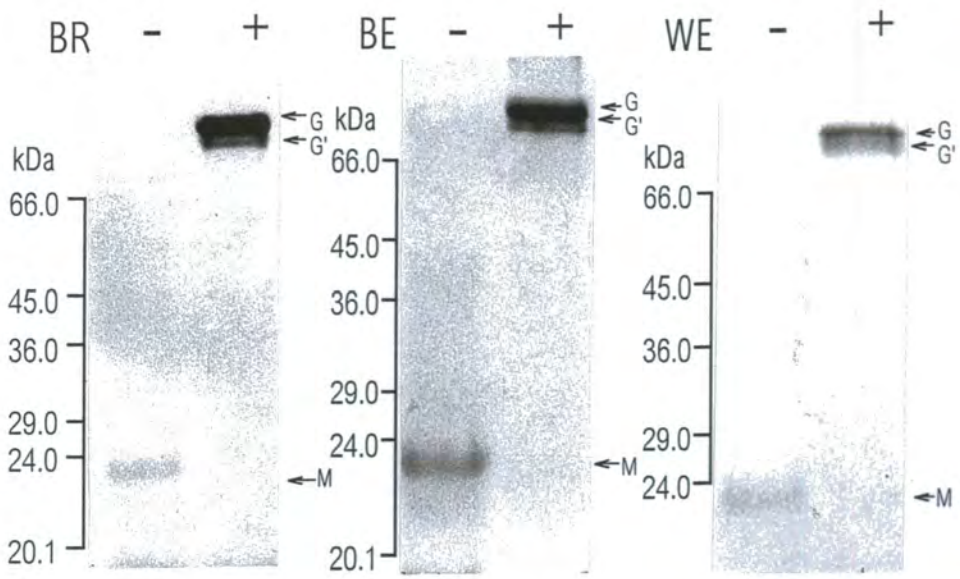


Figure 7.3. DE52 cellulose anion exchange chromatography traces.
Barley root and transgenic 3S1 oilseed rape chromatography traces. 10 ml fractions were collected from the column (2 ml / min flow rate) using 50 mM sodium phosphate buffer pH 7.5 and a 0.5 M NaCl gradient, represented by the dashed line. Production of the gradient was controlled by a Pharmacia Biotech Gradifrac system.

Figure 7.4. Silver stained SDS-PAGE gel of barley seedling oxalate oxidase.
Samples (10 ng) of non-heat (-) and heat treated (+, 2 min at 100 °C) commercial barley seedling oxalate oxidase (Sigma, Poole, Dorset, U.K.) were loaded into the wells of a 12% (w/v) SDS-PAGE gel. Proteins were detected using the Bio-Rad silver staining kit. MW, molecular weight marker. The arrows indicate oligomeric and monomeric oxalate oxidase.

Figure 7.5. Silver stained SDS-PAGE gels of native and transgenic oxalate oxidases.
Oxalate oxidase enzymes were isolated from barley roots (BR), barley embryos (BE), wheat embryos (WE) transgenic oilseed rape (3S1), transgenic tobacco (C26 and SGS5). Samples of non-heat (-) and heat treated (+, 2 min at 100 °C) lyophilised DE52 fractions were analysed on 12% (w/v) SDS-PAGE gels and proteins were detected using the Bio-Rad silver staining kit. The arrows indicates the oligomers of the G and G' isoforms and the monomers (M).


-
+
MW
Oligomer
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Monomer


BR
-
+
G
G'
kDa
66.0
45.0
36.0
29.0
24.0
20.1
M
BE
-
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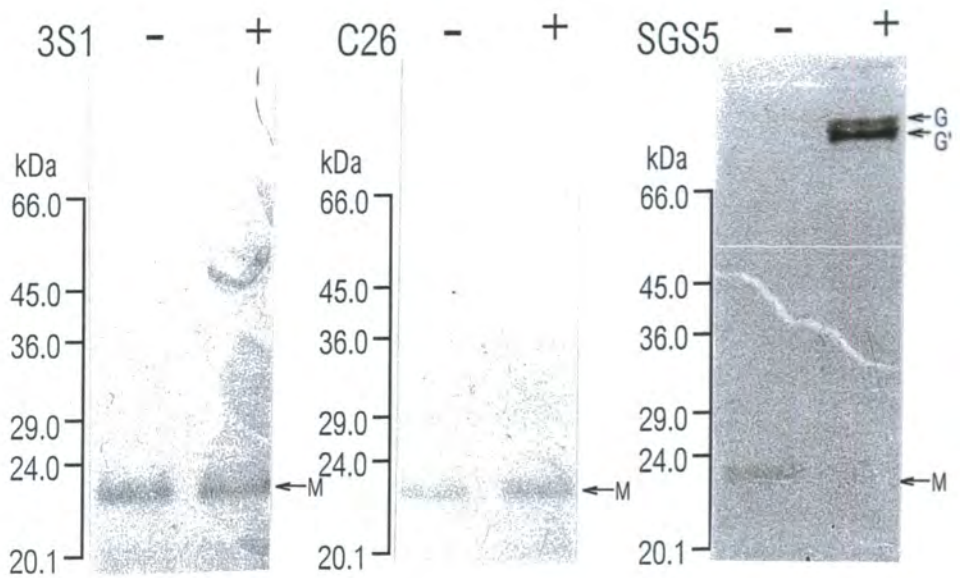
3S1
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66.0
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24.0
20.1
M
SGS5
-
+
G
G'
kDa
66.0
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36.0
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24.0
M

heat treated samples following pepsin digestion (Fig. 7.5. BE- and WE-, respectively), which was in agreement with the results obtained by Grzelczak and Lane [1984, 1985] following proteolysis of wheat embryo oxalate oxidase.

Oxalate oxidase purification from barley root was probably the most successful, resulting in the isolation of several mg of pure protein. The single purifications of each of the 6 oxalate oxidase proteins provided sufficient protein for all of the structural and kinetic studies. Table 7.1 illustrates the complete purification procedure for barley root oxalate oxidase.

Fraction	Volume (ml)	Protein (mg/ml)	Enzyme (units/ ml)	Total enzyme (units)	Specific activity (u/mg)	Step purification factor	Step yield (%)
A	725	0.260	10.5	7613	40.4	1	100
B	7	9.210	3822.0	26754.0	415.0	10.27	351.4
C	30	0.861	395.0	11850.0	458.8	1.11	44.3
D	35	0.704	458.5	16048.0	651.3	1.42	135.0
E	27.25	0.066	128.0	3488.0	1939.4	2.98	21.7
F	35.5	0.051	572.5	20324.0	11225.5	5.79	582.6

Table 7.1. Barley root oxalate oxidase purification table.
A, crude total protein extract; B, 70% (relative saturation) $(NH_4)_2SO_4$ resuspended pellet; C, G100 column combined eluant fractions; D, dialysed G100 column eluant fractions; E, DE52 fractions; F, dialysed DE52 fractions.

One of the major problems encountered using this purification procedure was the reliability of the monitoring of the levels of enzyme retrieved compared with the levels of contaminating proteins present. The fluctuation in oxalate oxidase enzyme activity at the different purification steps was probably due to the presence of NaCl as the major buffer component at each of the purification stages. Cloride ions were previously reported to be an inhibitor of oxalate oxidase enzyme activity [Suzuki and Meeuse 1965]. For example, total oxalate oxidase activity in the initial protein extracts (Table 7.1. row A) should have been at their highest levels than at any other stage in the purification. However, the levels of detectable enzyme activity in these extracts were in some case (i.e. in the cereal total extracts) almost non-existent, presumably as a result of using extraction buffer containing 400 mM NaCl. The lack of detectable enzyme activity may also have occurred due to the complexing of oxalate oxidase with compound released into the total extracts, resulting in enzyme inhibition. Chiriboga [1966] also encountered problems with measuring enzyme activity during purification, particularly in crude extracts of barley roots. NaCl was included in the G100 column buffer to prevent protein-matrix interactions which were previously observed when protein samples containing oxalate oxidase were analysed on a superose 12 gel filtration column with a low NaCl buffer concentration (50 mM). The presence of 200 mM NaCl in the G100 column buffer was sufficient to prevent these interactions, which would have

resulted in loss of yield and column contamination at this purification step. Enzyme activity levels were found to increase once the column fractions were dialysed against buffer which did not contain NaCl (e.g. specific activities of 1936 and 11226 in the DE52 combined wash fractions before and after dialysis against distilled water, respectively).

Protein	Specific activity (u / mg)	Purification factor	Yield (%)
barley root	11225.5	282.8	267.0
barley embryo	519.6	56.8	121.3
wheat embryo	5132.8	69.7	166.6
3S1 oilseed rape	3477.0	261.8	23.7
C26 tobacco	11778.6	132.5	18.7
SGS5 tobacco	825523.8	49.6	82.5

Table 7.2. Overall purification data for dialysed DE52 fractions.

Table 7.2. illustrates the final specific activity, purification factor and yield (%) of each of the purified oxalate oxidase proteins, however these results are unreliable as a means of determining which of the purification steps are the most effective because of NaCl inhibition of the oxalate oxidases observed at various stages in the purification protocol. According to this data the purification of oxalate oxidase from transgenic 3S1 oilseed rape and C26 leaf INF were the least successful. The only reliable method for confirming the homogeneity of the purified oxalate oxidase proteins was to analyse them on SDS-PAGE gels (Fig 7.5.). Taking into account the problems of monitoring the efficiency of the various purification steps, the combination of methods used in this protocol appeared to be highly successful at purifying oxalate oxidase from all sources to homogeneity.

8. RESULTS AND DISCUSSION.

Structure and stability of the native and transgenic oxalate oxidases.

The results described in this chapter relate to experiments carried out to study the comparative structural properties and stability of the native cereal (barley root, barley embryo and wheat embryo) and the transgenic oxalate oxidases (3S1 oilseed rape, C26 and SGS5 tobacco). Reports published by other research groups were used as a basis for designing experiment to determine the structural properties of the native and transgenic oxalate oxidases. The majority of the published methods were adapted to optimise the experimental conditions.

8.1. Oxalate oxidase secondary structure.

This work was performed in collaboration with Peter Wilde and Fiona Husband at the Institute of Food Research (Norwich, U.K.).The secondary structure parameters of native barley root and transgenic 3S1 oilseed rape oxalate oxidase proteins were examined using circular dichroism (CD) spectroscopy. CD spectroscopy is highly sensitive to changes in the secondary and tertiary structure of proteins and therefore the effect of various treatments on the ordered conformation and stability of proteins can be examined, for example the effect of heat treatment and SDS. The CD results described in this chapter are likely to be more accurate than those previously published by McCubbins *et al.* [1987], because their CD spectra for wheat embryo oxalate oxidase only went down to a wavelength of 200 nm as opposed to the CD spectra in this study which went down to a wavelength of 180 nm. Improved accuracy regarding the secondary structure of proteins is obtained if data is available to wavelengths as short as 180 nm [Manning 1993]. The secondary structures of the proteins were described in terms of α-helix, β-sheet and aperiodic. The term aperiodic is used in preference to the term 'random' since the process of protein folding appears to follow folding pathways producing defined structures as opposed to 'random' which suggests that the protein structure is not defined [Fiona Husband, personal communication]. The selcon program used in this study to define protein secondary structure from the CD spectra included β-turns within the definition of aperiodic. The proteins used in these experiments were purified to homogeneity, as described in section 3.26. The results of these experiments made it possible to compare the secondary structures of native and transgenic oxalate oxidases. The initial experiments carried out involved taking measurements of the CD spectra of a range of concentrations of barley root oxalate oxidase at different pathlengths, to ensure that the optical densities (OD) of the protein samples were kept at around 1.0-1.5 OD units. Fig. 8.1. illustrates the CD spectra generated over the range of barley root oxalate oxidase concentrations and pathlengths evaluated.

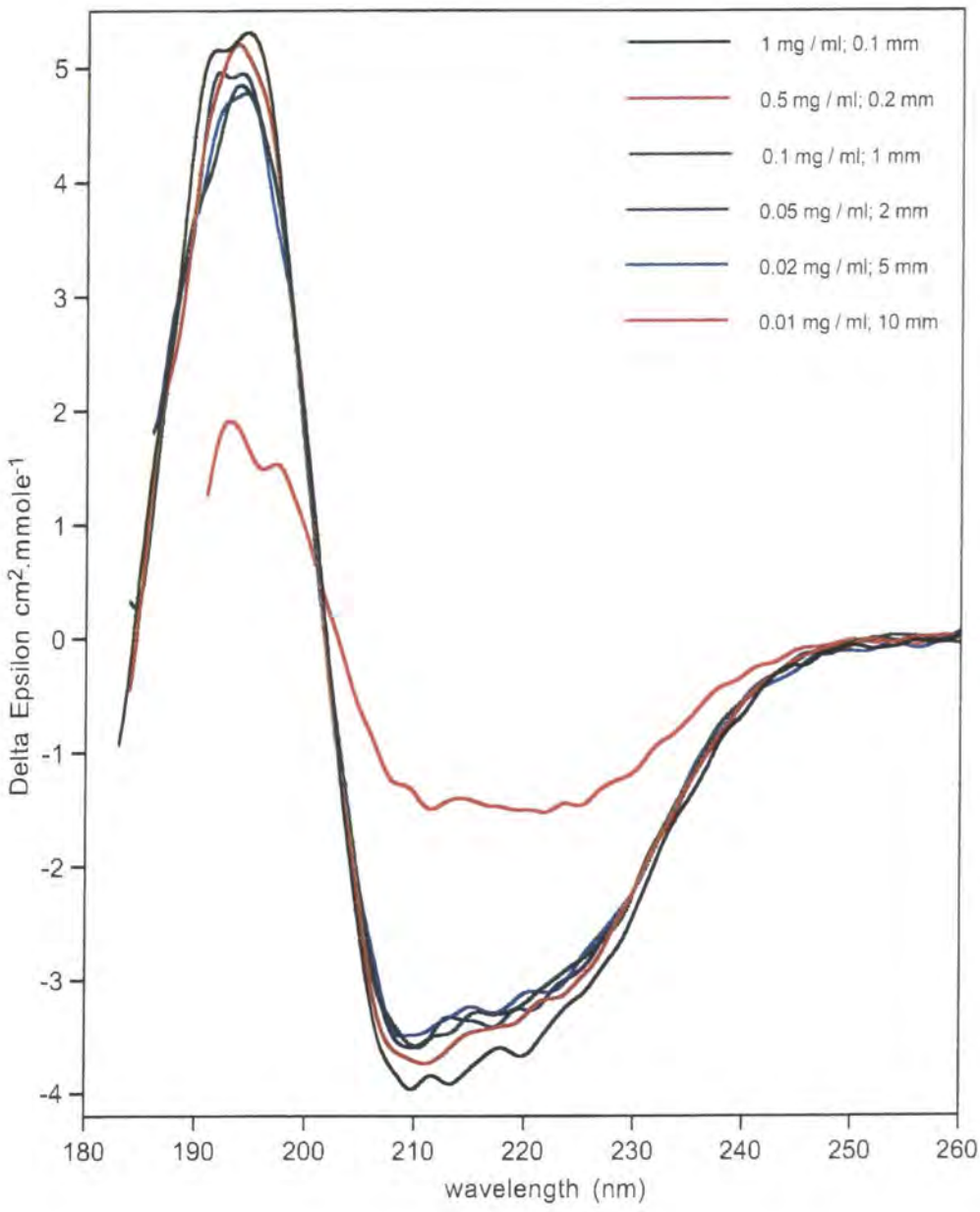


Figure 8.1. CD spectra of different barley root oxalate oxidase protein concentrations.
Barley root oxalate oxidase was diluted with 10 mM Na_2HPO_4 pH 7.0 to give a range of protein concentrations (0.01 - 1 mg / ml), which were measured at various pathlengths (0.1 - 10 mm). Measurements were taken over the range of 180 - 260 nm, as described in section 3.28.

Concentration (mg / ml)	Pathlength (mm)	α-helix (%)	β-sheet (%)	aperiodic (%)
1.0	0.1	34.2	18.0	47.4
0.5	0.2	34.3	18.7	47.1
0.1	1.0	33.7	19.7	47.9
0.05	2.0	36.2	19.2	48.0
0.02	5.0	36.2	17.7	45.1
0.01	10.0	16.4	27.2	36.5

Table 8.1. Effect of protein concentration on the predicted secondary structure of barley root oxalate oxidase.

Barley root oxalate oxidase consisted of an average of 35% α-helix 19% β-sheet and 47% aperiodic, over the higher range of protein concentrations (Table 8.1. 0.02 - 1 mg / ml). The CD for barley root oxalate oxidase at the lowest protein concentration (0.01 mg / ml) changed considerably to approximately 16% α-helix, 27% β-sheet and 37% aperiodic compared with the other protein concentrations tested (Fig. 8.1 and Table 8.1.). This apparent change in secondary structure is a phenomenon often observed with CD spectroscopy at low protein concentrations [Manning 1993]. Transgenic 3S1 oilseed rape consisted of approximately 27% α-helix, 27% β-sheet and 47% aperiodic (Fig. 8.2. and Table 8.2.). Barley root oxalate oxidase contained higher levels of α-helix and lower levels of β-sheet compared with 3S1 oilseed rape. McCubbins *et al.* [1987] reported a lower α-helix content for wheat embryo oxalate oxidase in 50 mM sodium phosphate buffer (pH 7.0) (20%) and a β-sheet content of about 33%. Considering wheat embryo oxalate oxidase [McCubbins *et al.* 1987] and barley root oxalate oxidase (used in this study) share 96% protein sequence identity the CD spectra generated for these two proteins were significantly different. This may have resulted from the fact that the CD spectra generated by McCubbins *et al.* [1987] only went down to 200 nm and so information regarding α-helix structures in the protein was lost, emphasising the β-sheet content of the wheat embryo protein.

A concentration of 0.5 mg / ml and a pathlength of 0.2 mm gave suitable OD readings for the barley root and 3S1 oilseed rape oxalate oxidases (Fig. 8.1. and 8.3.). CD measurements were also attempted on C26 and SGS5 transgenic tobacco oxalate oxidases. However, during the course of these experiments at IFR Norwich these were unsuccessful, as the proteins appeared to precipitate out of solution in the presence of 10 mM sodium phosphate pH 7.0. A phenomenon which was not previously observed with any of these protein preparations at Durham.

CD was also carried out on the native and transgenic oxalate oxidases following heat treatment (30 min at 22, 60 or 80 °C). These experiments were performed to determine if

there were any differences in the stability of the native and transgenic protein secondary structures following heat treatment.

Protein sample	temperature (°C)	α-helix (%)	β-sheet (%)	aperiodic (%)
barley root	22	34.3	18.7	47.1
barley root	60	21.6	35.7	53.6
barley root	80	21.4	25.8	58.0
3S1 oilseed rape	22	27.2	27.4	46.6
3S1 oilseed rape	60	21.4	31.8	54.0
3S1 oilseed rape	80	22.8	28.9	52.3

Table 8.2. Effect of heat treatment on the predicted secondary structure of barley root and transgenic 3S1 oilseed rape oxalate oxidases.
Measurements were taken using a fixed protein concentration (0.5 mg / ml) and pathlength (0.2 mm). The protein samples were heat treated for 30 min at 22, 60 or 80 °C.

Heat treatment had a more significant effect on the secondary structure of barley root oxalate oxidase (Fig. 8.2.) compared with that from 3S1 oilseed rape (Fig. 8.3.). For both proteins an increase in temperature led to a loss of α-helix and an increase in the proportion of β-sheet and aperiodic structure (Table 8.2.). The levels of α-helix in the native and transgenic proteins following heat treatment at 60 and 80 °C were very similar. The levels of β-sheet in the two proteins were both higher following 60 °C treatment compared with the levels calculated in the proteins treated at 80 °C for 30 min. The change in secondary structure of the oxalate oxidases with increasing temperature presumably resulted from dissociation of the proteins. The effect of heat treatment on the structure of the native and transgenic oxalate oxidase is discussed later in this chapter. According to the results of McCubbins *et al.* [1987] following heat treatment of wheat embryo oxalate oxidase (73 °C for 10 min and 100 °C for 2 min) the computed secondary structure contents of this protein did not change.

CD was also carried out on the native and transgenic oxalate oxidases following treatment with SDS (0, 0.05. 0.1 or 0.2% (w/v)). Barley root oxalate oxidase was previously shown to be enzymatically stable to this treatment when analysed on an SDS-PAGE gel (in 1 x loading buffer containing 1% (w/v) SDS, Fig. 7.5. BR-) with only low levels of dissociation of the pentamer to the monomer in non-heat treated samples, compared with 3S1 oxalate oxidase which completely dissociated in 1% (w/v) SDS (Fig 7.5. 3S1-). These CD experiments were performed to determine if there were any changes in the stability of the native and transgenic oxalate oxidase protein secondary structures following treatment with SDS.

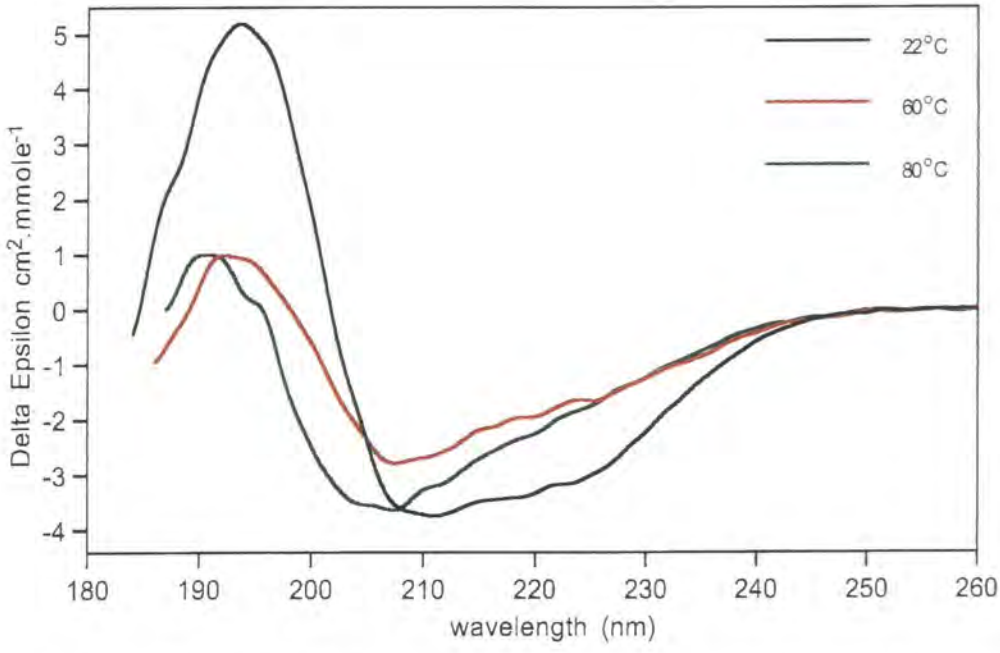


Figure 8.2. Effect of heat treatment on the secondary structure of barley root oxalate oxidase.
Barley root oxalate oxidase (0.5 mg / ml) was measured over the range of 180 - 260 nm (0.2 mm pathlength), as described in section 3.28. The protein samples were heat treated for 30 min at the 22, 60 or 80 °C.

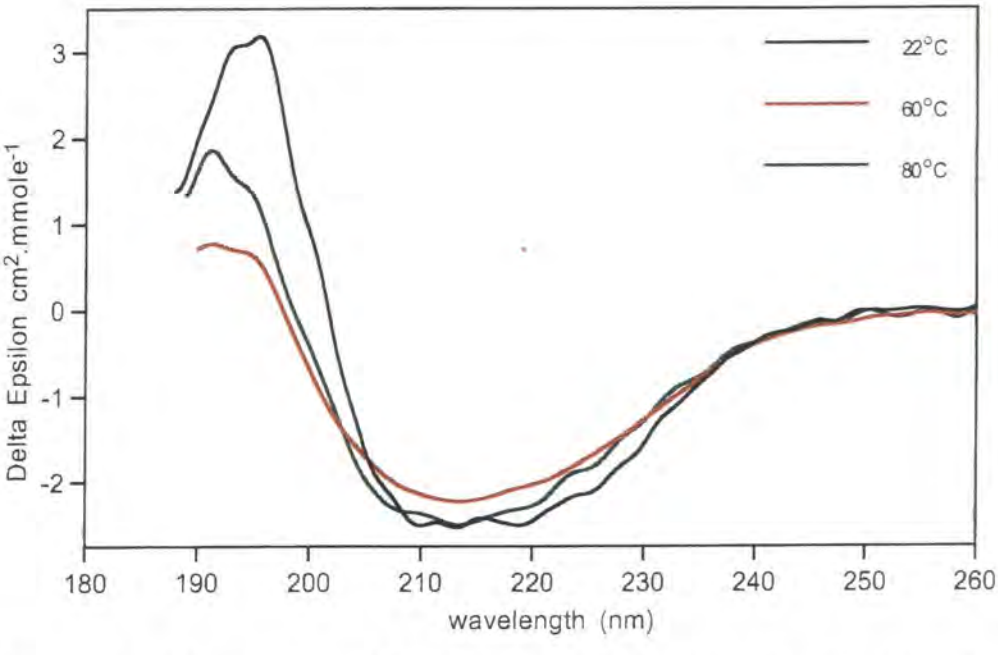


Figure 8.3. Effect of heat treatment on the secondary structure of transgenic 3S1 oilseed rape oxalate oxidase.
Transgenic 3S1 oilseed rape oxalate oxidase (0.5 mg / ml) was measured over the range of 180 - 260 nm (0.2 mm pathlength), as described in section 3.28. The protein samples were heat treated for 30 min at the 22, 60 or 80 °C.

Protein sample	SDS (% (w/v))	α-helix (%)	β-sheet (%)	Aperiodic (%)
barley root	0.0	34.3	18.7	47.1
barley root	0.05	31.5	18.0	48.0
barley root	0.1	28.2	23.7	48.0
barley root	0.2	32.1	19.7	49.5
3S1 oilseed rape	0.0	27.2	27.4	46.6
3S1 oilseed rape	0.05	27.8	22.6	48.5

Table 8.3. Effect of SDS treatment on the predicted secondary structure of barley root and transgenic 3S1 oilseed rape oxalate oxidases.
Measurements were taken using a fixed protein concentration (0.5 mg / ml) and pathlength (0.2 mm). SDS was added to the protein immediately prior to measuring the sample.

These results showed that barley root oxalate oxidase was very resistant to structural change following SDS treatment between 0-0.2% (w/v) and there was no significant change in secondary structure of this protein (Fig. 8.4. and Table 8.3.). 3S1 oilseed rape oxalate oxidase was also largely unaffected by 0.05% (w/v) SDS treatment (Fig. 8.5. and Table 8.3.). However, increasing the concentration of SDS above 0.05% (w/v) led to the precipitation of the protein, which meant that no further data was generated regarding the effect of SDS on this protein. McCubbins *et al.* [1987] did not report any significant changes in the secondary structure of wheat embryo oxalate oxidase following SDS (about 0.75% (w/v)) treatment at pH 6. However, at pH 2 SDS treatment of the protein led to a significant increase in α-helix content (61%) and a reduced β-sheet content (7%). Some proteins that are rich in β-sheet can actually be converted into helices in excess SDS solutions particularly at low pH, which make it difficult to interpret changes in protein conformation [Wu *et al.* 1981; Manning 1993].

The CD data generated suggested that there were differences in the secondary structure of the native barley root and transgenic 3S1 oilseed rape oxalate oxidases. In general, the transgenic protein had a lower α-helix content compared with the native protein. This difference in the secondary structure may have accounted for the reduced stability of the transgenic 3S1 oxalate oxidase compared with the native barley root protein, as the precise secondary structure content of the native protein presumably plays an important role in maintaining the unusual structural stability of oxalate oxidase.

8.2. Molecular weight determination of the native and transgenic oxalate oxidases.

8.2.1. Gel filtration chromatography and SDS-PAGE analysis.

An important question that was addressed in this study concerned the molecular weights of the native and transgenic oxalate oxidases and the level of oligomerisation of these proteins. Superose 12 gel filtration column chromatography using the Pharmacia Smart

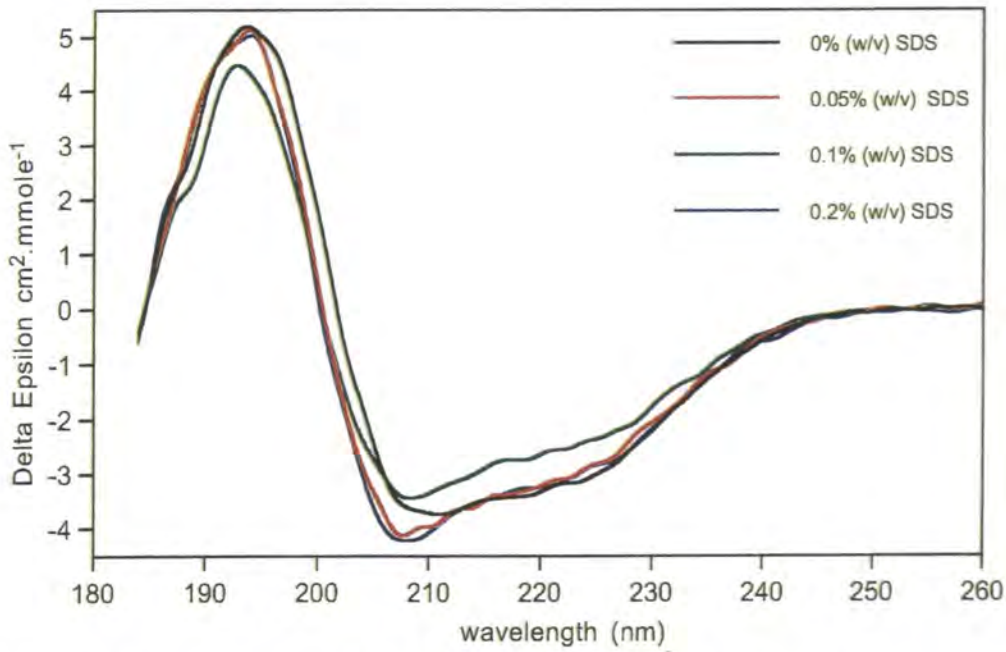


Figure 8.4. Effect of SDS treatment on the secondary structure of barley root oxalate oxidase.
Barley root oxalate oxidase (0.5 mg / ml) was measured over the range of 180 - 260 nm (0.2 mm pathlength), as described in section 3.28. The protein samples were treated with 0, 0.05. 0.1 or 0.2% (w/v) SDS.

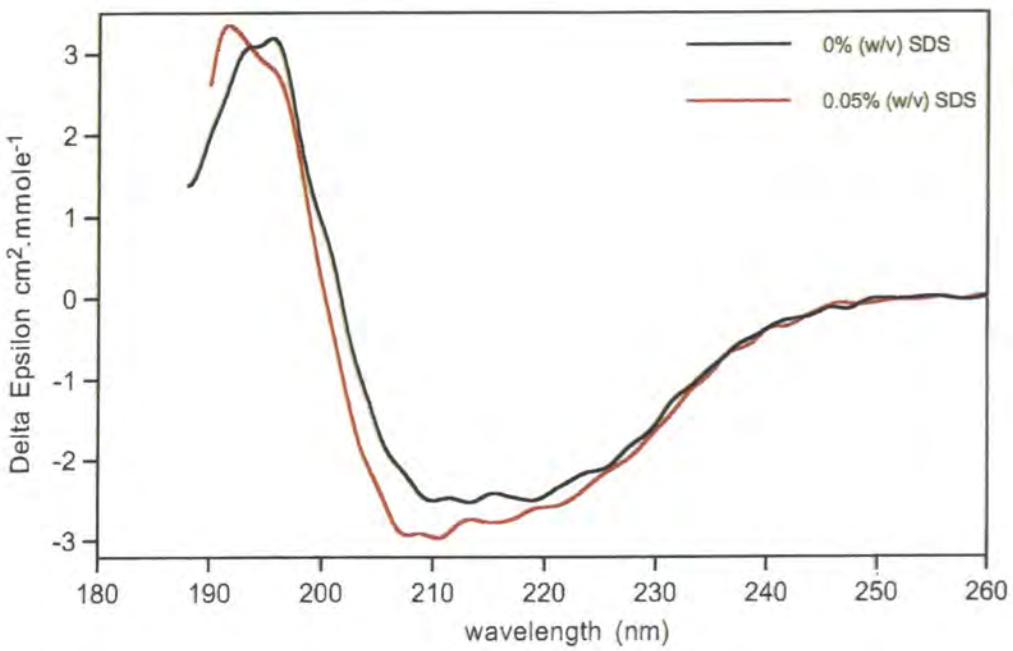


Figure 8.5. Effect of SDS treatment on the secondary structure of 3S1 transgenic oilseed rape oxalate oxidase.
3S1 transgenic oilseed rape oxalate oxidase (0.5 mg / ml) was measured over the range of 180 - 260 nm (0.2 mm pathlength), as described in section 3.28. The protein samples were treated with 0 or 0.05% (w/v) SDS.

system (FPLC) provided evidence to demonstrate that all of the native and transgenic oxalate oxidases had similar molecular weights of approximately 110-120 kDa and therefore that all of these proteins existed as oligomers. Figure 8.6. shows the OD trace (at 280 nm) obtained when a sample of barley root extract was separated on a superose 12 gel filtration column. The eluted fractions were tested for oxalate oxidase activity (Fig. 8.7.A) using the microtitre plate enzyme assay method, as described in section 3.11.4. Fig. 8.7.A illustrated that the strongest activity for barley root oxalate oxidase was detected in fractions 12 and 13 corresponding to a molecular weight of approximately 110-120 kDa, which corresponded to the oligomeric form of the enzyme. The molecular weights of the oxalate oxidases were calculated from a series of standard proteins with known molecular weights which were chromatographed on the superose 12 column under identical experimental conditions. Fig. 8.7.B illustrates a dot blot of the superose 12 column fractions of chromatographed 3S1 INF, stained to detect oxalate oxidase activity, which was detected in column fractions 12-14. The gel filtration experiments demonstrated that all the native and transgenic proteins had similar molecular weights, as each oxalate oxidase protein was eluted from the column in fractions 11-14. All of the native and transgenic oxalate oxidases exhibited highest enzyme activity in fractions 12-13, corresponding to a molecular weight of approximately 110-120 kDa. Gel filtration chromatography had demonstrated that all of the native and transgenic enzymes were oligomeric and exhibited oxalate oxidase activity . However, the results of the gel filtration chromatography only provided a very rough estimation of the molecular weights of the oxalate oxidase oligomers (i.e. between 110 and 120 kDa)

The superose 12 gel filtration fractions which exhibited high levels of oxalate oxidase activity (fractions 12 - 15) were pooled. The pooled column fractions were then analysed on 12% (w/v) SDS-PAGE gels. The gels were either, silver stained to detect general protein composition (Fig. 8.8.A, 8.9.A and 8.10.A), electroblotted onto nitrocellulose membranes and stained to detect oxalate oxidase enzyme activity (Fig. 8.8.B, 8.9.B and 8.10.B) or western blotted and reacted with anti-oxalate oxidase antibodies to detect the oxalate oxidase polypeptides (Fig. 8.8.C, 8.9.C and 8.10.C).

Fig. 8.8. shows the barley root (BR) and barley embryo (BE) oxalate oxidase proteins. These experiments enabled a comparison of the expression of oxalate oxidase at different developmental stages in this single species (3 day old germinated embryo and 10 day old barley roots). It was clear that these two tissues both appeared to express the G and G' isoforms, the two differentially glycosylated oxalate oxidase isoforms found in germinating wheat embryos [Lane *et al.* 1986 1987], particularly in the heat treated samples (Fig. 8.8.A BR+ and BE+, indicated by the arrow labelled monomers) where a tight doublet was detected in the region of 22-23 kDa. Both the barley root and embryo proteins exhibited oxalate oxidase activity on an electroblot in the non-heat treated samples (Fig. 8.8.B BR- and BE-, indicated by arrow labelled pentamers). The proteins also strongly reacted with the anti-oxalate oxidase antibodies and there did not appear to be any cross-reaction with any other proteins in these samples (Fig. 8.8.C). The pentamers (Fig. 8.8.C BR- and BE-) and the monomers (Fig. 8.8.C BR+ and BE+) appeared to react with the antibodies to more or less the same extent. There

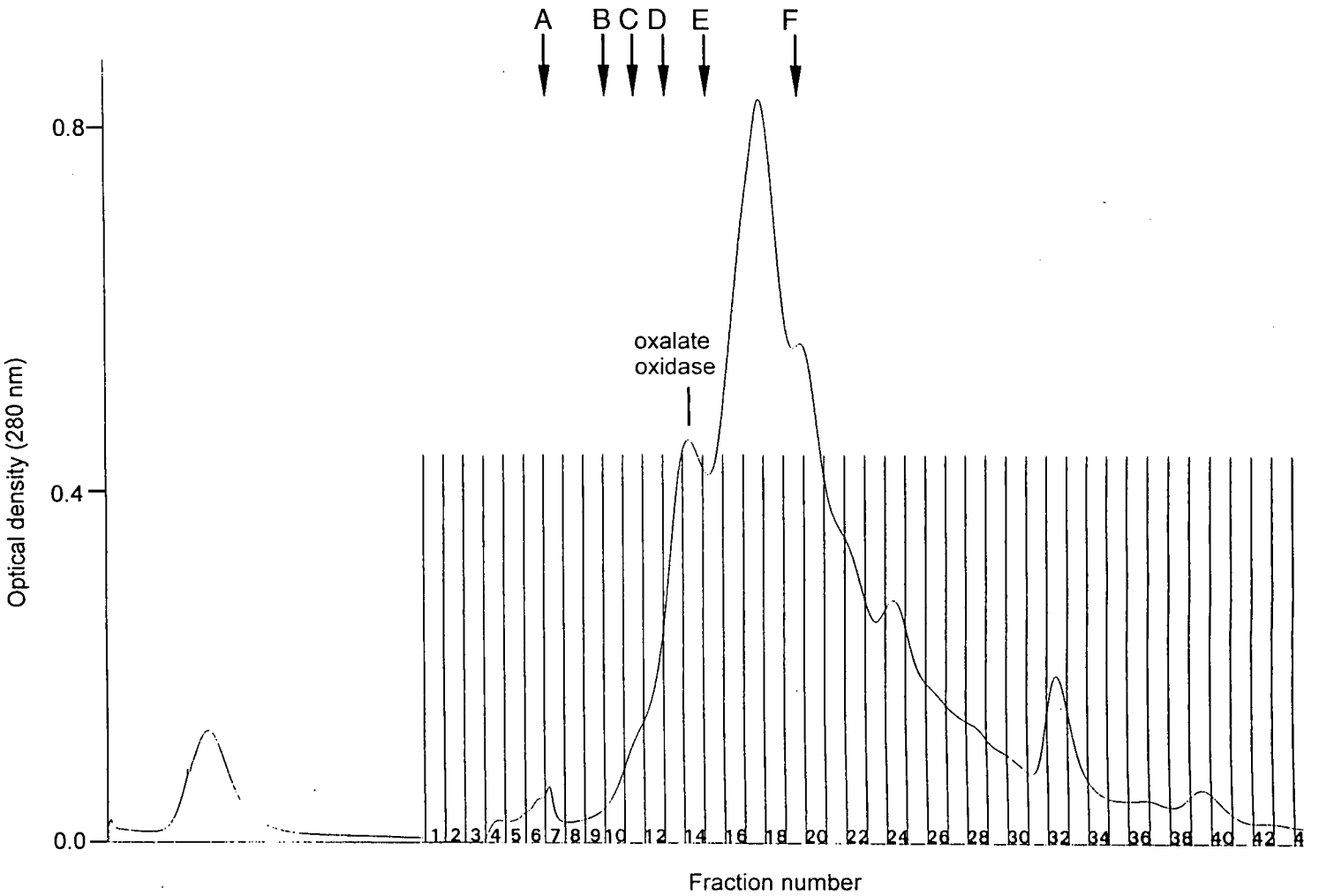


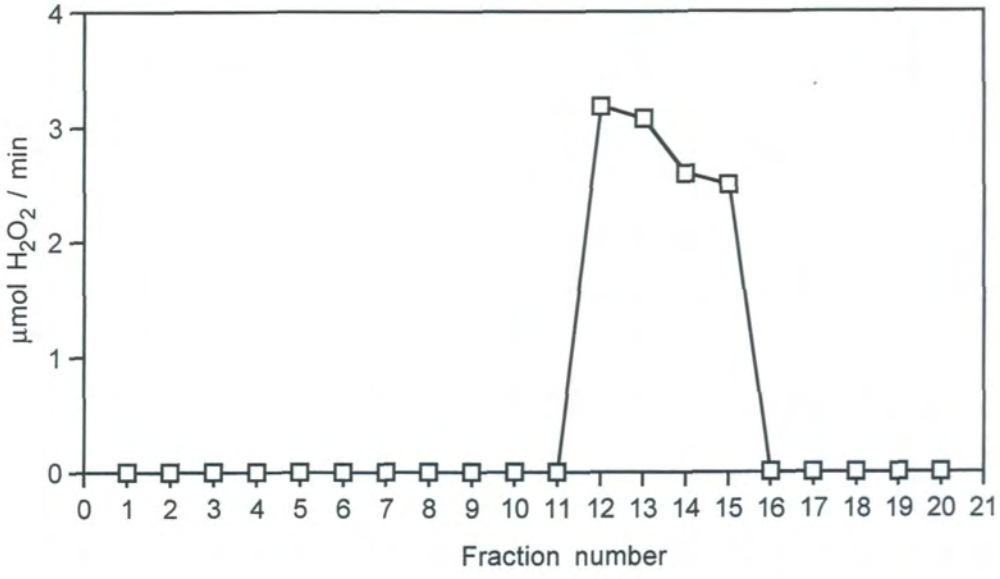
Figure 8.6. Oxalate oxidase analysis by gel filtration column chromatography.
Approximately 10 mg of the protein fraction precipitated at 70% (relative saturation) ammonium sulphate from barley root total protein extract was loaded onto a superose 12 gel filtration column at a flow rate of 40 µl / min. 50 µl fractions were collected from the column. The elution of protein from the column was monitored at 280 nm. The column was callibrated using; thyroglobulin (669 kDa, A), apoferrilin (443 kDa, B), β-amylase (200 kDa, C), alcohol dehydrogenase (150 kDa, D), BSA (66 kDa, E), ribonuclease A (13.7 kDa, F).

Figure 8.7. Detection of oxalate oxidase in gel filtration chromatography fractions.

A, Approximately 500 µg of the proteins precipitated at 70% (relative saturation) ammonium sulphate from a barley root extract was loaded onto a superose 12 gel filtration column at a flow rate of 40 µl / min. 50 µl fractions were collected from the column. 2.5 µl aliquots of each column fraction were assayed for oxalate oxidase activity using the cuvette solution enzyme assay method, described in section 3.11.2.

B, Approximately 230 µg of transgenic 3S1 oilseed rape extracellular proteins were loaded onto a superose 12 gel filtration column at a flow rate of 40 µl / min. 50 µl fractions were collected from the column. 2.5 µl of each column fraction were dot blotted onto a nitrocellulose membrane, which was stained to detect oxalate oxidase enzyme activity as described in section 3.11.1. Rows A, B, C and D columns 1-10 represent fractions 1-10, 11-20, 21-30 and 31-40, respectively.

A


µmol H_2O_2 / min
0
1
2
3
4
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Fraction number

B

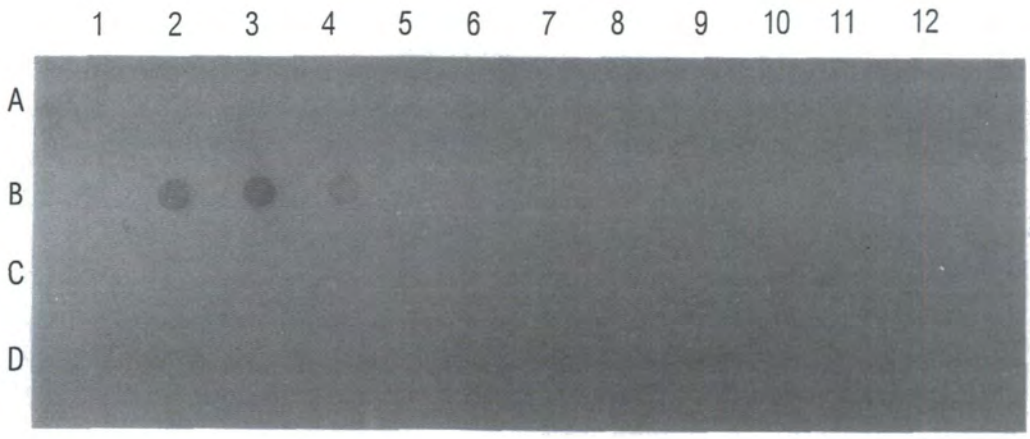

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D

Figure 8.8. Size estimation of barley root and barley embryo oxalate oxidases.
Approximately 3.19 µg of barley root (BR) and 3.11 µg of barley embryo (BE) protein fractions were analysed on a 12% (w/v) SDS-PAGE gel. The proteins were partially purified according to the superose 12 gel filtration chromatography method described in section 3.25. The proteins were loaded onto the gel with (+) and without heat treatment (-). M, molecular weight marker. The arrows indicate the oligomers and monomers of the G and G' oxalate oxidase isoforms, X indicates the additional oxalate oxidase detected in the barley root preparation.
A, silver stained SDS-PAGE analysis.

B, electroblot stained for oxalate oxidase enzyme activity, as described in section 3.11.1.

C, Western blots: anti-oxalate oxidase antiserum was used as the primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. The blot was exposed to x-ray film for 1 week at -80 °C.

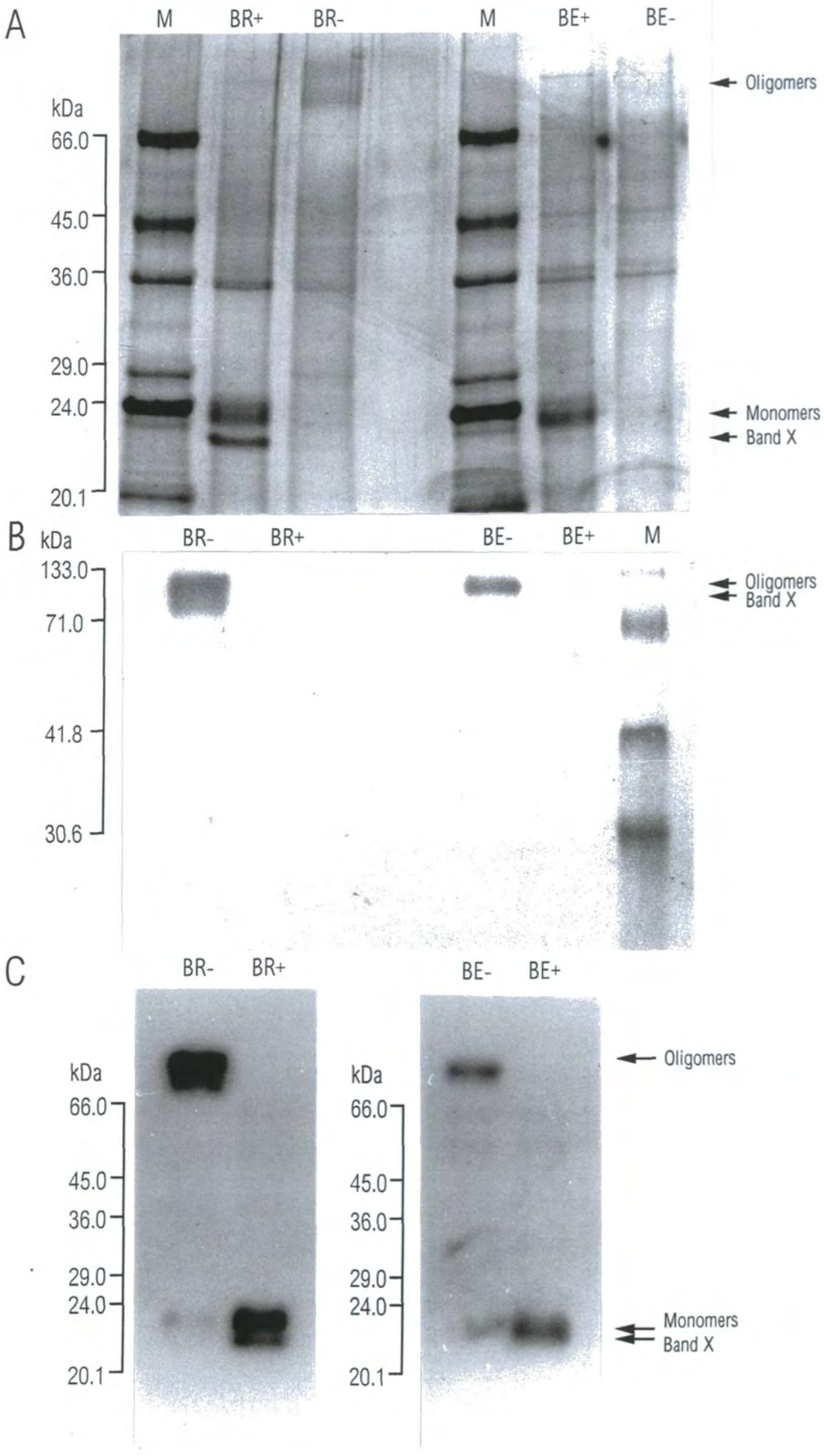

A
M
BR+
BR-
M
BE+
BE-
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Oligomers
Monomers
Band X
B
kDa
BR-
BR+
BE-
BE+
M
133.0
71.0
41.8
30.6
Oligomers
Band X
C
BR-
BR+
BE-
BE+
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Oligomers
Monomers
Band X

Figure 8.9. Size estimation of wheat embryo and transgenic SGS5 tobacco oxalate oxidases.

Approximately 3.41 µg of wheat embryo (WE) and 0.13 µg of transgenic SGS5 tobacco protein fractions were analysed on a 12% (w/v) SDS-PAGE gel. The proteins were partially purified according to the superose 12 gel filtration chromatography method described in section 3.25. The proteins were loaded onto the gel with (+) and without heat treatment (-). M, molecular weight marker. The arrows indicate the oligomers and monomers of the G and G' oxalate oxidase isoforms.

A, silver stained SDS-PAGE analysis.

B, electroblot stained for oxalate oxidase enzyme activity, as described in section 3.11.1.

C, Western blots: anti-oxalate oxidase antiserum was used as the primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. Blots were exposed to x-ray film for 1 week at -80 °C.

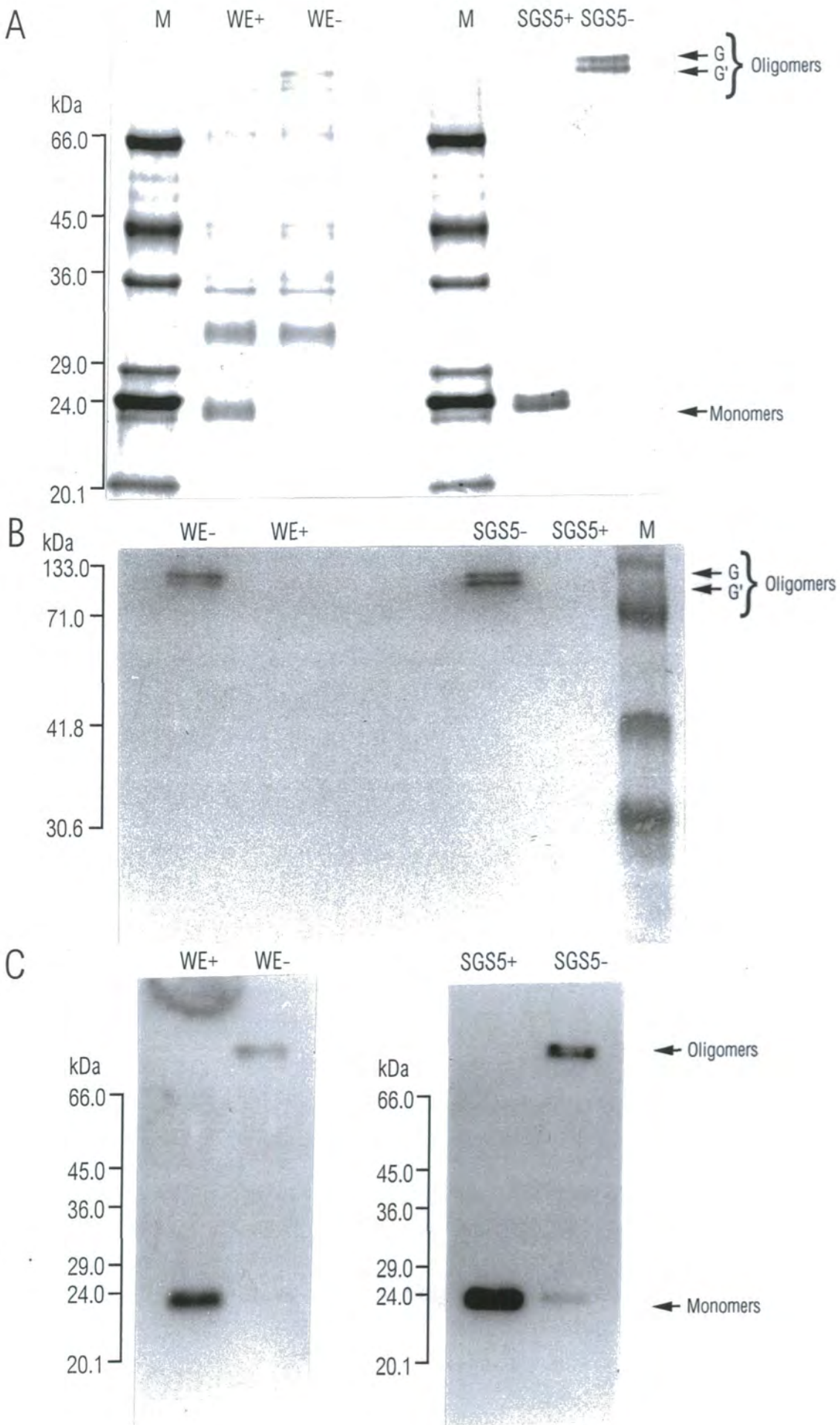

A
M
WE+
WE-
M
SGS5+
SGS5-
G
G'
Oligomers
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Monomers
B
kDa
WE-
WE+
SGS5-
SGS5+
M
133.0
71.0
41.8
30.6
G
G'
Oligomers
C
WE+
WE-
SGS5+
SGS5-
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Oligomers
Monomers

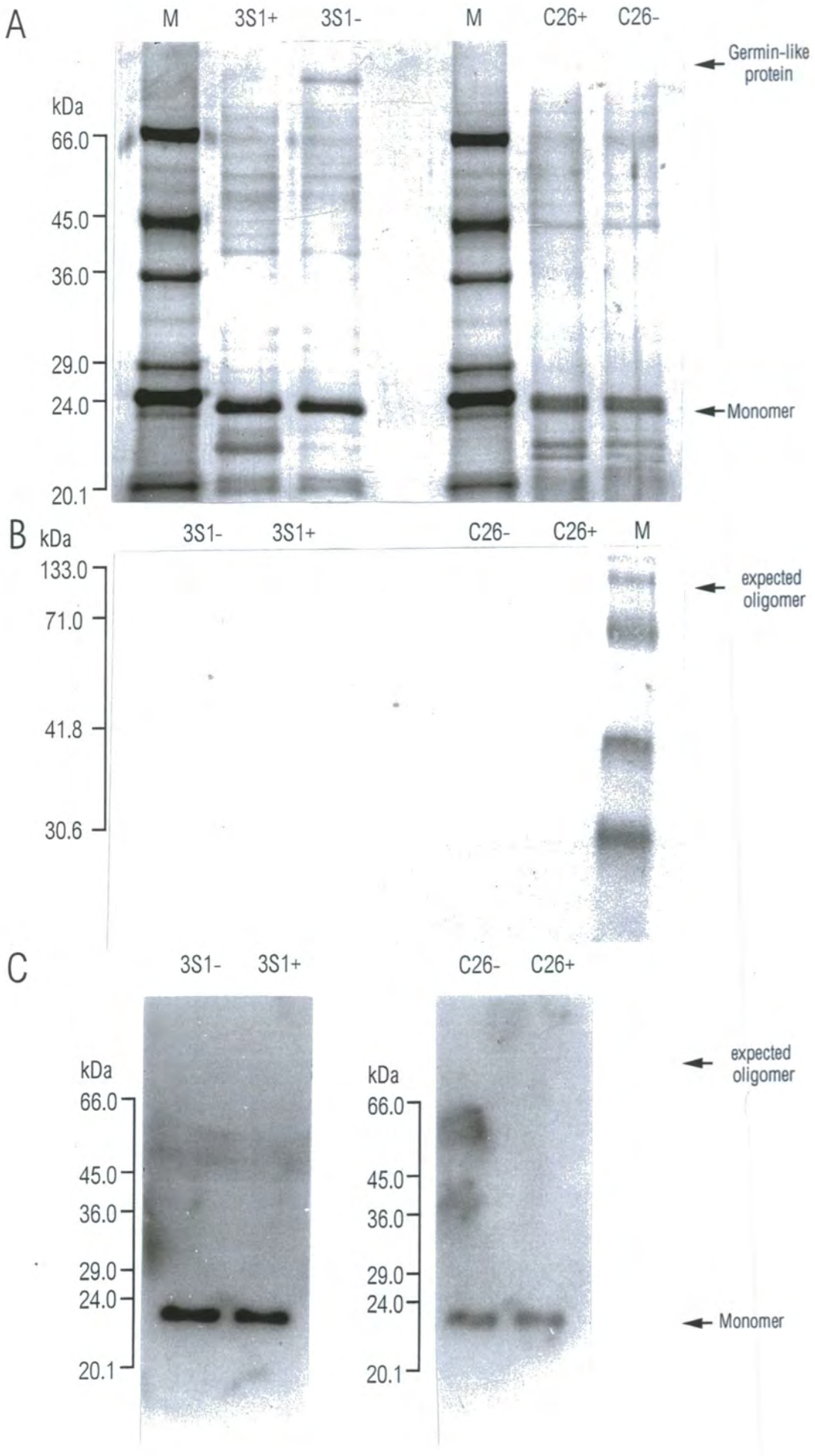
Figure 8.10. Detection of transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases.

Approximately 0.4 µg of transgenic 3S1 oilseed rape and 0.62 µg of transgenic C26 tobacco protein fractions were analysed on a 12% (w/v) SDS-PAGE gel. The proteins were partially purified according to the superose 12 gel filtration chromatography method described in section 3.25. The proteins were loaded onto the gel with (+) and without heat treatment (-). M, molecular weight marker. The arrows indicate the oxalate oxidase monomer, the expected position of the oligomer in non-heat treated samples and a oxalate oxidase-like protein.

A, silver stained SDS-PAGE analysis.

B, electroblot stained for oxalate oxidase enzyme activity, as described in section 3.11.1.

C, Western blots: anti-oxalate oxidase antiserum was used as the primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. Blots were exposed to x-ray film for 1 week at -80 °C.


A
M
3S1+
3S1-
M
C26+
C26-
Germin-like protein
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Monomer
B
kDa
3S1-
3S1+
C26-
C26+
M
133.0
71.0
41.8
30.6
expected oligomer
C
3S1-
3S1+
C26-
C26+
kDa
66.0
45.0
36.0
29.0
24.0
20.1
kDa
66.0
45.0
36.0
29.0
24.0
20.1
expected oligomer
Monomer

was one major difference observed between the proteins expressed in the two different barley tissues and this was the presence of an additional lower molecular weight oxalate oxidase protein (about 22 kDa) in the barley root sample (Fig. 8.8.A BR+, indicated by arrow X). This protein exhibited oxalate oxidase activity (Fig. 8.8.B BR-) and reacted with the anti-oxalate oxidase antibodies (Fig. 8.8.C BR- and +). It was also present at a concentration comparable to the G and G' monomeric isoforms. A corresponding protein was not detected in barley embryo following oxalate oxidase enzyme activity staining (Fig. 8.8.B BE-) or western blotting with anti-oxalate oxidase antibodies (Fig. 8.8.C BE- and +) even with a prolonged exposure (2 weeks). The separation of the G and G' isoforms of the barley samples was not particularly good on 12% (w/v) SDS-PAGE gels (Fig. 8.8.) compared with the separation of these two isoforms in the wheat embryo and SGS5 transgenic oxalate oxidases. Although previous SDS-PAGE analysis had confirmed the presence of the G and G' isoforms in barley embryos and roots (Fig. 7.5. BE and BR, respectively). The presence of this additional oxalate oxidase isoform in barley roots may have resulted from differential gene expression during development from embryo to seedling, which will be discussed further in chapter 11.

Fig. 8.9. shows the wheat embryo (WE) and transgenic SGS5 tobacco oxalate oxidase proteins. The gf-2.8 (oxalate oxidase) gene expressed in the SGS5 transgenic tobacco line originated from wheat embryo and so these experiments enabled a direct comparison of the expression of the same gene in the native and foreign host plants. The transgene was also expressed in a dicotyledonous plant and therefore a basic comparison of the protein processing mechanisms was possible, i.e. would the transgenic protein have the same structural properties when expressed in a dicotyledonous plant as in the native monocotyledonous plant. The two wheat embryo oxalate oxidase isoforms (G and G') were visible but not easily discernible as two separate bands on the silver stained SDS-PAGE gel (Fig. 8.9.A WE- and +). Whereas the two transgenic oxalate oxidase isoforms were clearly visible in the transgenic SGS5 tobacco sample (Fig. 8.9.A SGS5) in both the heat (+) and non-heat treated (-) samples (as indicated by arrows G and G'). The native and transgenic oxalate oxidases had molecular weights of approximately 22-23 kDa for the monomers and 110-120 for the pentamers. Fig. 8.9.B illustrates an electroblot stained to detect enzyme activity in the wheat embryo and transgenic SGS5 proteins, where both the G and G' isoforms were clearly demonstrated to exhibit oxalate oxidase activity. The anti-oxalate oxidase antibodies reacted much more strongly with the wheat embryo and SGS5 tobacco oxalate oxidase monomers (Fig. 8.9.C WE+ and SGS5+) compared with the pentamers (Fig. 8.9.C WE- and SGS5-), even though they were both present at the same concentration on the electroblot. These antibodies were originally raised against the monomeric (heat treated) form of barley root oxalate oxidase and may therefore have reacted more strongly with the denatured protein compared with the folded / oligomerised protein. One noticeable difference between the native and transgenic oxalate oxidases was that the G and G' isoforms in the transgenic SGS5 tobacco appeared to be expressed at similar levels (Fig. 8.9.C SGS5- and +) and they both exhibited similar levels of enzyme activity. Whereas, the G' isoform in wheat embryo was

always shown to be expressed at lower levels than G (Fig. 8.9. C WE- and +) (< 50%), which explained the lower level of enzyme activity staining of G' on the electroblot (Fig. 8.9.B WE-).

Fig. 8.10. shows the transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidase proteins. These experiments enabled a comparison of the expression of the same transgene in two different plant species. The extensin-oxalate oxidase gene construct was identical in both transgenic species. The two transgenic proteins were shown to be present at high concentrations (Fig. 8.10.A 3S1 and C26), with monomer molecular weights of about 22-23 kDa. The estimated 3S1 and C26 pentameric molecular weights from chromatography were about 110-120 kDa. In contrast to the situation with native and transgenic SGS5 proteins the 3S1 and C26 pentamers underwent complete dissociation to the monomers when analysed on SDS-PAGE gels (Fig. 8.10.A, the arrow indicates the approximate position of where the pentamers were expected to be). The 3S1 and C26 transgenic plants only appeared to express a single transgenic protein (Fig. 8.10.A 3S1 and C26- and +) and not the G and G' doublet. Oxalate oxidase enzyme activity was not detected in the non-heat treated transgenic protein samples (Fig. 8.10.B 3S1- and C26-), due to the dissociation of the active pentamers to monomers in the presence of SDS. The anti-oxalate oxidase antibodies reacted strongly with the transgenic 3S1 and C26 monomeric proteins. The antibodies did not react with any proteins corresponding to the pentamers (i.e. 110-120 kDa) in the non-heat treated samples (Fig. 8.10.C 3S1- and C26-). Even though the results of the SDS-PAGE gels and western blots suggested that both the transgenic 3S1 and C26 oxalate oxidase proteins existed only as monomers (Fig. 8.10. A and C), the results of the gel filtration column chromatography had already confirmed that these two oxalate oxidases were pentameric and active (Fig. 8.7.B).

All of the native and transgenic oxalate oxidases had apparent molecular weights of approximately 22-23 kDa for the monomers and approximately 110-120 kDa for the oligomers (as determined by SDS-PAGE analysis and gel filtration chromatography). It was therefore concluded that all of these proteins existed as pentamers in the plants, since five identical monomeric subunits with molecular weights of about 22-23 kDa would generated homopentamers of about 110-120 kDa.

For all three of the native cereal and transgenic SGS5 tobacco oxalate oxidases brief heat treatment (100 °C for 2 min) resulted in the conversion of all of the pentamers to monomers, which was clearly seen when the proteins were detected on western blots with anti-oxalate oxidase antibodies (Fig. 8.8.C BR+ and BE+ and 8.9.C WE+ and SGS5+, respectively). Transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases were only ever observed as monomers following analysis on SDS-PAGE gels regardless of whether the proteins were heat treated or not (Fig. 8.10.C 3S1 and C26), which clearly demonstrated the instability of the 3S1 and C26 oxalate oxidase oligomers compared with the native cereal and transgenic SGS5 oxalate oxidases.

Enzyme assay staining of the electroblots was a more sensitive, rapid and convenient method of detecting active oxalate oxidase in non-heat treated samples, compared with western blotting with anti-oxalate oxidase antibodies. However, western blotting was necessary where the 3S1 and C26 transgenic proteins were concerned because of the lack of

detectable enzyme activity for these oxalate oxidases following analysis by SDS-PAGE and electroblotting (Fig 8.10.B 3S1- and C26-, respectively). There did not appear to be any significant cross-reaction of the anti-oxalate oxidase antibodies with other proteins present in these samples (Fig. 8.8., 8.9. and 8.10.C).

Gel filtration chromatography (Fig. 8.6. and 8.7.A and B) and SDS-PAGE analysis (Fig. 8.8., 8.9. and 8.10.A) enabled a reasonable estimation of the molecular weights of the oxalate oxidase monomers (about 23 kDa) and pentamers (about 120 kDa). However, this was only accurate to about ± 1 kDa for the monomers and ± 5 kDa for the pentamers and the estimations could be several mass unit (kDa) away from the true molecular weights of these proteins. However, in all the estimates carried out the molecular weights of all of the native and transgenic oxalate oxidases were about 10 kDa lower than those previously reported for the pentamer (about 125 kDa) [Lane *et al.* 1987; McCubbins *et al.* 1987; Lane *et al.* 1993; Kotsira and Clonis 1997]. The earliest published reports regarding the structure of oxalate oxidase suggested that the protein was a dimer with a molecular weight of about 150 kDa [Sugiura *et al.* 1979; Pietta *et al.* 1982]. Schmitt [1991] reported the purification and detection of an oxalate oxidase from barley roots which was active as the monomer, however during this study no enzyme activity was ever associated with the monomers. More recently oxalate oxidases were identified in barley with molecular weights of about 100 kDa (i.e. tetramers) from 10 day old roots [Dumas *et al.* 1993] and roots of barley plants infected with powdery mildew [Zhang *et al.* 1995]. Lane *et al.* [1992] reported the presence of an isoform of oxalate oxidase in wheat about 25 days postanthesis which appeared to be tetrameric with a molecular weight of about 105 kDa.

McCubbins *et al.* [1987] calculated the constituent oxalate oxidase polypeptide to have a molecular weight of about 20 kDa, according to the primary amino acid sequence. Therefore the pentamer would have a mass of about 100 kDa. Jaikaran *et al.* [1990] calculated the oligosaccharide chain attached to each monomers to have a mass of about 2-3 kDa (calculated from SDS-PAGE analysis), which contributed about 10-15 kDa to the mass of the pentamers. Therefore according to this data the oxalate oxidase pentamers had a molecular weight of about 110-115 kDa, which was in close agreement with the molecular weights of the oxalate oxidases studied during this work.

Gane *et al.* [in press] used computer modelling to generate a three dimensional model of the structure of oxalate oxidase, based on the structure of vicilin. Baulein *et al.* [1995], Shutov *et al.* [1995] and Braun *et al.* [1996] suggested that oxalate oxidase and vicilin-like seed storage proteins may have originated from a common evolutionary origin. Warwicker and O'Connor [1995] also used computer modelling to generate a three dimensional structure for the storage vicilin of cocoa. Assuming that there is a statistically significant similarity between the amino acid sequences of different proteins it is possible to generate a convincing three dimensional structure using known crystallography data. If the proteins share greater than 20% sequence homology then the levels of the various secondary, tertiary and quaturnary structure exhibited by the proteins are likely to be quite similar. Current published evidence shows oxalate oxidase to be pentameric. However, based on their computer modelling data

Gane *et al.* [in press] suggested an alternative structure for oxalate oxidase – a dimer of trimers. It is likely that only once the crystallographic structure of oxalate oxidase has been determined will the question of the level of oligomerisation of this protein be truly answered.

8.2.2. Mass spectrometry of oxalate oxidase.

This work was carried out in collaboration with Ammelia Johnson at KRATOS Analytical (Manchester, U.K.). To try to resolve the size situation further the more highly resolving technique of matrix assisted laser desorption ionisation-time of flight (MALDI-ToF) mass spectrometry was carried out on native and transgenic oxalate oxidases. This technique involved mixing the resuspended protein sample with a matrix (i.e. sinapinic acid) which absorbs the laser light (337 nm), prevents protein-protein interactions and assists in ion formation by donating protons to the analyte. The ions generated all have the same kinetic energy, which enter the time of flight (ToF) mass analyser and fly down the evacuated tube until they reach the detector. The mass of a protein is determined by measuring velocity i.e. the time it takes for the protein to travel down the ToF mass analyser [KRATOS Analytical technical data]. Useful data was generated for all of the oxalate oxidases except wheat embryo oxalate oxidase. Fig. 8.11. illustrates the trace obtained for the barley root oxalate oxidase where there appeared to be contamination in the protein sample. However, silver staining of a 12% (w/v) SDS-PAGE gel of the purified barley root protein sample had shown that the oxalate oxidase was purified to apparent homogeneity (Fig. 7.5. BR). The additional peaks present in the sample apparently resulted from the presence of chemical ions in the protein samples and as a result of the fragmentation of some of the oxalate oxidase protein during the mass spectrometry procedure. In Fig. 8.11. the arrows indicate the peaks corresponding to the oxalate oxidase monomers in the barley root sample. The oxalate oxidase isoforms had molecular weights of 22.70 kDa (G') and 22.87 kDa (G), as well a peak at 22.58 kDa (X), possibly corresponding to the additional lower molecular weight oxalate oxidase protein expressed in barley roots. The barley embryo oxalate oxidase sample provided a much clearer spectrum containing two peaks with molecular weights of 22.81 and 22.92 kDa (Fig. 8.12.) The transgenic SGS5 tobacco oxalate oxidase sample contained a peaks at 22.61 kDa and 22.81 kDa (Fig. 8.13.).The native and transgenic SGS5 oxalate oxidases examined had very similar molecular weights (22.6 - 22.9 kDa), which corresponded quite closely to the masses of the proteins initially measured following resolution on SDS-PAGE gels. It was therefore assumed that all of the native and transgenic SGS5 oxalate oxidase proteins had monomer molecular weights of about 22.8 kDa (± 0.2 kDa) and thus estimated pentamer molecular weights of about 114 kDa (± 1 kDa). Mass spectrometry analysis of purified transgenic 3S1 and C26 oxalate oxidases (Fig. 8.14. and 8.15., respectively) confirmed the presence of single proteins with molecular weights of 22.78 and 22.68, respectively. These results provided the most accurate determination to date of the exact molecular weights of the oxalate oxidase monomers and they confirmed the initial size determination results of the SDS-PAGE analysis and gel filtration chromatography.

8.3. Oxalate oxidase oligomerisation.

Dimethyl suberimidate (DMS) was used to cross-link the monomer subunits in the oxalate oxidase oligomers to further confirm the existence of the native and transgenic proteins as pentamers following SDS-PAGE analysis, as described in section 3.22. The conditions used were selected on the basis of similar cross-linking experiments performed by McCubbins *et al.* [1987] on wheat embryo oxalate oxidase. However, it was found to be necessary to adapt these conditions to improve the ratio of the higher oligomeric oxalate oxidase protein species in the heat treated samples. Sheehan *et al.* [1990] demonstrated that the degree of thermal stability of the oligomer was dependant on the degree of cross-linking with optimal stabilisation occurring when approximately half of all available amino groups were covalently attached to DMS. Obviously the chance of two monomers being cross-linked to form dimers was much greater than the frequency with which the five monomeric units of an individual pentamer could be cross-linked. The frequency of cross-linked pentamers in the heat treated samples as a consequence was very low and this oligomeric form could only be seen on western blots after long exposures (about 2 weeks at -80 °C). Maintaining the concentration of DMS at 0.8 mg / ml was sufficient to cross-link a significant proportion of the pentamers but it was not high enough to cause detectable cross-linkage between different proteins in the treated crude protein samples, which occurred at >5 mg / ml of DMS. Therefore, instead of varying the concentration of DMS it was decided to increase the incubation time of the proteins with DMS from 2 to 6 hr at room temperature. This significantly improved the yield of fully cross-linked oxalate oxidase pentamers.

Fig. 8.16.A shows a western blot of native and transgenic oxalate oxidases (0.8 mg / ml) cross-linked with DMS at room temperature (for 6 hr), then treated with SDS and β-mecaptoethanol (1% (v/v) final concentrations) at 37 °C (for 2 hr), and analysed on a 12% (w/v) SDS-PAGE gels following heat treatment (2 min, 100 °C). A series of protein species with molecular weights corresponding to integral multiples of the monomer molecular weight (about 22.8 kDa) were generated from the native cereal oxalate oxidase (barley root, barley embryos and wheat embryo) (i.e. dimer, trimer, tetramer and pentamer), confirming that all of these oxalate oxidases existed as pentamers (Fig. 8.16.A, BR, BE and WE, respectively). These formed the same protein band pattern as that reported by McCubbins *et al.* [1987] when they cross-linked pulse labelled wheat embryo oxalate oxidase with DMS, where the monomers and dimers were in greatest abundance. Transgenic SGS5 tobacco oxalate oxidase was similarly cross-linked with DMS to generate five protein species with molecular weights of approximately 23, 46, 69, 92 and 115 kDa (molecular weights were determined from protein size markers separated on the SDS-PAGE gel). The autoradiograph was over exposed to enable the detection of the higher molecular weight cross-linked protein species (i.e. trimer, tetramer and pentamer).

Cross-linked oxalate oxidase proteins were not detected in either of the transgenic 3S1 oilseed rape or C26 tobacco DMS treated samples (Fig. 8.16.A 3S1 and C26, respectively). The experiment was therefore repeated with increased 3S1 and C26 protein concentrations (2 µg) and increased DMS concentration (2 mg / ml) with an incubation time of

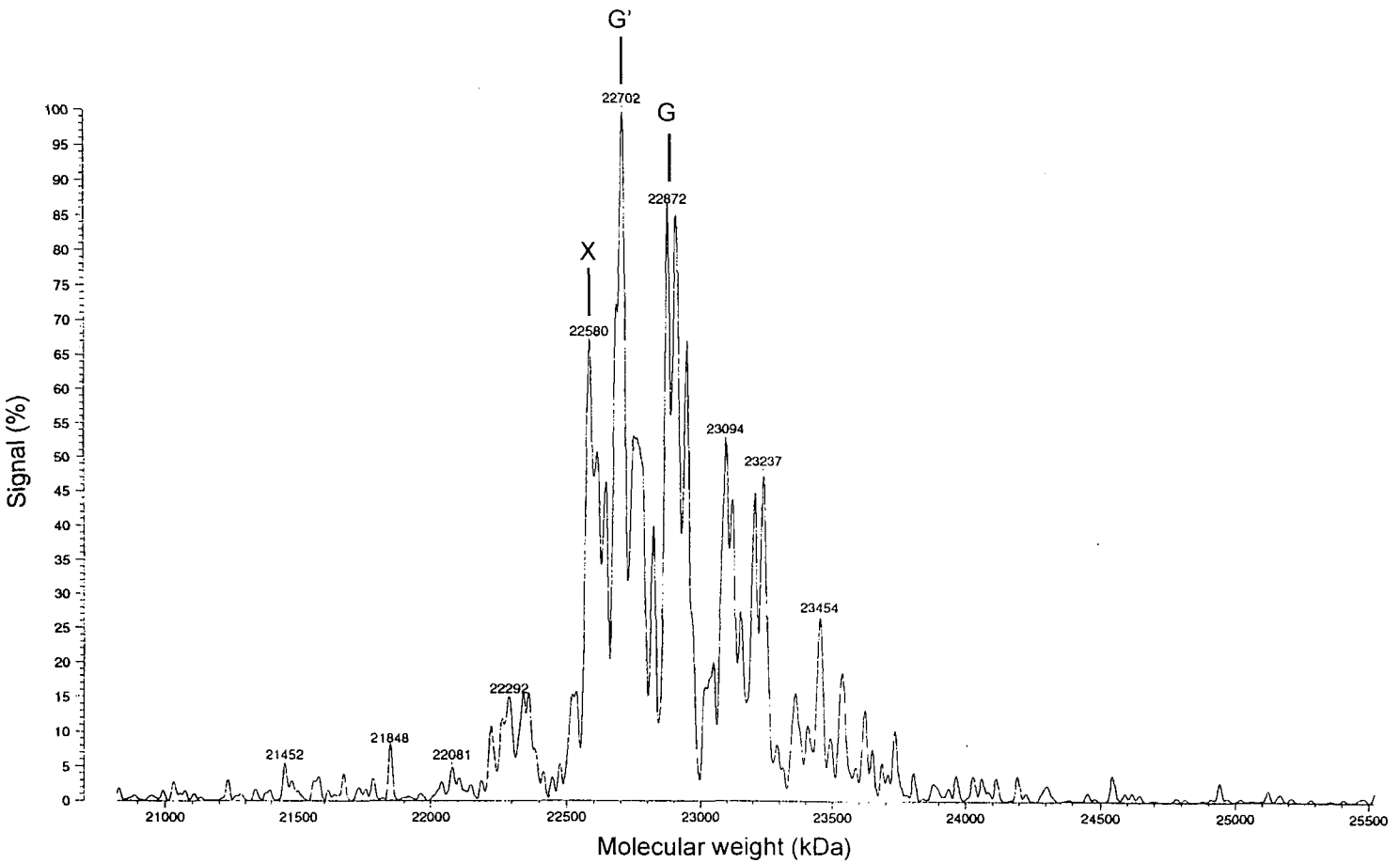


Figure 8.11. Molecular weight determination of the barley root oxalate oxidase monomers.
Mass spectrometry was carried out using a Kompact MALDI 2 with a 77 cm flight tube. The protein was dissolved (1 mg/ml) in 0.1% (v/v) trifluoroacetic acid. The matirix used was sinapinic acid at 10 mg/ml in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. 0.5 ml of the sample and 0.5 ml of the matrix were added to the sample well on the sample slide. The sample was then crystalised by drying and inserted into the instrument .100% signal = 1 mV[sum = 193 mV].

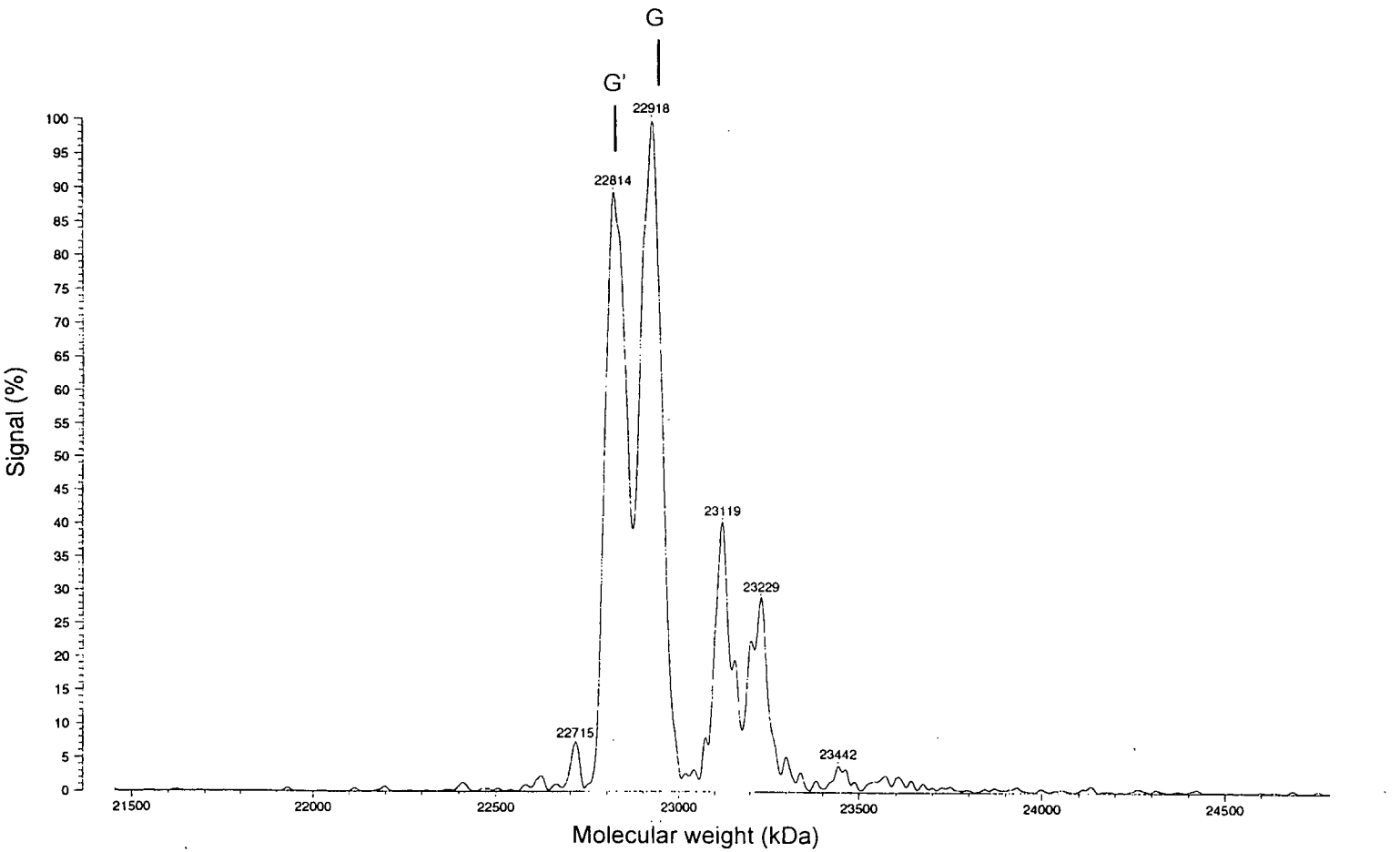


Figure 8.12. Molecular weight determination of the barley embryo oxalate oxidase monomers.
Mass spectrometry was carried out using a Kompact MALDI 2 with a 77 cm flight tube. The protein was dissolved (1 mg/ml) in 0.1% (v/v) trifluoroacetic acid. The matirix used was sinapinic acid at 10 mg/ml in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. 0.5 ml of the sample and 0.5 ml of the matrix were added to the sample well on the sample slide. The sample was then crystalised by drying and inserted into the instrument .100% signal = 7 mV[sum = 1084 mV].

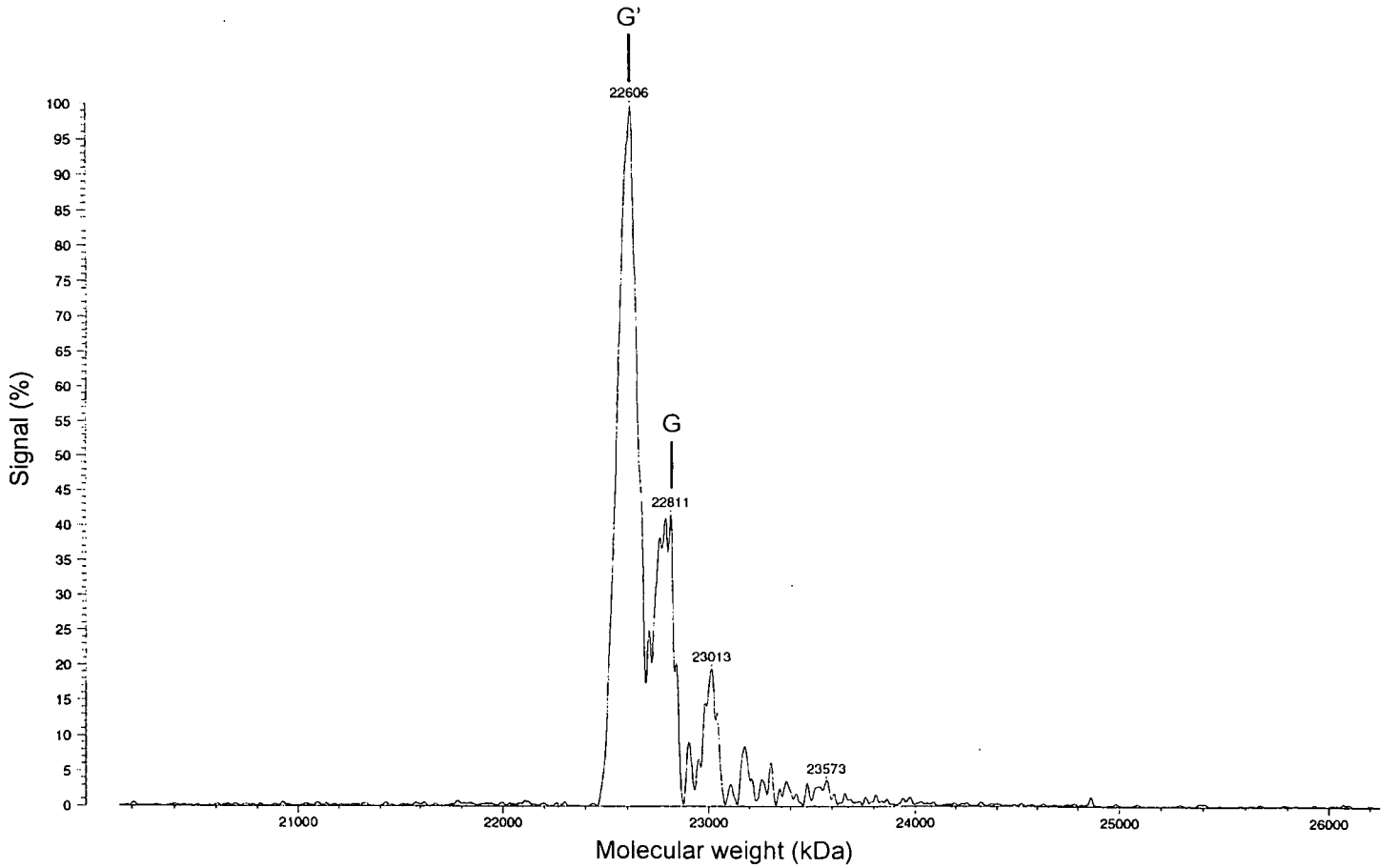


Figure 8.13. Molecular weight determination of the transgenic SGS5 tobacco oxalate oxidase monomers. Mass spectrometry was carried out using a Kompact MALDI 2 with a 77 cm flight tube. The protein was dissolved (1 mg/ml) in 0.1% (v/v) trifluoroacetic acid. The matirix used was sinapinic acid at 10 mg/ml in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. 0.5 ml of the sample and 0.5 ml of the matrix were added to the sample well on the sample slide. The sample was then crystalised by drying and inserted into the instrument .100% signal = 13 mV[sum = 1468 mV].

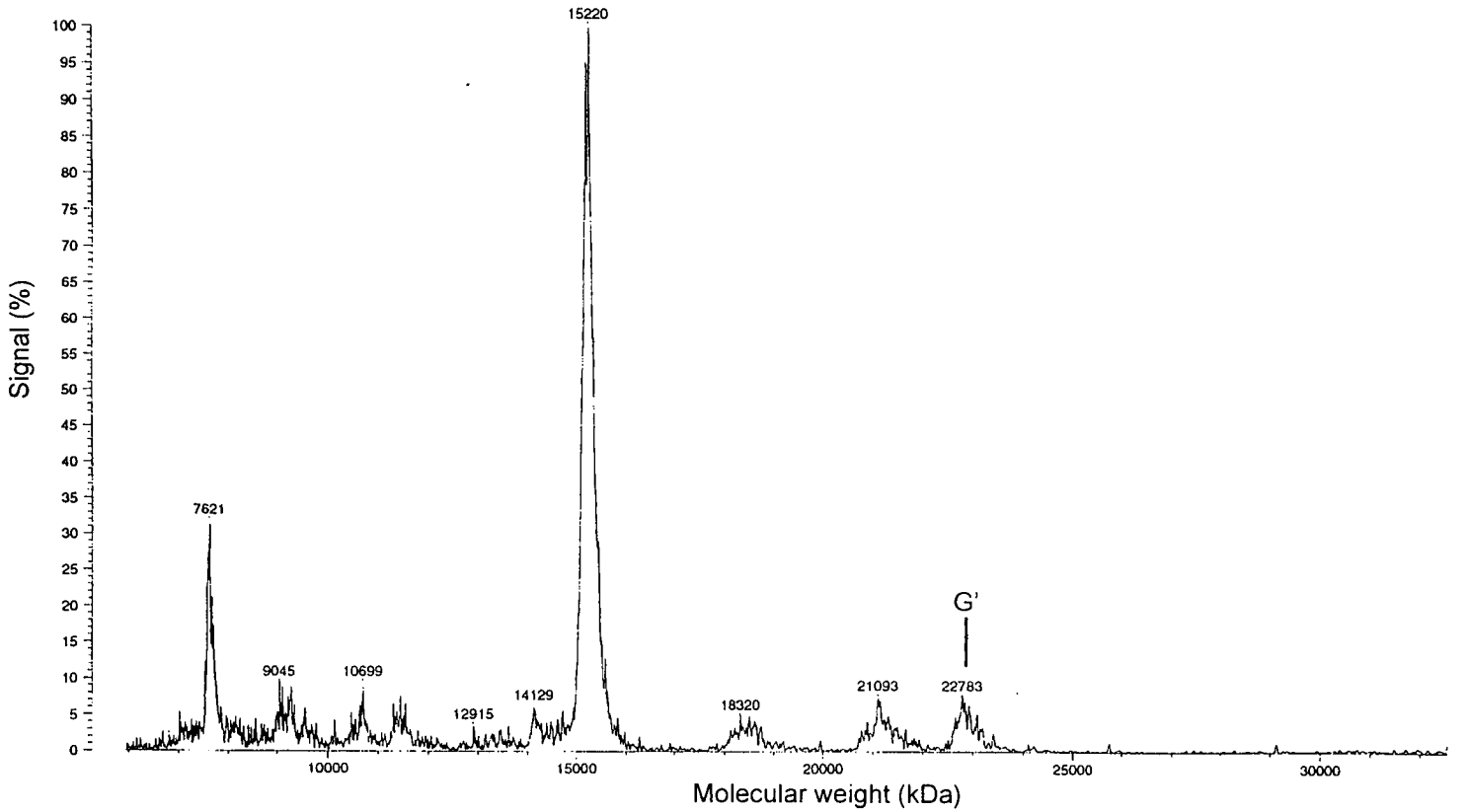


Figure 8.14. Molecular weight determination of the transgenic 3S1 oilseed rape oxalate oxidase monomer. Mass spectrometry was carried out using a Kompact MALDI 2 with a 77 cm flight tube. The protein was dissolved (1 mg/ml) in 0.1% (v/v) trifluoroacetic acid. The matirix used was sinapinic acid at 10 mg/ml in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. 0.5 ml of the sample and 0.5 ml of the matrix were added to the sample well on the sample slide. The sample was then crystalised by drying and inserted into the instrument .100% signal = 4 mV[sum = 698 mV].

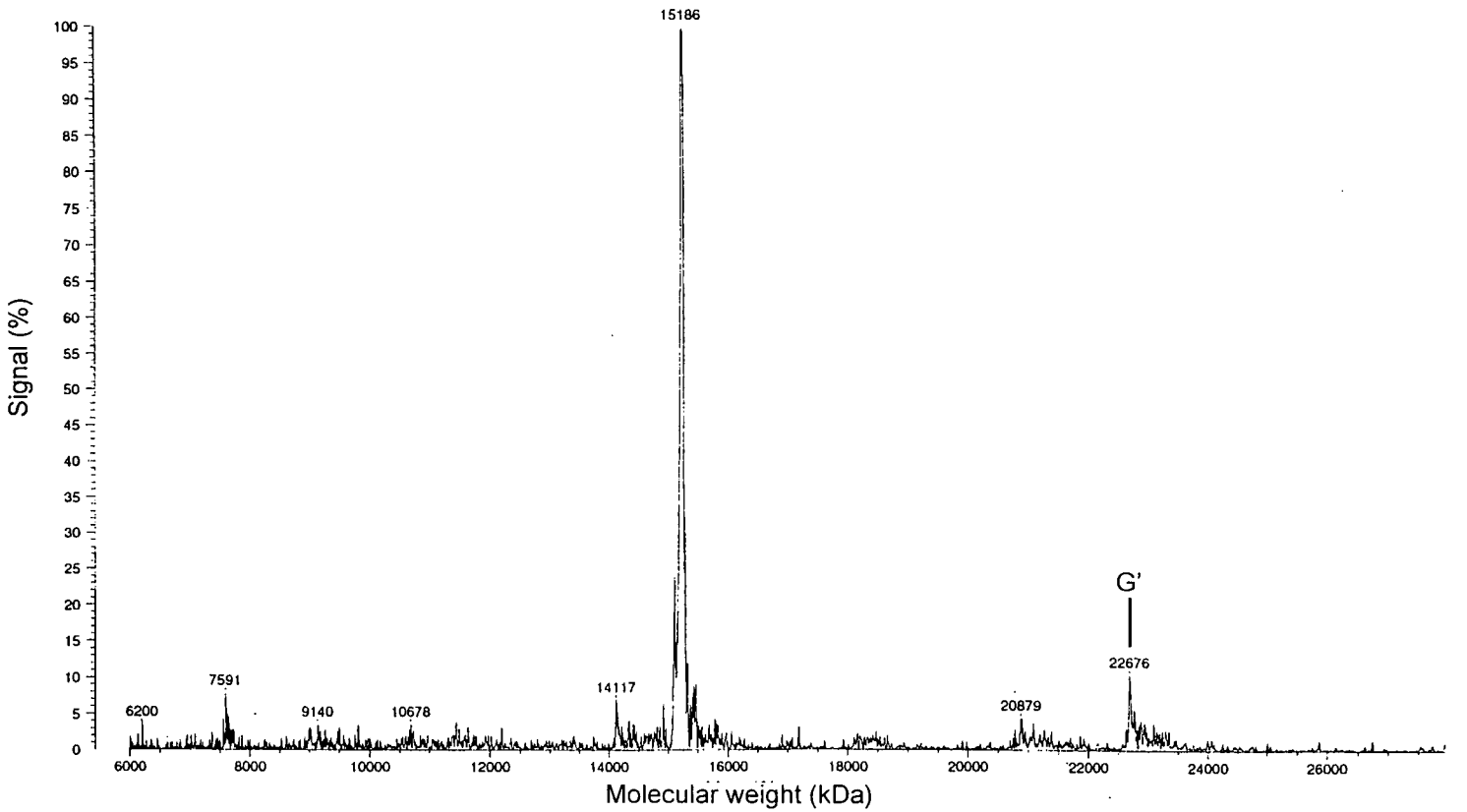


Figure 8.15. Molecular weight determination of the transgenic C26 tobacco oxalate oxidase monomer. Mass spectrometry was carried out using a Kompact MALDI 2 with a 77 cm flight tube. The protein was dissolved (1 mg/ml) in 0.1% (v/v) trifluoroacetic acid. The matirix used was sinapinic acid at 10 mg/ml in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. 0.5 ml of the sample and 0.5 ml of the matrix were added to the sample well on the sample slide. The sample was then crystalised by drying and inserted into the instrument .100% signal = 3 mV[sum = 537 mV].

6 hr at room temperature. Fig. 8.16.B shows the presence of the cross-linked protein species for both of these transgenic oxalate oxidases. Cross-linked oligomers with molecular weights of about 115 kDa were detected in both the heat (+) and non-heat treated samples (-), which were comparable with the molecular weights of the native cereal and transgenic SGS5 oxalate oxidase pentamers. Bands representing the dimers (about 46 kDa) were barely visible on the western blot but there were no visible bands representing the trimers or tetramers. Although, the 3S1 and C26 transgenic oxalate oxidases were normally unstable and dissociated to their constituent monomers in the presence of SDS and without heat treatment this experiment had confirmed that these two transgenic oxalate oxidases existed as pentamers with molecular weights of about 110-120 kDa (Fig. 8.10.A 3S1- and C26-).

8.4. Oxalate oxidase glycosylation.

Jaikaran *et al.* [1990] have published data regarding the glycosylation of wheat embryo oxalate oxidase, including the extent of differential glycosylation of the two protein isoforms (G and G') and a suggestion as to the glycans present on the oligosaccharide side chains (G monomer, $(GlcNAc_2(Man)_2(Man\text{-}Xyl)(GlcNac)(GlcNAc\text{-}Fuc))$; G' monomer $((Man)_2(Man\text{-}Xyl)(GlcNAc)(GlcNAc\text{-}Fuc)))$ [Jaikaran *et al.* 1990]. These experiments were used as the basis of experiments carried out to examine glycosylation of the native and transgenic oxalate oxidases.

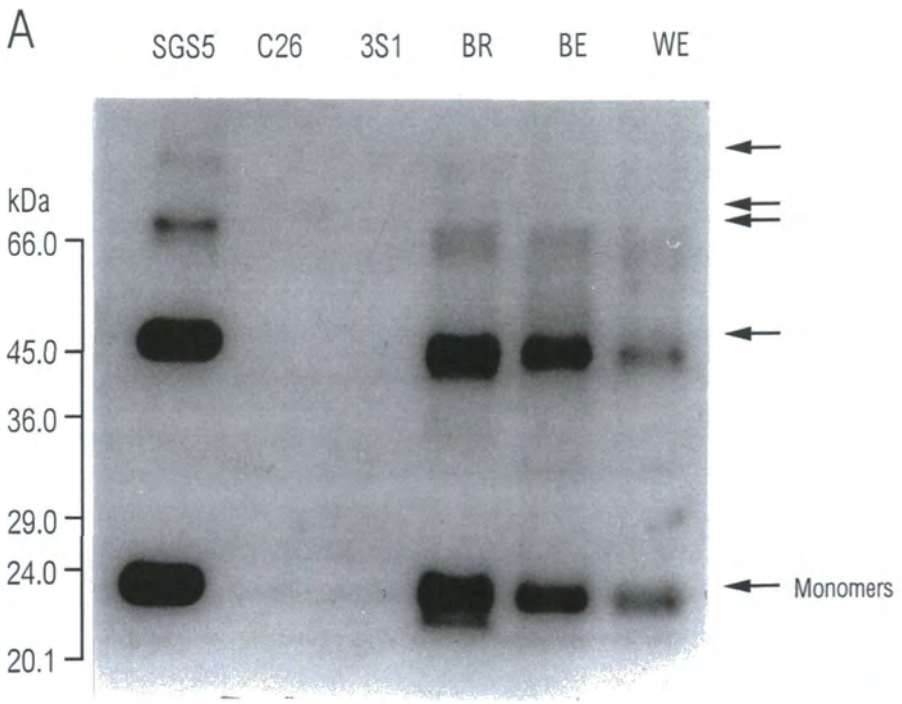
8.4.1. SDS-PAGE analysis of oxalate oxidase glycosylation.

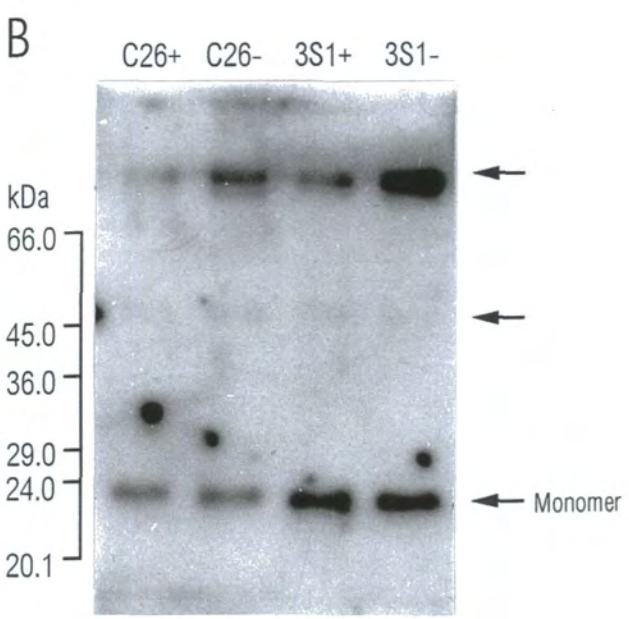
Native and transgenic oxalate oxidases were analysed by SDS-PAGE, the gels were stained with Schiff's reagent for the detection of glycoproteins. All 6 of the oxalate oxidase proteins were visible on the SDS-PAGE gel after staining. However, the low levels of staining made it impossible to obtain adequate photographs of the gels (results not shown). Therefore, other staining method were used to confirm glycosylation of these oxalate oxidases. Periodic acid silver staining was shown to be a reasonably reliable method of 'in gel' glycoprotein detection, using the method of Dubray and Bezard [1982]. However, on several occasions this staining procedure resulted in the non-specific staining of all the proteins present on the gel. Fig. 8.17. illustrates an SDS-PAGE gel stained using the periodic acid silver staining method, on which samples of extracts from the native and transgenic plants were analysed. The pentameric proteins in the barley root, barley embryo, wheat embryo and transgenic SGS5 tobacco and the monomeric proteins in transgenic 3S1 oilseed rape and C26 tobacco in non-heat treated samples were intensely stained (as indicated by the arrows). This gel also illustrated the similarity in the molecular weights of the native and transgenic oxalate oxidase proteins. Low levels of the native and transgenic SGS5 oxalate oxidase monomers were detected on the gel, resulting from dissociation of the pentamer in the presence of SDS. A high molecular weight protein (about 100-110 kDa) in the 3S1 oilseed rape samples was intensely stained, the significance of this germin-like protein will be discussed later in chapter 10.

Figure 8.16. SDS-PAGE analysis and western blotting of oxalate oxidases cross-linked with dimethyl suberimidate.

A, Partially purified protein samples were treated with 0.8 mg / ml dimethyl suberimidate for 6 hr at room temperature, as described in section 3.22. The approximate protein concentration in the treated samples were, 0.13 µg transgenic SGS5 tobacco, 0.62 µg transgenic C26 tobacco, 0.4 µg transgenic 3S1 oilseed rape, 3.19 µg barley root (BR), 3.11 µg barley embryo (BE) and 3.41 µg wheat embryo (WE). After cross-linking the protein samples were heat treated at 100 °C for 2 min prior to separation on a 12% (w/v) SDS-PAGE gel. The proteins were electroblotted onto a nitrocellulose membrane. Anti-oxalate oxidase antiserum was used as the primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. The blot was exposed to x-ray film for 2 weeks at -80 °C. The different oxalate oxidase protein species are indicated by the arrows.

B, Partially purified transgenic protein samples were treated with 2 mg / ml dimethyl suberimidate for 6 hr at room temperature, as described in section 3.22. The approximate protein concentration in the treated samples were, 2.0 µg of transgenic 3S1 oilseed rape and C26 tobacco. The protein were separated on a 12% (w/v) SDS-PAGE gel with (+) and without heat treatment (-). The proteins were electroblotted onto a nitrocellulose membrane. Anti-oxalate oxidase antiserum was used as the primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. The blot was exposed to x-ray film for 2 weeks at -80 °C. The oxalate oxidase monomers and oligomers are indicated by the arrows.


A
SGS5
C26
3S1
BR
BE
WE
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Monomers


B
C26+
C26-
3S1+
3S1-
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Monomer

8.4.2. Concanavalin A sepharose analysis of oxalate oxidase.

Concanavalin A (ConA) is a lectin isolated from *Concanavilis ensiformis* (jackbean), which selectively binds terminal mannose, glucose and α-N-acetylglucosamine residues on glycoproteins. Confirmation of the gylcosylation of the oxalate oxidases was achieved by passing protein extracts through a ConA sepharose B column. The bound proteins were eluted from the column using α-mannopyranoside, which has a very high affinity for the lectin. Fig. 8.18.A shows a graph of the µmol of H_2O_2 formed / min for each of the wash (W 1 - 10) and eluant (E 1-10) fractions (0.5 ml) collected from the ConA column for transgenic SGS5 tobacco INF. No enzyme activity was detected in any of the wash fractions, confirming that all the oxalate oxidase bound to the ConA. Oxalate oxidase was eluted from the column in fraction E4 (E1 to E3 being the column void volume). Fig. 8.18.B shows a 12% (w/v) SDS-PAGE gel stained with a Coomassie blue of INF and ConA sepharose column fractions W6 and E6. The transgenic oxalate oxidase protein was present in the sample prior to loading onto the column (INF - and +). Oxalate oxidase was absent in the wash fraction (W - and +)and eluted from the column in the eluant (E - and +). All of the native and transgenic oxalate oxidases bound to the lectin and were eluted with α-mannopyranoside, which confirmed that the proteins all possessed an oligosaccharide side chain(s) containing terminal mannose, glucose and /or α-N-acetylglucosamine residues.

8.4.3. Inhibition of glycosylation *in planta*.

Having confirmed that the transgenic enzymes were also glycosylated the degree of glycosylation was examined *in planta* by inhibiting glycosylation of a proportion of these proteins. This was achieved by treating germinating seeds (barley, transgenic 3S1 oilseed rape, C26 and SGS5 tobacco) and isolated quiescent embryos (wheat and barley) with tunicamycin. Tunicamycin is an antibiotic which blocks the first step in the lipid saccharide pathway by inhibiting N-acetylglucosamine transferase [Elbein 1987], preventing the transfer of the primary oligosaccharide side chain to the nascent protein.

The initial experiment carried out involved the germination of isolated mature quiescent embryos in the presence of tunicamycin (10 µg / ml) and chloramphenicol (10 µg / ml) for 3 days, as described in section 3.23. Chloramphenicol was used to prevent the growth of bacteria / fungi on the germinating embryos. Chloramphenicol did not appear to have any effect on the germination or growth of the isolated embryos at a concentration of 10 µg / ml. A concentration of 10 µg / ml of tunicamymin was used in all experiments compared with 5 µg / ml, used by Jaikaran *et al.* [1990] in a similar study. Fig. 8.19.A illustrates the effect of tunicamycin treatment on oxalate oxidase isolated from 3 day germinated wheat and barley embryos (WE and BE), grown in the presence (T+) or absence (T-) of tunicamycin. The protein isoforms were detected on an oxalate oxidase enzyme assay stained electroblot. Treatment of the embryos with tunicamycin led to the production of oxalate oxidase oligomers containing different ratios of glycosylated and unglycosylated monomeric subunits in non-heat treated protein samples (Fig. 8.19.A WE and BE T+). The lowest molecular weight oligomer consists entirely of five unglycosylated monomers progressing to the highest molecular weight

Figure 8.17. SDS-PAGE analysis and detection of oxalate oxidase oligosaccharides by periodic acid silver staining.

Partially purified protein samples were analysed on a 12% (w/v) SDS-PAGE gel, without heat treatment. Approximately 0.13 µg transgenic SGS5 tobacco, 0.62 µg transgenic C26 tobacco, 0.4 µg transgenic 3S1 oilseed rape, 3.19 µg barley root (BR), 3.11 µg barley embryo (BE) and 3.41 µg wheat embryo (WE) were loaded onto the gel. Proteins were detected using the periodic acid silver staining method of Dubray and Bezard [1982]. MW, molecular weight marker. The arrows indicate the oligomers and monomers of the G and G' oxalate oxidase isoforms.

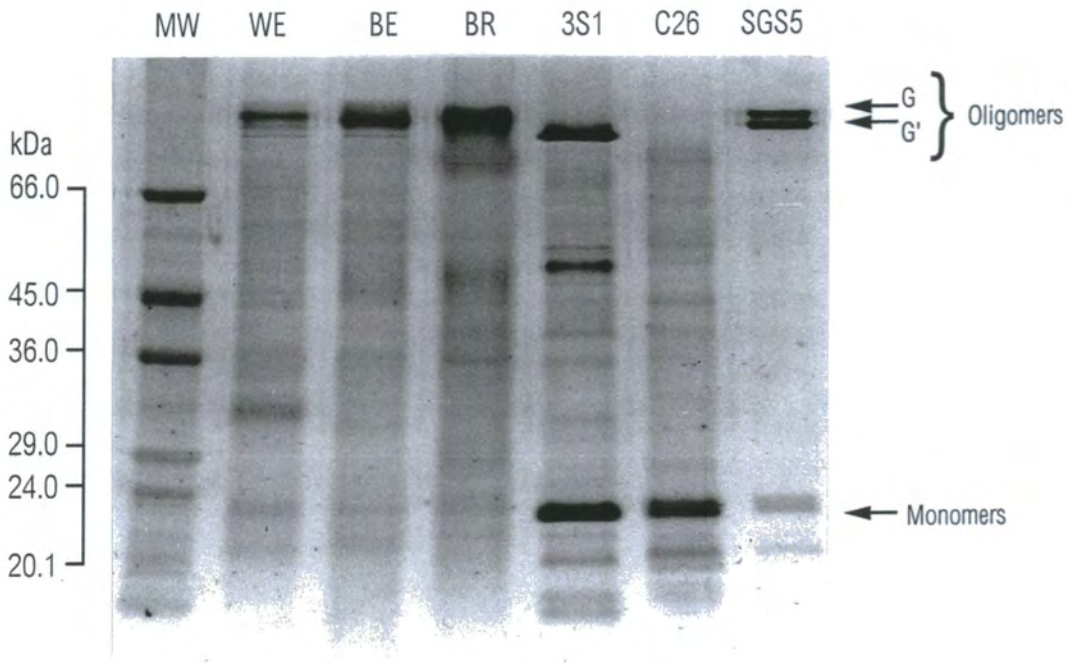

MW
WE
BE
BR
3S1
C26
SGS5
kDa
66.0
45.0
36.0
29.0
24.0
20.1
G
G'
Oligomers
Monomers

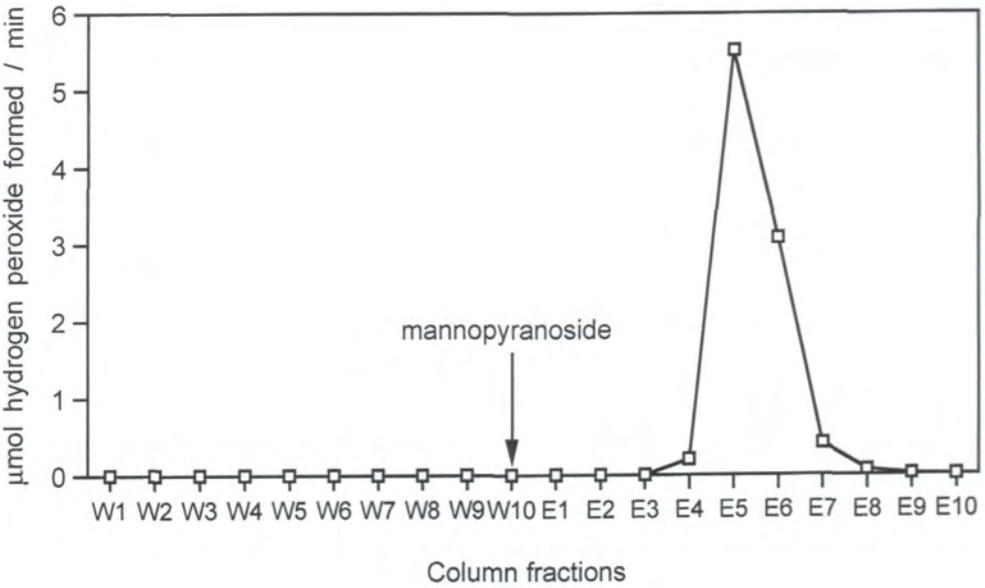
Figure 8.18. Analysis of transgenic oxalate oxidase by Concanavalin A sepharose B column chromatography.

Transgenic SGS5 tobacco INF containing approximately 80 µg of total protein was loaded onto a 1 ml Con A sepharose B column. The column was washed with CaMg TBS buffer and the bound proteins were eluted with CaMg TBS containing 5 mM α-mannopyranoside (see arrow). 1 ml fractions were collected from the column.

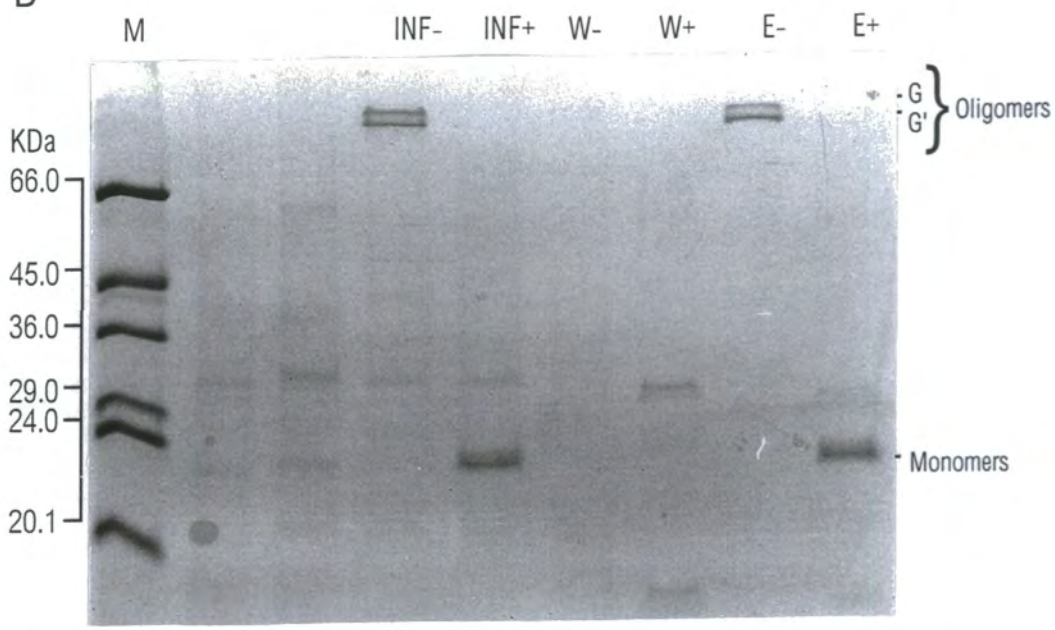
A, 5 µl aliquots of each of the wash (W) and eluant (E) fractions were assayed for oxalate oxidase enzyme activity using the enzyme assay described in section 3.11.2.

B, Approximately 6 µg of INF, wash fraction number 6 (W) and eluant fraction number 6 (E) collected from the Con A sepharose B column were analysed on a 12% (w/v) SDS-PAGE gel, with (+) and without (-) heat treatment (2 min, 100 °C). Proteins were detected by Coomassie blue staining. M, molecular weight marker. The arrows indicate the oligomers and monomers of the G and G' oxalate oxidase isoforms.

A


µmol hydrogen peroxide formed / min
0
1
2
3
4
5
6
mannopyranoside
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 E1 E2 E3 E4 E5 E6 E7 E8 E9 E10
Column fractions

B


M
INF-
INF+
W-
W+
E-
E+
KDa
66.0
45.0
36.0
29.0
24.0
20.1
G
G'
Oligomers
Monomers

oligomer, which consists entirely of five glycosylated monomers. According to McCubbins *et al.* [1987] the oligosaccharide chain present on each monomer had a molecular weight of about 2-3 kDa. It was therefore expected that the different 'glycoforms' of the oligomers would exhibit a size difference of about 2-3 kDa i.e. a difference of about 10-15 kDa between the lowest and highest molecular weight oligomeric proteins. Analysis of the treated protein samples on a 17.5% (w/v) SDS-PAGE gels gave sufficient separation to enable the detection of all of the differentially glycosylated oligomers.

Glycosylation was affected in both cereal roots and embryos resulting in the oxalate oxidase 'ladder' of oligomers in the non-heat treated samples. For barley root oxalate oxidase it was not possible to calculate the number of protein bands present in the 'ladder' because of poor separation on the gel (result not shown). The separation of the various oligomers in the wheat and barley embryo total protein extracts were much clearer and it was possible to identify eleven (enzyme assay stained) differentially glycosylated oxalate oxidase pentameric species in both of these tunicamycin treated samples (Fig. 8.19.A WE and BE T+, respectively). Eleven oligomers were generated from the two differentially glycosylated oxalate oxidase isoforms present in the germinated embryos (G and G'). The lower molecular weight isoform of oxalate oxidase (G') present in the germinated embryos always appeared to be expressed at lower levels compared with the higher molecular weight isoform (G) (< 50%) (Fig. 8.19.A WET- and BET-). This meant that although the oligomeric protein species generated from the G' isoform were visible on the original stained electroblot they only appeared as smears on the photographed electroblot (Fig 8.19.A WE and BE T+, indicated by the bracket labelled 'glycoforms'). The G and G' oxalate oxidase isoforms were clearly visible in the untreated wheat embryo extract (Fig. 8.19.A WE T-, as indicated by the arrows). Jaikaran *et al.* [1990] only reported the detection of seven oxalate oxidase bands following tunicamycin treatment of wheat embryos, presumably resulting from the more highly expressed G isoform.

One of the major drawbacks of the tunicamycin treatment was that protein glycosylation was universally affected and the effect this 'stress' was having on the germinating embryos was unknown. Furthermore tunicamycin treatment was by no means completely effective at preventing polypeptide glycosylation; if it was then the embryos presumably would not have germinated at all. It was observed that as the concentration of tunicamycin was increased the degree of embryo germination and growth declined. 10 µg / ml of tunicamycin was selected as a suitable concentration to prevent sufficient glycosylation for detection of the unglycosylated oxalate oxidase subunits, but still allowing enough protein glycosylation for growth and development.

The second experiment performed involved the exposure of mature quiescent barley and wheat embryos, barley grains, transgenic 3S1 oilseed rape, C26 and SGS5 tobacco seeds to tunicamycin (10 µg / ml) from the start of imbibition. The barley and oilseed rape seedlings were grown for 10 days and the embryos for 3 days in the presence (T+) or absence (T-) of tunicamycin. Unfortunately due to the slow rate of germination and growth of tobacco and the extremely small size of the initial seedlings this particular strategy for studying

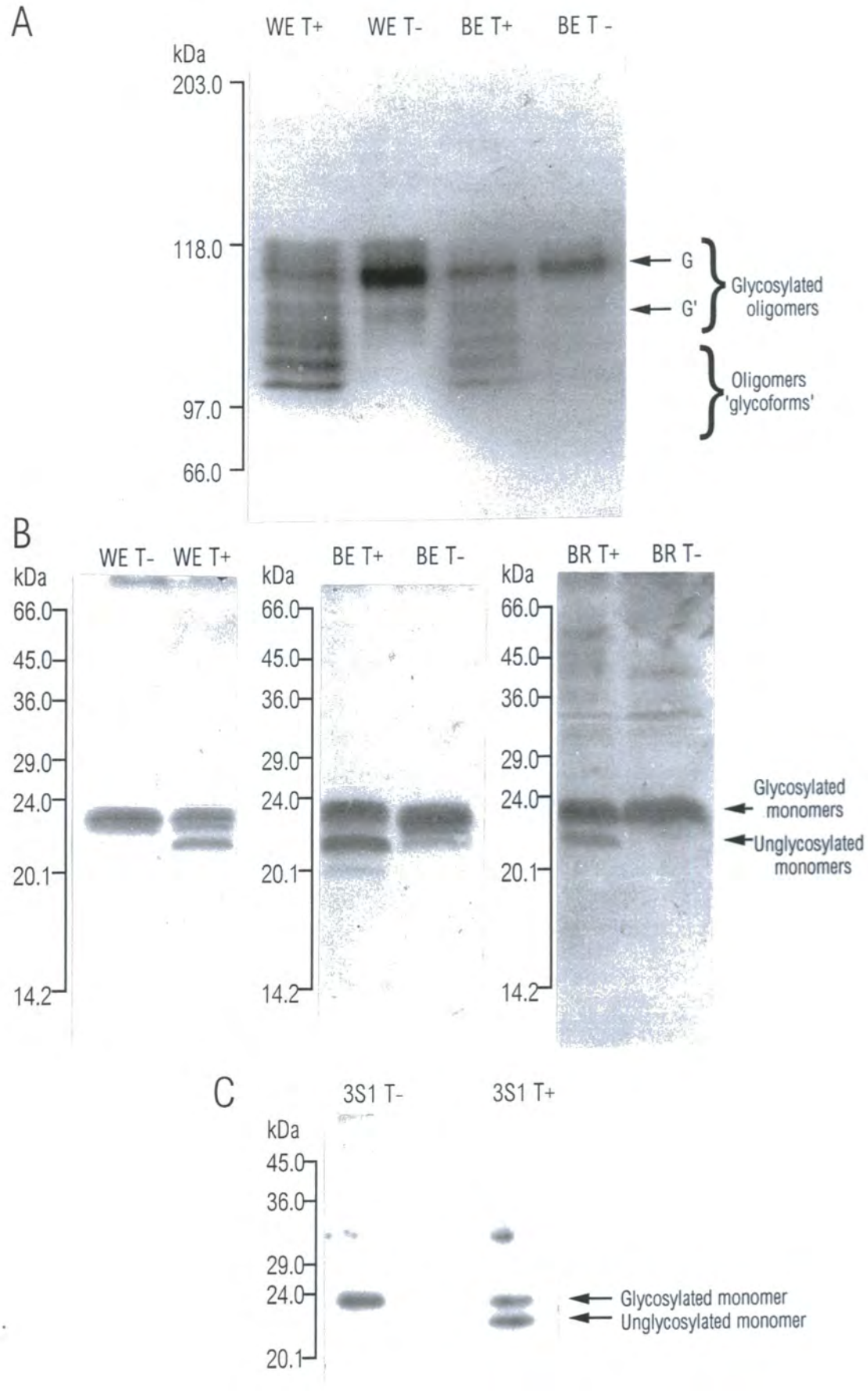
Figure 8.19. Effect of tunicamycin on the glycosylation of oxalate oxidase.

Isolated mature quiescent barley and wheat embryos, barley grains and transgenic 3S1 oilseed rape seeds were germinated and grown in the presence of sterile distilled water containing chloramphenicol (10 µg / ml) and tunicamycin (10 µg / ml). The embryos were grown for 3 days and the barley and oilseed rape seedlings for 10 days, as described in section 3.23. Total protein extracts were prepared as described in section 3.3.

A. Electroblot of SDS-PAGE analysis. Non-heat treated samples (20 µg) of wheat embryo (WE) and barley embryo (BE) total protein extracts prepared from embryos grown in the presence (T+) or absence (T-) of tunicamycin. The proteins were analysed on a 17.5% (w/v) SDS-PAGE gel. The proteins were electroblotted onto a nitrocellulose membrane, which was assayed for oxalate oxidase enzyme activity, as described in section 3.11.1. The arrows indicate the G and G' glycosylated oligomers. The bracket illustrates the protein bands representing the differentially glycosylated oligomers following tunicamycin treatment.

B. Electroblot of SDS-PAGE analysis. Heat treated samples (20 µg) of barley root (BR), barley embryo (BE) and wheat embryo (WE) total protein extracts prepared from seedling roots and embryos grown in the presence (T+) or absence (T-) of tunicamycin, were analysed on 12% (w/v) SDS-PAGE gels. The proteins were electroblotted onto a nitrocellulose membranes and reacted with anti-oxalate oxidase antiserum as primary antibodies (1:10,000 dilution) and alkaline phosphatase-conjugated donkey anti-rabbit antibodies as secondary antibodies (1:1,000 dilution). The western blots were treated as described in section 3.19.2. for the colorimetric detection of oxalate oxidase. The arrows indicate the glycosylated and unglycosylated monomers.

C. Electroblot of SDS-PAGE analysis. Heat treated samples (10 µg) of transgenic 3S1 oilseed rape total protein extracts of entire seedlings grown in the presence (+) or absence (-) of tunicamycin were analysed on a 12% (w/v) SDS-PAGE gel. The proteins were electroblotted onto a nitrocellulose membrane and reacted with anti-oxalate oxidase antiserum as primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. The blot was exposed to x-ray film for 1 week at -80 °C. The arrows indicate the glycosylated and unglycosylated monomers.


A
WE T+
WE T-
BE T+
BE T -
kDa
203.0
118.0
97.0
66.0
G
G'
Glycosylated oligomers
Oligomers 'glycoforms'
B
WE T-
WE T+
BE T+
BE T-
BR T+
BR T-
kDa
66.0
45.0
36.0
29.0
24.0
20.1
14.2
Glycosylated monomers
Unglycosylated monomers
C
3S1 T-
3S1 T+
kDa
45.0
36.0
29.0
24.0
20.1
Glycosylated monomer
Unglycosylated monomer

the glycosylated nature of the transgenic tobacco oxalate oxidases was not successful. A significant difference in the rate of growth was observed between barley and 3S1 oilseed rape seedlings grown in the presence or absence of tunicamycin. Germination was delayed by at least two days in the presence of tunicamycin and the seedlings were only about half the size of the barley and oilseed rape seedlings grown only in the presence of chloramphenicol. Seedlings grown in the presence of tunicamycin (10 µg / ml) were also yellow and etiolated, chlorophyll development appeared to be delayed up to day 8 compared with the untreated seedlings which formed green cotyledons or coleoptiles by day 4 (for oilseed rape and barley, respectively).

Fig. 8.19.B illustrates the effect of heat treatment (2 min, 100 °C) on the tunicamycin treated (T+) and untreated (T-) wheat and barley embryos (WE and BE) and barley root (BR) total protein extracts. The glycosylated and unglycosylated oxalate oxidase monomers were clearly visible in the tunicamycin treated extracts when analysed on 12% (w/v) SDS-PAGE gels, which were electroblotted onto a nitrocellulose membrane and western blotted (Fig. 8.19.B WE, BE and BR T+, respectively). The G' isoforms were only really visible in the barley embryo extracts, where both the glycosylated and unglycosylated G and G' isoforms were present at detectable levels (Fig. 8.19.B BE- and +). Fig. 8.19.C shows the presence of the glycosylated and unglycosylated oxalate oxidase proteins in the tunicamycin treated transgenic 3S1 oilseed rape seedling total protein extracts.

Western blots of the heat treated protein samples shown in Fig. 8.19.B and C were used to determine the approximate molecular weights of the glycosylated and unglycosylated oxalate oxidase monomers. The native (WE, BE and BR) and transgenic (3S1) gylcosylated monomers all appeared to have molecular weights of about 22-23 kDa, compared with the unglycosylated monomers which had molecular weights of about 20-21 kDa. The molecular weights of the native and transgenic 3S1 unglycosylated monomers (about 20-21 kDa) closely resembled the molecular weight of the oxalate oxidase polypeptide (20 kDa), as determined from the primary amino acid sequence [McCubbins *et al.* 1987]. The glycans attached to the oxalate oxidase monomers had molecular weights of about 2-3 kDa, which meant that the glycans were contributing approximately 10-15 kDa to the molecular weight of the oxalate oxidase pentamers, which was in agreement with the results of Jaikaran *et al.* [1990] for wheat embryo oxalate oxidase.

Glycosylation did not appear to be a necessary requirement for the assembly and maintenance of the pentameric structure of the cereal oxalate oxidases, which is in agreement with the results obtained by Jaikaran *et al.* [1990] for oxalate oxidase isolated from tunicamycin treated wheat embryos. Fig. 8.19.A illustrates the effect of tunicamycin treatment on the wheat and barley embryo oxalate oxidases in non-heat treated samples. The lowest molecular weight oligomers in these two samples represent pentamers consisting entirely of five unglycosylated monomers. These unglycosylated proteins were structurally stable (i.e. pentameric) and they retained their enzyme activity (in the presence of 1% (w/v) SDS), which demonstrated that the oligosaccharide side chains did not appear to play a significant role in proteins stability or activity. Jaikaran *et al.* [1990] had also demonstrated the resistance of the

unglycosylated pentamer to dissociation in SDS containing media and to digestion by pepsin. The western blots in Fig. 8.19.B and C also confirmed that the anti-oxalate antibodies raised against the glycosylated, heat treated protein reacted against the oxalate oxidase apoprotein of the unglycosylated monomer subunits generated during the tunicamycin treatment.

8.5. Structural stability of oxalate oxidases.

8.5.1. Thermal stability.

This work was performed in collaboration with Tim Mann and Paul Gabbott at Perkin Elmer Thermal Analysis (Buckinghamshire, U.K.). The thermal stability of the native barley root transgenic SGS5 tobacco oxalate oxidases were studied using differential scanning calorimetry (DSC). This technique involved measuring the uptake / release of energy from the proteins when exposed to increasing temperature (50-150 °C at a rate of 10 °C / min). A significant uptake of energy takes place when proteins are denatured, producing a characteristic scan (represented by a peak in reaction temperature on an endo up (mW) scale) for a given protein. Denaturation is essentially a melting process which involves the breaking of hydrogen bonds, which is an endothermic reaction. When oligomeric proteins dissociate into their constituent subunits as a result of thermal instability there is a release of energy (i.e. an exothermic reaction) characterised by a drop in reaction temperature. This technique allowed the measurement of the temperature at which the oxalate oxidases proteins unfolded / dissociates as well as sensitive measurement of the amount of energy uptake / release. Figs. 8.20. and 8.21. illustrate the traces obtained following DSC of barley root and SGS5 tobacco oxalate oxidases, respectively. Barley root (Fig. 8.20.) and transgenic SGS5 tobacco oxalate oxidases (Fig. 8.21.) exhibited similar patterns of energy release, at temperatures of about 81.7 and 79.8 °C, respectively. These results illustrated the close structural similarity of the barley root and transgenic SGS5 tobacco oxalate oxidases with regard to their thermal stability. The release of energy from the proteins probably resulted from the dissociation of the pentamer and the subsequent unfolding of the monomer with increasing temperature. It was possibly that the exothermic reaction resulting from pentamer dissociation was greater and therefore masked the smaller endothermic reaction resulting from protein denaturation. This technique did not generate meaningful data for the transgenic 3S1 oxalate oxidase.

The effect of heat treatment on barley root oxalate oxidase structure was examined following heat treatment for 30 min at various temperatures (Fig. 8.22. 40, 60, 80 and 90 °C). Barley root oxalate oxidase appeared to be completely stable and retained its pentameric structure following incubations at 40 °C and 60 °C for 30 min. The oxalate oxidase monomer was not detectable in either of these two samples. When the protein was incubated at 80 °C for 30 min monomer began to appear, which suggested that the pentamer was starting to dissociate / denature under these conditions. Following heat treatment at 90 °C for 30 min all of the pentamer was converted to monomer. These results agreed with those obtained from DSC, which showed the temperature of dissociation / denaturation of barley root oxalate oxidase to be about 82 °C. Fig. 8.23. illustrates the effect of incubating barley root oxalate

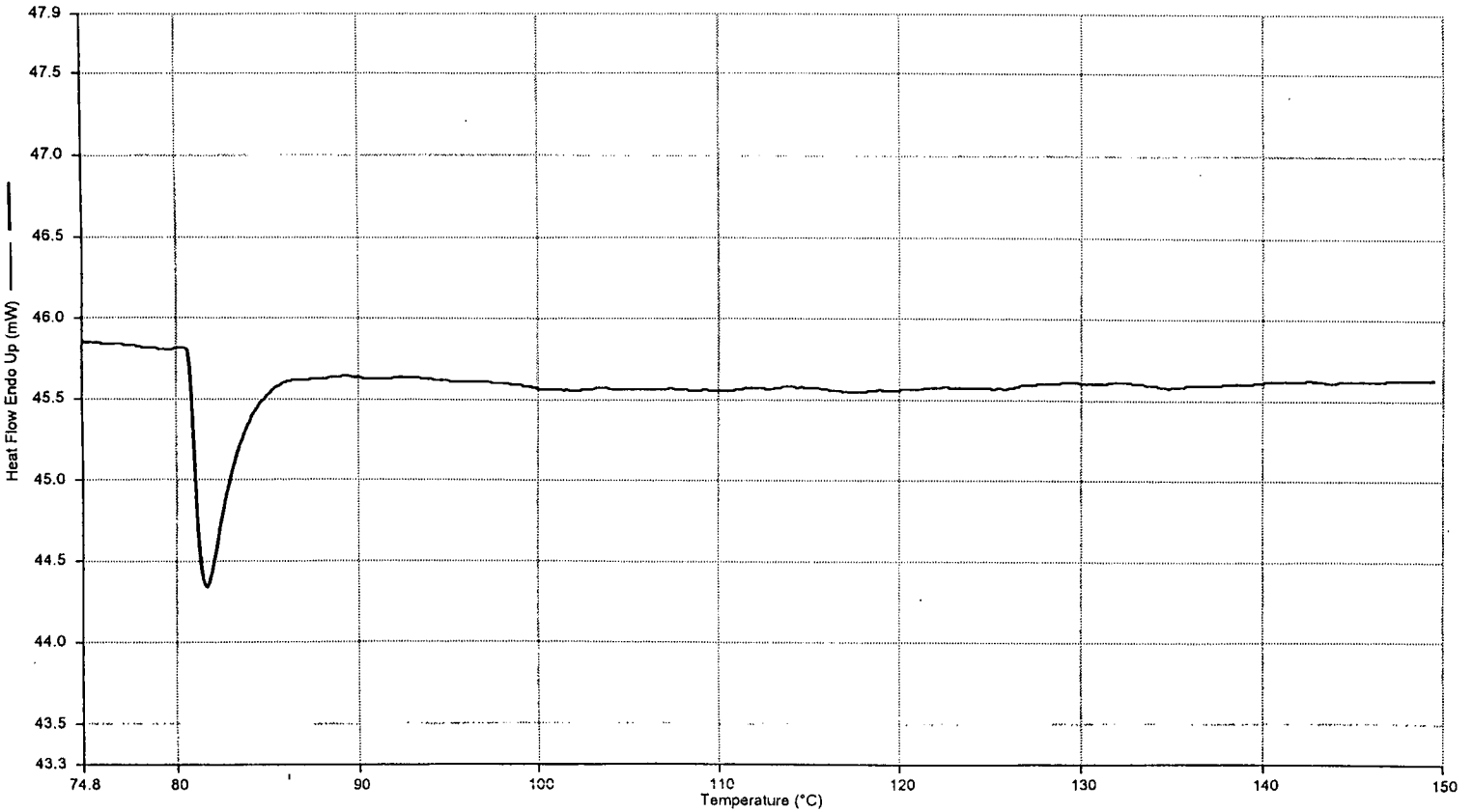


Figure 8.20. Differential scanning calorimetry of barley root oxalate oxidase.
0.103 mg of purified barley root oxalate oxidase was resuspended in MilliQ water and analysed using a Perkin Elmer Pyris 1 in a stainless steel large volume pan.Temperature increase occurred at a rate of 10°C / min, over a temperature range of 50-150°C.

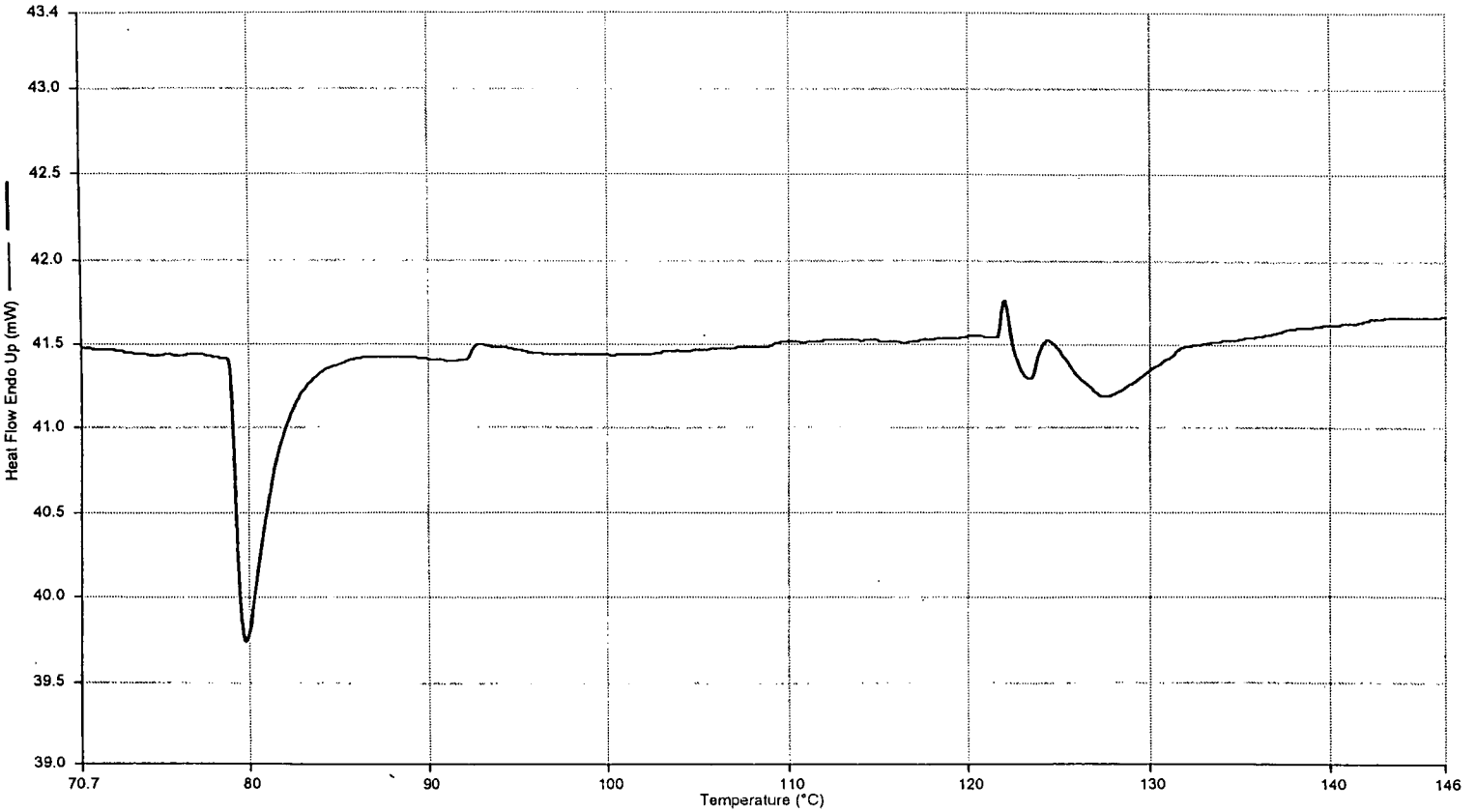


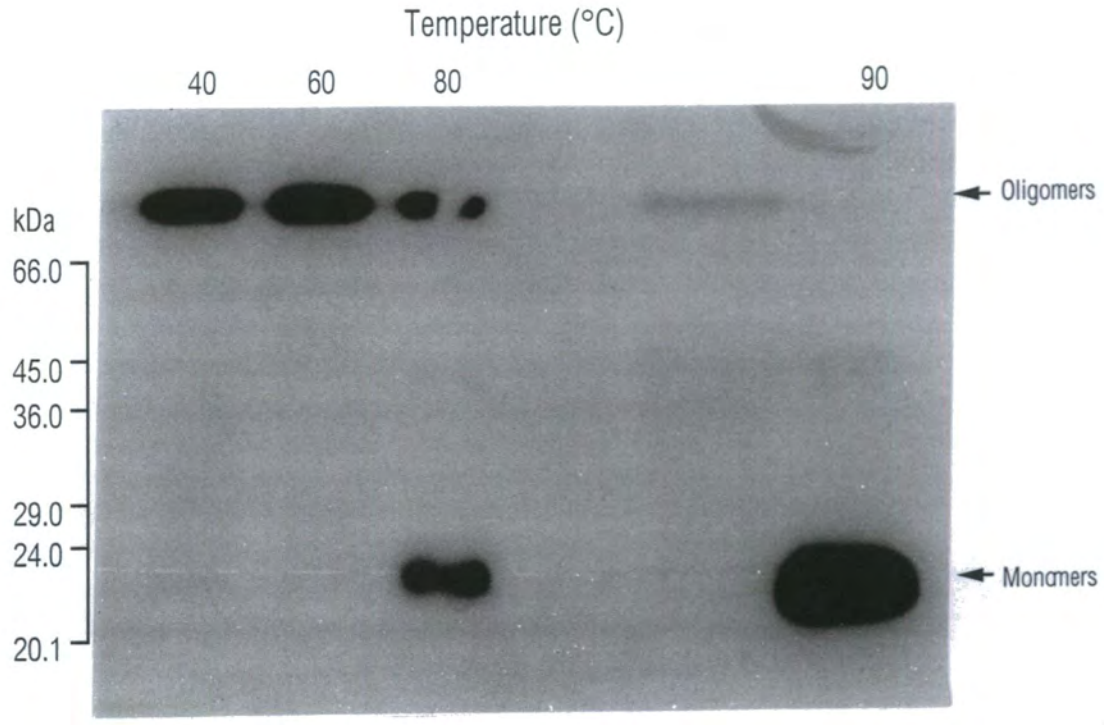
Figure 8.21. Scanning calorimetry of transgenic SGS5 oxalate oxidase.
0.168 mg of purified transgenic SGS5 oxalate oxidase was resuspended in MilliQ water and analysed using a Perkin Elmer Pyris 1 in a stainless steel large volume pan.Temperature increase occurred at a rate of 10°C / min, over a temperature range of 50-150°C.

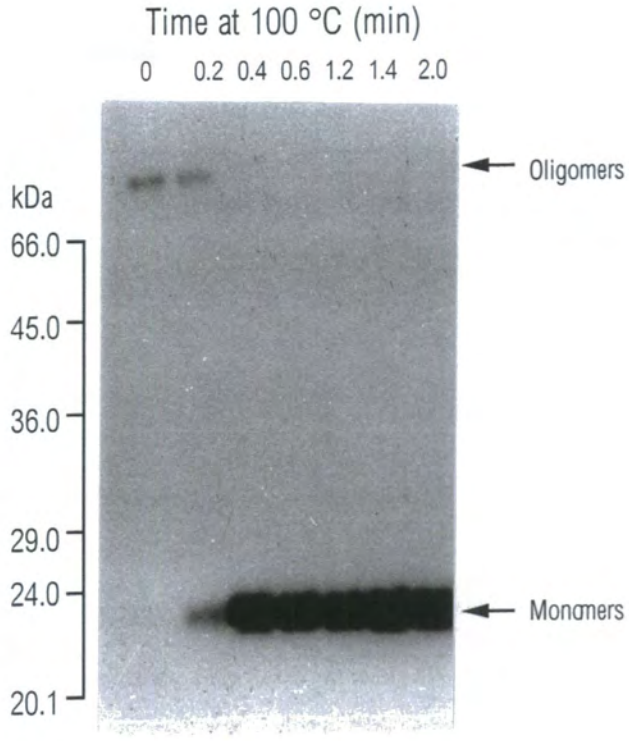
Figure 8.22. Effect of temperature on the stability of the barley root oxalate oxidase oligomer.

Western blot of SDS-PAGE analysis of total protein extracts of 10 day old barley roots prepared as described in section 3.3. were heat treated at 40, 60, 80 and 90 °C for 30 min. Approximately 20 µg of total proteins were analysed on a 12% (w/v) SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. The electroblot was reacted with anti-oxalate oxidase antiserum as primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. The blot was exposed to x-ray film for 1 week at -80 °C. The arrows indicate the oligomers and monomers.

Figure 8.23. Thermostability of barley root oxalate oxidase.

Western blot of SDS-PAGE analysis of total protein extracts of 10 day old barley roots prepared as described in section 3.3., were heat treated at 100 °C for increasing time periods (0 - 2 min, at 20 sec increments). Approximately 20 µg of total proteins were analysed on a 12% (w/v) SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. The electroblot was reacted with anti-oxalate oxidase antiserum as primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey-anti-rabbit antibodies as secondary antibodies (185 kBq) for the detection of oxalate oxidase. The blot was exposed to x-ray film for 1 week at -80 °C. The arrows indicate the oligomers and monomers.


Temperature (°C)
40
60
80
90
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Oligomers
Monomers


Time at 100 °C (min)
0
0.2
0.4
0.6
1.2
1.4
2.0
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Oligomers
Monomers

oxidase at 100 °C for increasing periods of time (0 - 2 min). Without heat treatment, barley root oxalate oxidase was only present in its pentameric form. Following 20 sec at 100 °C a significant proportion of the pentamer was converted to monomer. At 40 sec the protein was only present in its monomeric form. Barley root oxalate oxidase appeared to have a half-life of approximately 20 sec at 100 °C, the same as wheat embryo oxalate oxidase [Lane *et al.* 1992].

8.5.2. Effect of SDS on the stability of oxalate oxidase.

The effect of SDS treatment on the structural integrity of oxalate oxidase was clearly seen when the native and transgenic enzymes were analysed on SDS-PAGE gels (Fig. 8.8.A, 8.9.A and 8.10.A). All the protein samples separated on SDS-PAGE gels were mixed with 2 x loading buffer which gave a final concentration of 1% (w/v) SDS. Barley root, barley embryo and wheat embryo oxalate oxidases were largely stable in the presence of 1% (w/v) SDS and the two isoforms of each of these proteins retained their pentameric structures (Fig. 8.8.A WE and BE and 8.9.A BR, respectively). Oxalate oxidase expressed in transgenic SGS5 tobacco was also shown to be resistant to SDS (1% (w/v)) dissociation and was structurally stable (i.e. retaining its pentameric isoforms, Fig. 8.9.A SGS5). The pentameric isoforms of the cereal and transgenic SGS5 tobacco oxalate oxidases appeared to undergo low levels of dissociation from the pentamers to the monomers in the presence of 1% (w/v) SDS, as judged by the appearance of the monomers in non-heat treated samples (Fig. 8.8.A WE and BE and 8.9.A BR and SGS5). Dissociation occurred at similar levels for the two oxalate oxidase isoforms regardless of their nature (i.e. native or transgenic) or species of origin of the proteins. These enzymes also retained their activity in the presence of 1% (w/v) SDS (Fig. 8.8.B WE and BE and 8.9.B BR and SGS5). Under equivalent conditions the presence of 1% (w/v) SDS in samples of transgenic 3S1 oilseed rape and C26 tobacco protein extracts resulted in the complete dissociation of the oxalate oxidase pentamers and loss of enzyme activity (Fig. 8.10.A 3S1 and C26).

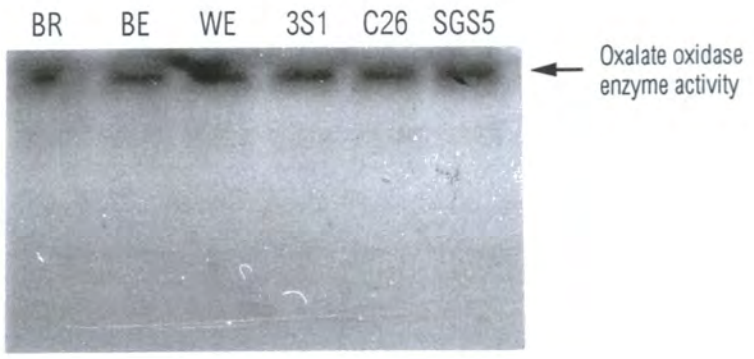
3S1 oilseed rape and C26 tobacco oxalate oxidases which lost their activity in the presence of SDS were shown to retain their enzyme activity (and presumably their pentameric structure) on an electroblot of a native PAGE gel (Fig. 8.24. 3S1 and C26, respectively). This demonstrated that it was not the separation of the transgenic proteins by PAGE (or the presence of 1% (v/v) β-mecaptoethanol in the native loading buffer) but the presence of 1% (w/v) SDS in the loading buffer used for SDS-PAGE, which resulted in the loss of quaternary structure and enzyme activity of these two transgenic proteins. The barley root, barley embryo and wheat embryo and transgenic SGS5 tobacco oxalate oxidases also exhibited enzyme activity on the electroblot of the native PAGE gel (Fig. 8.24. BR, BE, WE and SGS5, respectively).

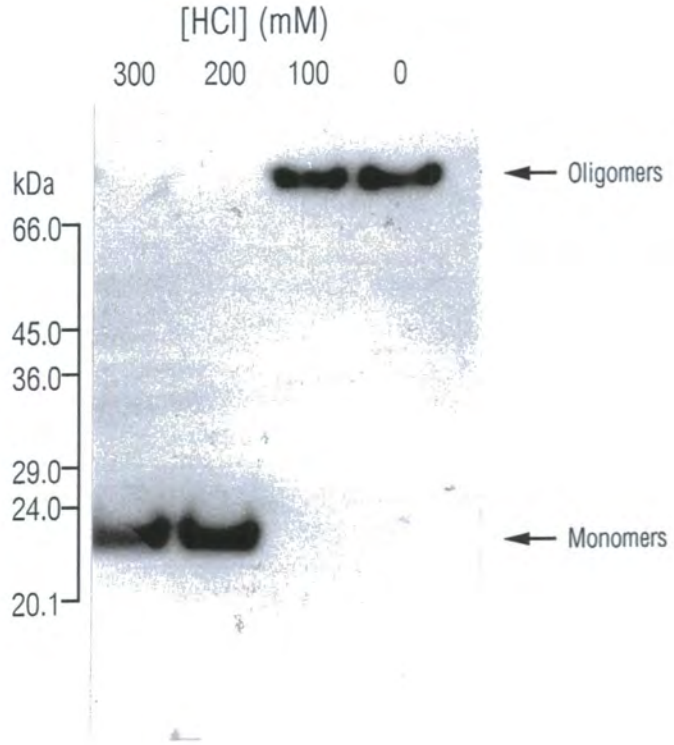
8.5.3. Effect of protease digestion on the structural stability of oxalate oxidase.

Digestion of crude extracts containing oxalate oxidase is a simple and quick method for purifying oxalate oxidase [Grzelczak and Lane 1984 1985]. Several experiments were

Figure 8.24. Structural integrity of oxalate oxidase during native PAGE analysis.
Transgenic 3S1 oilseed rape (0.80 µg), C26 (1.24 µg) and SGS5 tobacco (0.26 µg) INF and barley root (BR, 6.38 µg), barley embryo (BE, 6.22 µg) and wheat embryo (WE, 6.82 µg) crude total protein extracts were analysed on a 12% (w/v) native PAGE gel containing no SDS and without heat treatment. The proteins were electroblotted onto a nitrocellulose membrane, which was assayed for oxalate oxidase enzyme activity, as described in section 3.11.1. The arrow indicates the presence of oxalate oxidase enzyme active proteins.

Figure 8.25. Effect of hydrochloric acid on the stability of the wheat embryo oxalate oxidase oligomer.
Western blot of SDS-PAGE analysis of proteins precipitated at 70% (relative saturation) $(NH_4)_2SO_4$ from wheat embryo extract, which were incubated in the presence of increasing concentrations of HCl (0, 100, 200 and 300 mM) (75 µg protein / treatment). The proteins were analysed on a 12% (w/v) SDS-PAGE gel, without heat treatment and electroblotted onto a nitrocellulose membrane. The electroblot was reacted with anti-oxalate oxidase antiserum as primary antibodies (1:10,000 dilution) and ^{125}I-conjugated donkey anti-rabbit antibodies as a source of secondary antibodies (185 kBq) for the detection of oxalate oxidase. The blot was exposed to x-ray film for 1 week at -80 °C. The arrows indicate the oligomers and monomers of oxalate oxidase.


BR
BE
WE
3S1
C26
SGS5
Oxalate oxidase
enzyme activity


[HCl] (mM)
300
200
100
0
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Oligomers
Monomers

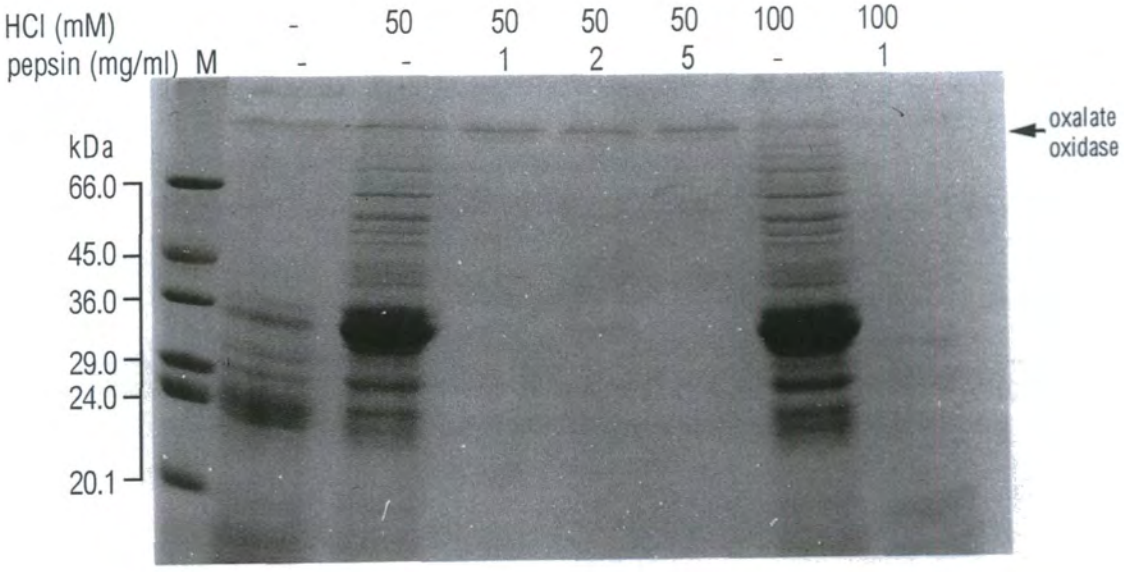
carried out to examine the stability and activity of oxalate oxidase following digestion of protein extracts with pepsin. The purpose of these experiments was to determine if the oxalate oxidases isolated from the three transgenic plant lines exhibited the same structural stability towards pepsin digestion as was previously reported to be one of the unusual characteristics of wheat embryo oxalate oxidase [Grzelczak and Lane 1984 1985]. Firstly the stability of the enzymes to acidification with HCl was examined. Fig. 8.25. illustrates the effect of increasing concentrations of HCl on the structural stability of wheat embryo oxalate oxidase. In the presence of 100 mM HCl the protein still retained its pentameric structure following analysis by SDS-PAGE. Treatment of the protein with 200 and 300 mM HCl resulted in the complete dissociation of the pentamer to the monomer, illustrating the instability of the native protein at HCl concentrations above 100 mM.

Various concentrations of HCl (50, 100 and 200 mM) and pepsin (1, 2 and 5 mg / ml) were tested on a wheat embryo protein extract to optimise the digestion process (Fig. 8.26.). An incubation period of 2 hr at 37 °C was used for all the digestion experiments. A concentration of 50 mM HCl was found to be sufficient to acidify the protein sample (about pH 1.8) to achieve an optimal rate of reaction for pepsin without causing dissociation of the oligomer. 1 and 2 mg / ml of pepsin were not sufficient to give complete digestion of sensitive proteins (75 mg protein / treatment) (Fig. 8.26. 50, 1 and 2, respectively) and so 5 mg / ml (Fig. 8.26. 50, 5) was used in all further experiments. The conditions described in section 3.24. with the extended incubation time (2 hr) were found to give the best results in terms of digesting contaminating proteins from the protein extracts. In the protein treatments illustrated in Fig 8.26., the presence of 50 mM HCl (50-) actually appeared to stabilise some of the proteins in the extract, which were absent in the protein sample incubated in water alone, particularly proteins in the size range of 40-80 kDa. High concentrations of protein in some of the samples affected the efficiency of the digestion process for a give incubation period. Pepsin treatment was not found to be particularly effective on crude total protein extracts (particularly those originating from germinated embryos) where significant levels of protein contamination were still present after 2 hr of incubation (Leaf total protein extracts were found to be more amenable to the digestion process described in section 3.24.). However, if partially purified cereal protein samples were treated, for example following $(NH_4)_2SO_4$ fractionation, then a 2 hr incubation period was quite sufficient to remove all contaminating proteins, to give a specimen almost exclusively consisting of oxalate oxidase (Fig. 8.26. 50 mM HCl, 5 mg / ml pepsin).

Virtually all other barley and wheat proteins from both root and embryo extracts were degraded by pepsin (Fig. 8.27. BR, BE and WE pep+). The wheat embryo (WE), barley embryo (BE) and barley root (BR) oxalate oxidases all appeared to be resistant to pepsin degradation and were structurally stable (Fig. 8.27. pep+). These oxalate oxidase proteins retained their pentameric structures when non-heat treated samples were analysed on SDS-PAGE gels (Fig. 8.27.). An electroblot of the pepsin treated and untreated (50 mM HCl and no pepsin) cereal extracts stained for oxalate oxidase activity revealed that even after the two hour incubation period with pepsin there did not appear to be any significant loss in oxalate

Figure 8.26. Effect of hydrochloric acid and pepsin concentration on the stability of wheat embryo oxalate oxidase.

SDS-PAGE analysis of proteins precipitated at 70% (relative saturation) $(NH_4)_2SO_4$ from wheat embryo extracts, which were incubated in the presence of increasing concentrations of HCl (50, 100 and 200 mM) and increasing concentrations of pepsin (1, 2 and 5 mg / ml stocks) (75 µg protein / treatment). The digests were prepared and incubated as described in section 3.24. The proteins were analysed on 12% (w/v) SDS-PAGE gels, without heat treatment. Proteins were detected by Coomassie Blue staining. M, molecular weight marker. The arrows indicate the oxalate oxidase oligomers.


HCl (mM) - 50 50 50 50 100 100
pepsin (mg/ml) M - - 1 2 5 - 1
kDa
66.0
45.0
36.0
29.0
24.0
20.1
oxalate oxidase

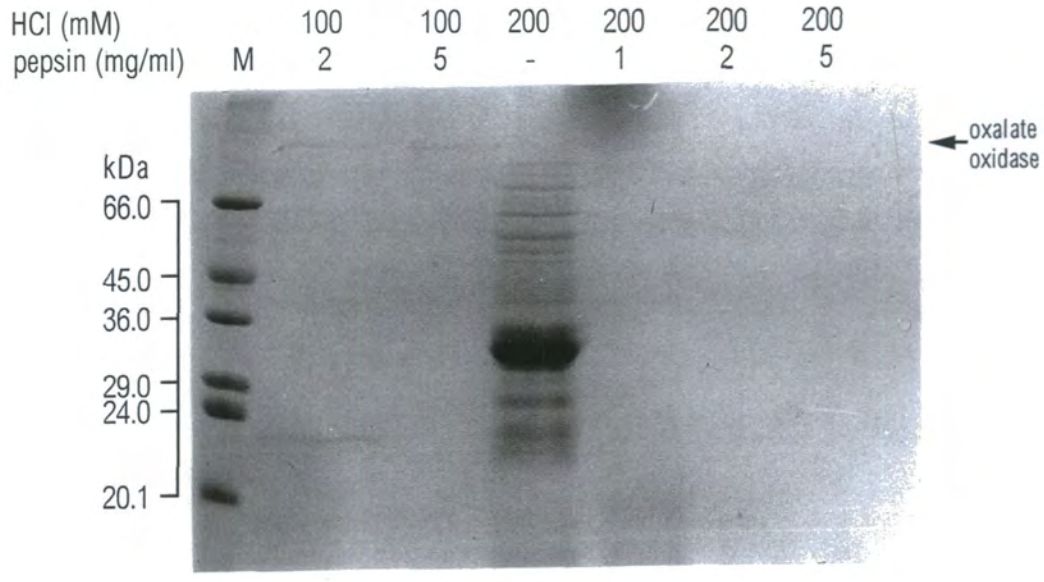

HCl (mM) 100 100 200 200 200 200
pepsin (mg/ml) M 2 5 - 1 2 5
kDa
66.0
45.0
36.0
29.0
24.0
20.1
oxalate oxidase

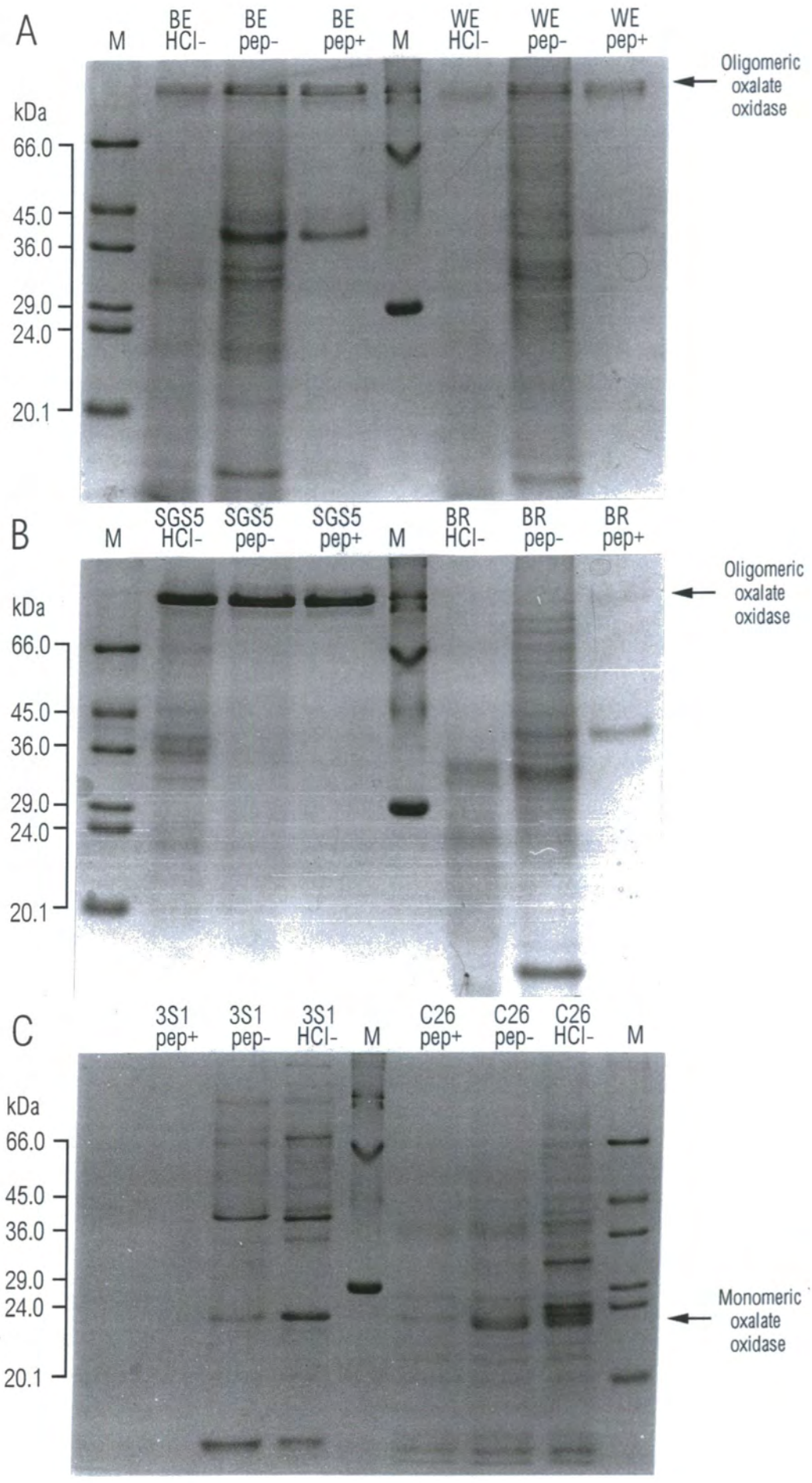
Figure 8.27. Effect of pepsin treatment on the stability of the native and transgenic oxalate oxidases.

SDS-PAGE analysis of proteins precipitated at 70% (relative saturation) $(NH_4)_2SO_4$ from wheat embryos (WE, 75 µg), barley embryos (BE, 79 µg) and barley roots (BR, 82 µg) and extracellular proteins in INF of transgenic 3S1 oilseed rape (46 µg), C26 (34 µg) and SGS5 tobacco (48 µg), which were incubated in the presence of 50 mM HCl and 5 mg / ml pepsin as described in section 3.24. The proteins were analysed on 12% (w/v) SDS-PAGE gels, without heat treatment. M, molecular weight marker. Proteins were detected by Coomassie Blue staining. HCl-, no HCl or pepsin; pep-, 50 mM HCl and no pepsin; pep+, 50 mM HCl and 5 mg / ml pepsin. The arrows indicate the oligomers and monomers of oxalate oxidase.

A. Barley and wheat embryo.

B. Transgenic SSG5 tobacco and barley root

C. Transgenic 3S1 oilseed rape and C26 tobacco.


A
M
BE HCl-
BE pep-
BE pep+
M
WE HCl-
WE pep-
WE pep+
Oligomeric oxalate oxidase
kDa
66.0
45.0
36.0
29.0
24.0
20.1
B
M
SGS5 HCl-
SGS5 pep-
SGS5 pep+
M
BR HCl-
BR pep-
BR pep+
Oligomeric oxalate oxidase
kDa
66.0
45.0
36.0
29.0
24.0
20.1
C
3S1 pep+
3S1 pep-
3S1 HCl-
M
C26 pep+
C26 pep-
C26 HCl-
M
kDa
66.0
45.0
36.0
29.0
24.0
20.1
Monomeric oxalate oxidase

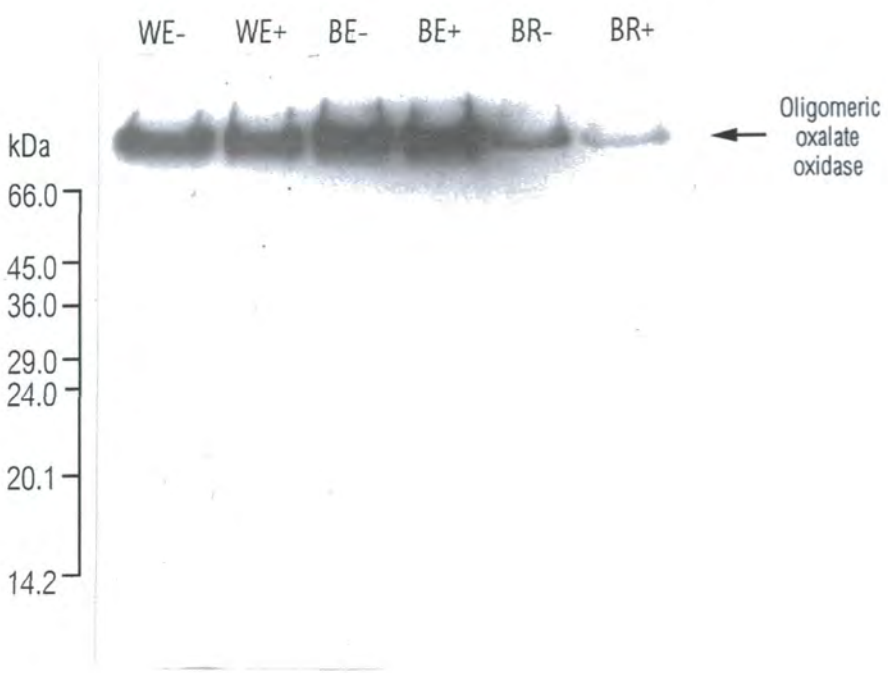
oxidase enzyme activity for the wheat and barley proteins (Fig. 8.28. WE+ and BE+) compared with the protein samples which were only acidified (Fig. 8.28. WE-, BE-). The pepsin treated barley root sample exhibited lower levels of activity compared with the sample which was only acidified (Fig. 8.28. BR+ and -, respectively).

Like the native proteins, oxalate oxidase isolated from transgenic SGS5 tobacco was shown to be resistant to digestion and structurally stable when incubated with pepsin for 2 hr (Fig. 8.27. SGS5 pep+). The protein retained its pentameric structure and enzyme activity in non-heat treated samples analysed on SDS-PAGE gels (result not shown). In contrast, transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases were structurally unstable and completely digested when incubated with pepsin for 2 hr (Fig. 8.27. 3S1 pep+ and C26 pep+, respectively), compared with the native barley root oxalate oxidase from which the gene originated. The transgenic 3S1 and C26 monomers were present in the acidified samples (50 mM HCl treatment) in the absence of pepsin (Fig. 8.27. PEP-) but at levels about 50% lower compared with the non-acidified sample (Fig. 8.27. HCl-). Even if these transgenic proteins were structurally stable following pepsin treatment they would have dissociated to their respective monomers when analysed on SDS-PAGE gels, resulting in the loss of enzyme activity. The presence of HCl in the INF samples actually appeared to result in the loss of a significant number of extracellular proteins when the samples were analysed on SDS-PAGE gels compared with the protein samples incubated with water alone (Fig 8.27. SGS5, 3S1 and C26 HCl-).

Due to the significant differences in stability of the native and transgenic proteins the effect of temperature and SDS treatment on the stability and enzyme kinetics of these oxalate oxidases was further examined; the results of these experiments are described in chapter 9. SGS5 tobacco expressed a transgenic protein which exhibited almost identical structural and stability properties comparable to those of the native cereal oxalate oxidases, particularly the wheat embryo protein, the gene for which was expressed in the transgenic SGS5 tobacco line. 3S1 oilseed rape and C26 tobacco on the other hand expressed transgenic proteins which did not possess the stable properties (i.e. resistance to SDS dissociation and pepsin degradation) of the native barley root oxalate oxidase. The integral structure of the monomer subunits (for all of the native or transgenic proteins) did not appear to be significantly affected by any of the treatments described in this chapter, i.e. there was no reduction in the apparent sizes of the basic monomer subunits resulting from these treatments. The relevance of the stability of the transgenic SGS5 tobacco oxalate oxidase and the structural instability of the transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidase will be discussed in chapter 11 with regard to the genes introduced and expressed in these plants.

Figure 8.28. Effect of pepsin treatment on the stability and activity of the cereal oxalate oxidases.

Electroblot of SDS-PAGE analysis of proteins precipitated at 70% (relative saturation) $(NH_4)_2SO_4$ from wheat embryos (WE, 75 µg), barley embryos (BE, 79 µg) and barley roots (BR, 82 µg), which were incubated in the presence of 50 mM HCl and 5 mg / ml pepsin as described in section 3.24. The proteins were analysed on 12% (w/v) SDS-PAGE gels, without heat treatment. The proteins were electroblotted onto a nitrocellulose membrane which was assayed for oxalate oxidase activity. -, 50 mM HCl and no pepsin; +, 50 mM HCl and 5 mg / pepsin.


WE-
WE+
BE-
BE+
BR-
BR+
kDa
66.0
45.0
36.0
29.0
24.0
20.1
14.2
Oligomeric
oxalate
oxidase

9. RESULTS AND DISCUSSION.

Enzyme kinetics of native and transgenic oxalate oxidases.

One of the most effective ways of studying potential subtle differences between the native and transgenic oxalate oxidases was to examine the kinetics of the reaction catalysed by the enzymes, including the determination of K_m and V_{max}. All of the native and transgenic oxalate oxidases are aggregates of five identical subunits so presumably each subunit contains an active site. All experiments were carried out using purified oxalate oxidase proteins as described in chapter 7, except for barley seedling oxalate oxidase which was purchased from Sigma (Poole, Dorset, U.K.) and used without further purification. This enzyme was initially used to optimise the various kinetic experiments prior to using the purified proteins. A Dynatech MR5000 microtitre plate reader was used to carry out the majority of the enzyme assays described in this chapter. The enzyme assay follows the catalytic breakdown of oxalic acid to CO_2 and H_2O_2, where one unit of oxalate oxidase is defined as the amount of enzyme forming 1 µmole of H_2O_2 from oxalic acid per minute at pH 3.8, at 37 °C. Horseradish peroxidase (HRPO) was used as a coupling enzyme which hydrolysed H_2O_2, resulting in the reaction between N-dimethyl alanine and amino antipyrine to form a purple dye, which was measured at 550 or 570 nm depending on the detection method used (see section 3.11.). The amount of dye formed was proportional to the amount of H_2O_2 produced as a result of oxalate oxidase activity. The OD of the assays were related to a standard curve of known H_2O_2 concentrations incubated with the assay developer to determine H_2O_2 produced and the units of enzyme present in the assay (Fig. 7.2.). The results of the enzyme kinetic experiments are described in this chapter.

9.1. Effect of oxalate oxidase concentration.

The first experiment carried out involved examining the relationship between oxalate oxidase and HRPO enzyme concentrations on the rate of the enzyme catalysed reactions, to establish the parameters of the assay. This experiment was performed in two parts, firstly varying the concentration of oxalate oxidase (barley seedling, 0.009-0.108 unit) with a fixed concentration of HRPO (2 units) and secondly varying the concentration of HRPO (1-16 units) with a fixed concentration of oxalate oxidase (0.036 unit). The combined substrate / developer assays were carried out at 37 °C in 1 ml cuvettes using the Unicam UV2 spectrophotometer, with readings taken every sec at 550 nm for 10 min. The enzyme assay procedure was different from the others carried out (as described in section 3.11.2) as it involved the combination of two enzyme assay reactions (oxalate oxidase and HRPO). Normally the oxalate oxidase was incubated with the oxalic acid substrate solution resulting in the generation and accumulation of H_2O_2 which was the substrate for the second enzyme HRPO, present in the enzyme assay developer. The hydrolysis of H_2O_2 by the coupling enzyme resulted in the generation of a purple dye which was measured at wavelengths of 550 or 570 nm, depending on the assay method and spectrophotometer used (section 3.11.2. or 3.11.4.). Fig. 9.1.A illustrates the results obtained when the concentration of oxalate oxidase enzyme

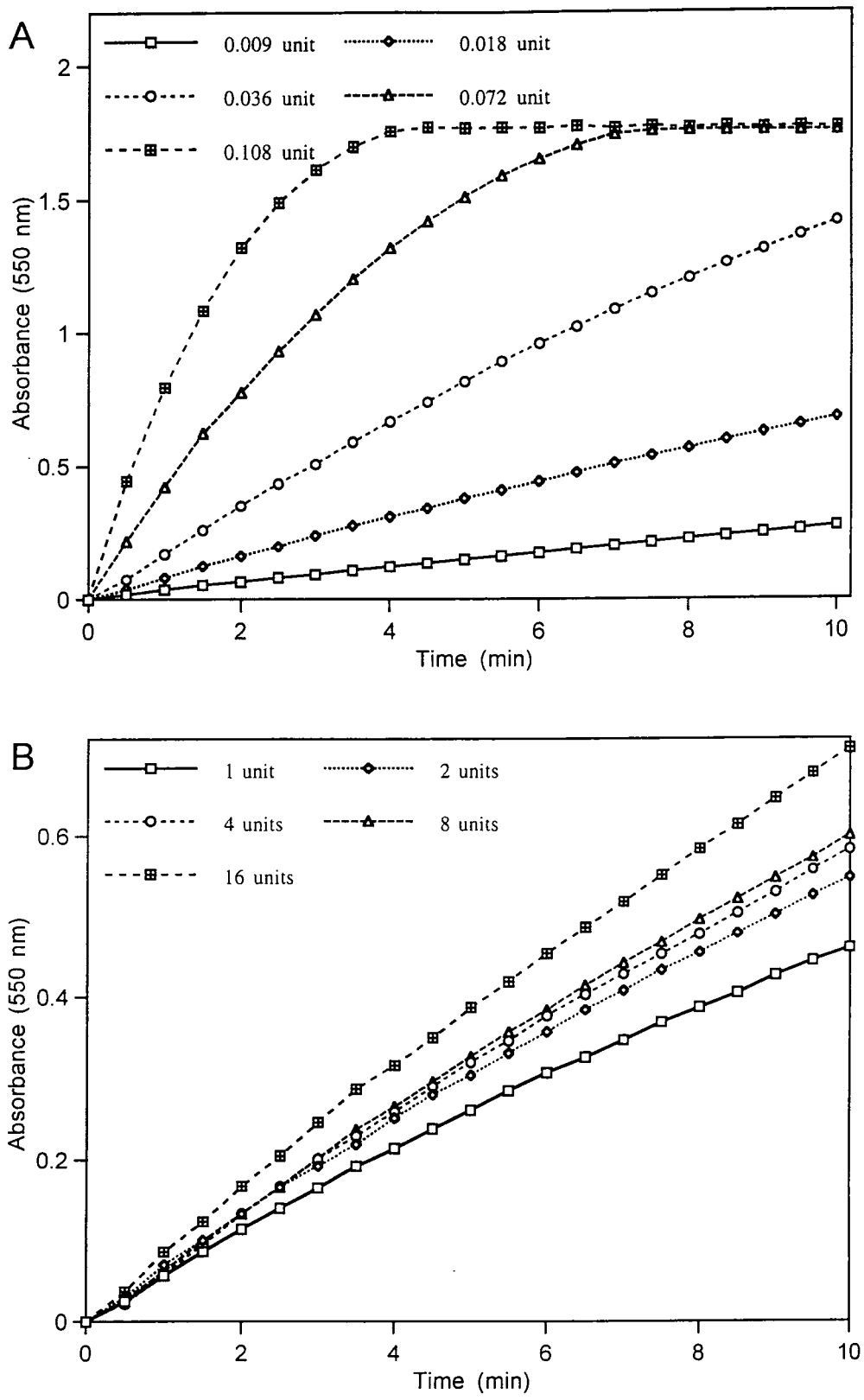


Figure 9.1. The effect of varying oxalate oxidase and HRPO concentrations. Cuvette enzyme assays were carried out by mixing oxalate oxidase, substrate solution (500 µl) and developer (300 µl) in a 1 ml cuvette and taking measurements for 10 min at 37 °C in a Unicam UV2 spectrophotometer at 550 nm. **A**, barley seedling oxalate oxidase concentration was varied (0.009-0.108 unit) with a fixed concentration of HRPO (2 units). **B**, barley seedling oxalate oxidase concentration was fixed (0.036 unit) and HRPO concentration was varied (1-16 units).

was varied with a fixed concentration of HRPO. The reaction retained linearity over the 10 min assay time period for levels at 0.009-0.036 units (u) of enzyme, under conditions where the substrate (oxalic acid) was in excess. Above 0.036 u enzyme the assay rapidly achieved a plateau of activity at an OD of about 1.7, resulting from the hydrolysis of the available substrate in the assay. Fig. 9.1.B illustrates the effect of varying the concentration of the coupling enzyme (HRPO) with a fixed concentration of oxalate oxidase. Over the 10 min incubation period the rate of reaction remained more or less linear for the 5 different concentrations of HRPO tested. As the concentration of HRPO increased the rate of the enzyme catalysed reaction also increased. However, unlike oxalate oxidase which achieved complete hydrolysis of all of the available substrate at the higher enzyme concentration (0.072 and 0.108 u) the activity of HRPO remained linear even at the highest concentrations tested (16 u). This meant that the hydrolysis of oxalic acid to CO_2 and H_2O_2 by oxalate oxidase in the combined assay was the rate determining step, which limited the activity of HRPO. As the concentration of HRPO increased the number of redundant active sites simply increased since the rate of reaction of the oxalate oxidase assay was insufficient to saturate the HRPO active sites with H_2O_2. Therefore as the concentration of HRPO was increased no significant increase in activity was observed. It was therefore considered adequate to use 2 u of HRPO / 800 µl reaction for all of the oxalate oxidase assays required. When using a coupling enzyme it is essential that the detection reaction occurs rapidly so that the enzyme-catalysed reaction is always rate limiting so that the initial rates measured correspond to the enzyme under investigation.

All further experiments were therefore carried out as described in section 3.11.4. where the enzyme was incubated with oxalic acid for 60 min followed by the addition of the developer (containing HRPO) immediately prior to taking readings at 570 nm in the Dynatech MR5000 microtitre plate reader (readings were taken within 60 sec of adding developer and 2.5 M Tris-HCl pH 7.5). The colour change observed upon the addition of the developer to the incubated enzyme / substrate solution was almost instantaneous, which highlighted the rapid rate of reaction of HRPO under the standard assay condition (pH 5.5 at room temperature). Tris-HCl was added to stop the reaction by altered the pH of the reaction to 7.5, which was outside the activity range of oxalate oxidase.

Fig. 9.2. shows the effect of varying enzyme concentration for each of the native and transgenic oxalate oxidases. The purpose of performing this experiment was to confirm linearity between initial rate and enzyme concentration to confirm the absence of activators and inhibitors in the enzyme preparations. The layout of the graphs enable a direct comparison between oxalate oxidase isolated from the same organs of different species (wheat and barley embryo); native (barley root) and transgenic (3S1 oilseed rape) plants expressing the same gene for the mature oxalate oxidase protein; and two transgenic tobacco plants (C26 and SGS5) which were expressing genes containing the same mature protein coding regions but different signal peptides. For all of the oxalate oxidases tested as the concentration of the enzyme increased the µmoles of H_2O_2 produced for a given incubation time (i.e. 60 min) increased. This was expected since the number of active sites present in the

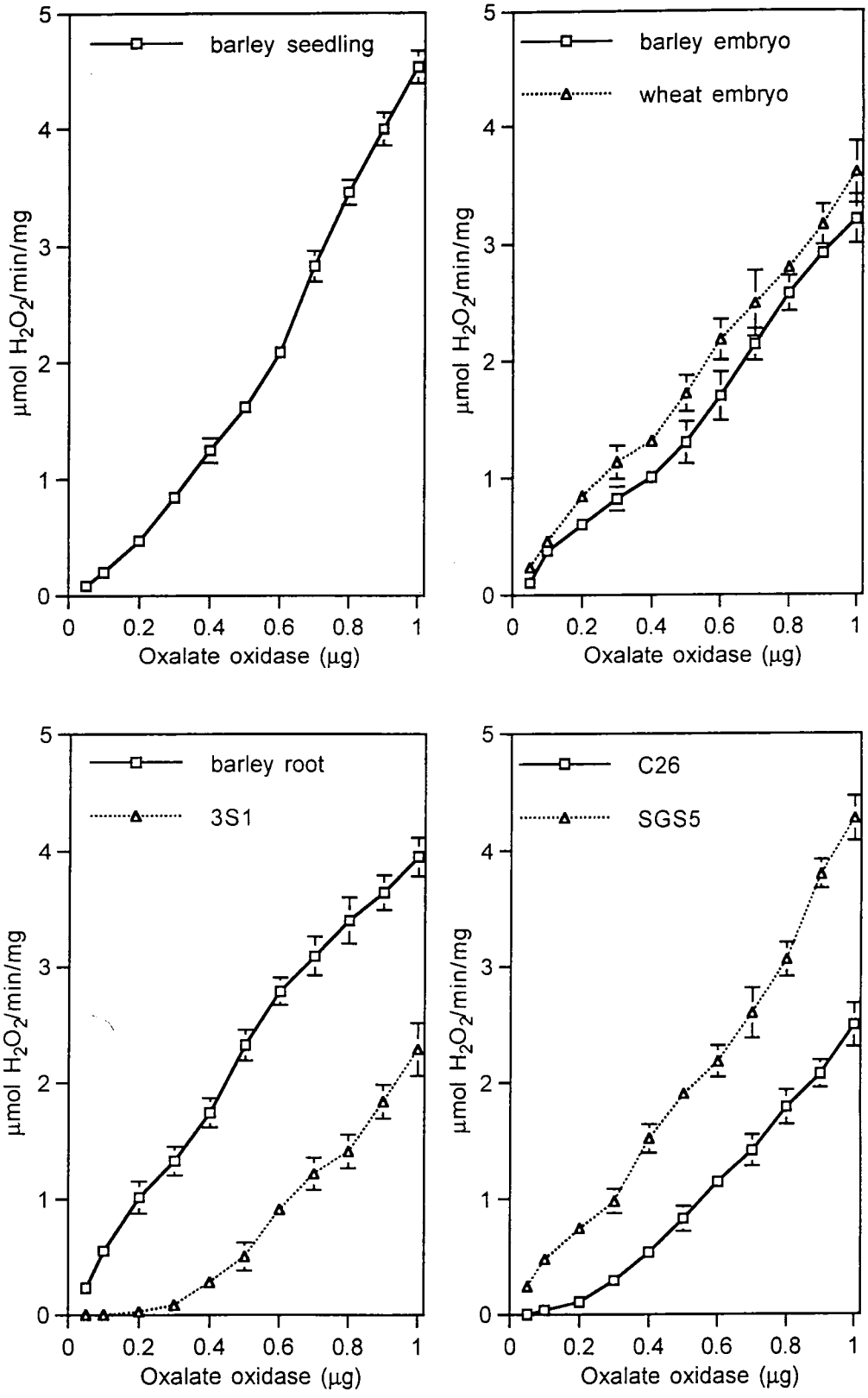


Figure 9.2. The effect of enzyme concentration on oxalate oxidase enzyme activity.
Native (barley seedling, root and embryo and wheat embryo) and transgenic (3S1 oilseed rape, C26 and SGS5 tobacco) oxalate oxidases were assayed using the standard microtitre plate enzyme assay protocol, as described in section 3.11.4. (i.e. 1mM oxalic acid, pH 3.5 at 37°C for 60 min) Enzyme concentration was altered as indicated. Error bars show mean ± standard errors.

assay were increasing with increasing enzyme concentration. The activity of all the enzymes remained linear over the concentration ranges tested which showed that the substrate (1 mM oxalic acid) was always in excess. Specific activities were calculated for the native and transgenic oxalate oxidase, which are shown in table 9.1. The specific activities of transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases were approximately 36-42% lower than those of the native barley root enzyme at comparable protein concentrations, under the standard enzyme assay conditions.

Enzyme	Specific activity (μmoles H_2O_2 /mg / sec)
barley seedling	0.075
barley root	0.066
barley embryo	0.053
wheat embryo	0.060
3S1 oilseed rape	0.038
C26 tobacco	0.042
SGS5 tobacco	0.071

Table 9.1. Specific activities of native and transgenic oxalate oxidases.

The concentrations of each of the oxalate oxidases tested were selected such that after a 60 min incubation period under standard assay conditions (as described in section 3.11.4.) OD's of about 0.4 were obtained. This gave enough scope for activity to increase or decrease when different assay conditions were tested. All of the different assay treatments were performed in triplicate, which enabled standard errors to be calculated (as shown by the error bars). The actual concentrations of each of the oxalate oxidases used were in the range of 0.2-0.6 μg / assay.

9.2. Effect of incubation time.

Fig. 9.3. illustrates the effect of incubation time on the rate of the enzyme catalysed hydrolysis of oxalic acid by native and transgenic oxalate oxidases. For each enzyme concentration tested the μmoles of H_2O_2 generated increased with increasing incubation time, indicating the stability of the enzyme catalysed reaction with time. The activity of all of the proteins remained reasonably linear over the 60 min assay period. None of the enzymes tested achieved complete hydrolysis of the available oxalic acid at the enzyme concentrations used over the 60 min assay period, i.e. the activity of the enzymes did not plateau. Therefore it was concluded that the enzyme concentrations selected for use in the subsequent assays (0.2-0.6 μg / assay) would always contain a molar excess of substrate.

A 60 min incubation period was used for all of the subsequent experiments. This incubation period meant that less of the purified enzymes were required to obtain sufficiently high OD readings (i.e. about 0.4 at OD_{570}). It would have been possible to increase the

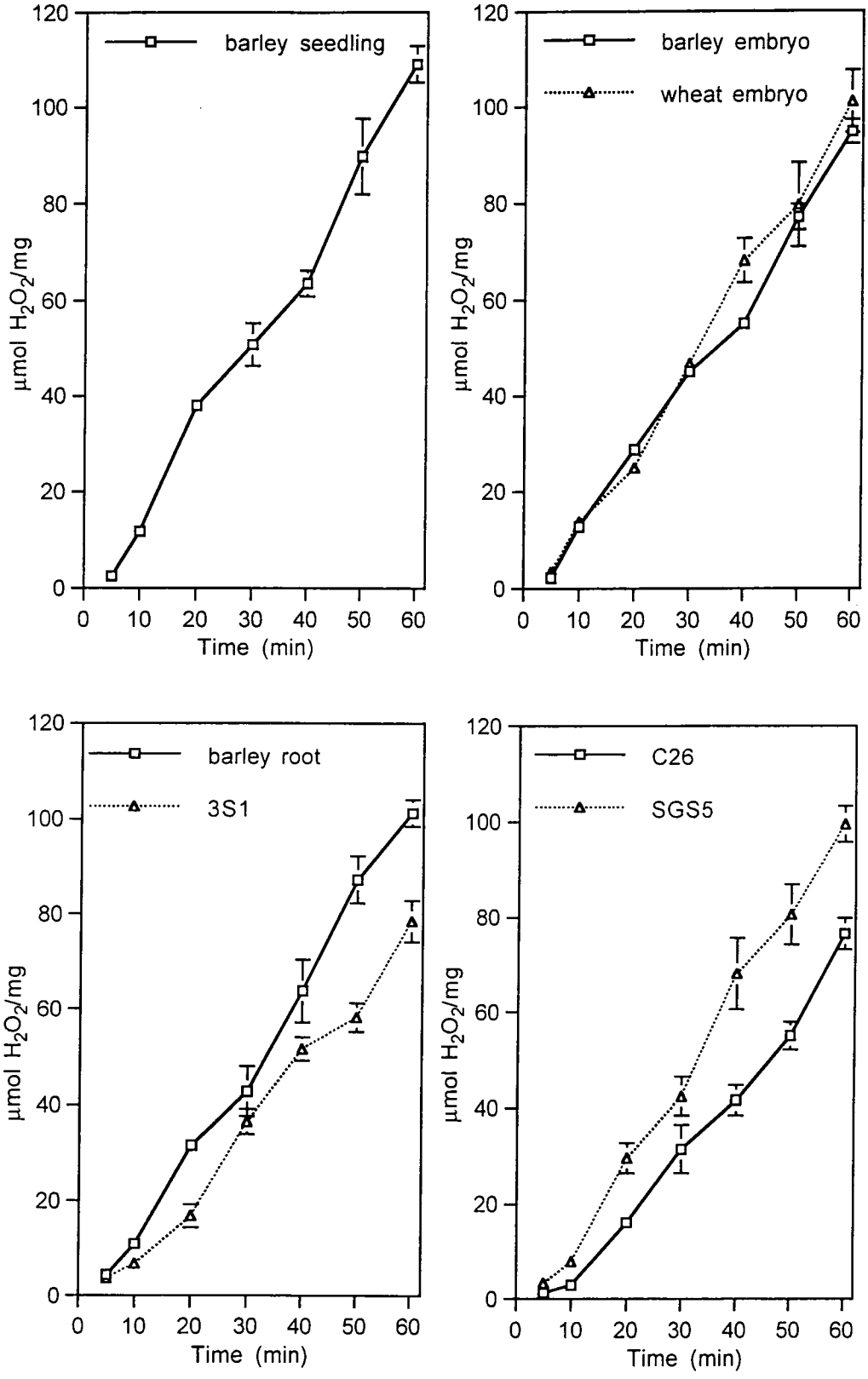


Figure 9.3. The effect of incubation time on oxalate oxidase activity.
Native (barley seedling, root and embryo and wheat embryo) and transgenic (3S1 oilseed rape, C26 and SGS5 tobacco) oxalate oxidases were assayed using the standard microtitre plate enzyme assay protocol, as described in section 3.11.4., except readings were taken after different incubation periods. Incubation time was altered as indicated. Error bars show mean ± standard errors.

enzyme concentration per assay and thereby reduce the incubation time to achieve a similar OD, if more of the purified enzymes had been available.

9.3. Effect of substrate concentration on oxalate oxidase activity.

The effect of increasing substrate concentration on the activity of the native and transgenic oxalate oxidases was studied to provide insight into the binding affinity and any inhibition effects on the enzymes by oxalic acid. The concentration of oxalic acid used in the standard assay was reduced from 2 mM used in the method described by Lane *et al.* [1993] to 1 mM, as preliminary experiments demonstrated that substrate concentrations above 1 mM caused significant inhibition of oxalate oxidase activity. Fig. 9.4. shows the substrate concentration curves generated when the native and transgenic oxalate oxidases were assayed in the presence of substrate concentrations up to 5 mM. The optimum oxalic acid concentrations to achieve maximum enzyme activity varied between about 0.4 mM for transgenic 3S1 oilseed rape and C26 tobacco, 0.6 mM for barley root, 0.8 mM for barley embryo, wheat embryo and transgenic SGS5 tobacco and 1.2 mM for barley seedling oxalate oxidase. Above the optimum oxalic acid concentrations all of the enzymes exhibited strong substrate inhibition. At a substrate concentration of 1 mM oxalic acid (standard assay concentration) the native cereal and transgenic SGS5 oxalate oxidases exhibited approximately 95-97% of the activity observed at their optimum substrate concentrations (0.6-1.2 mM). Transgenic 3S1 and C26 oxalate oxidases exhibited lower levels of activity at 1 mM oxalic acid (about 88%) compared with levels of activity at their substrate optimums (about 0.4 mM). The inhibition occurring above the optimum substrate concentrations for all of the oxalate oxidase may have results from substrate binding at other sites on the enzyme or as a result of steric hindrance caused by the substrate attempting to bind at all the active on the enzyme at the higher substrate concentrations, which may alter the configuration of other active sites on the enzyme molecule, resulting in inhibition. Pundir and Nath [1984], Trinchant and Rigaud [1996] and Kotsira and Clonis [1997] also reported substrate inhibition of oxalate oxidase at high oxalic acid concentrations.

Fig. 9.5. illustrates the Lineweaver-Burk plots of the substrate concentration data shown in Fig. 9.4. This is a double reciprocal plot of 1/v against 1/[S], which gives a straight line of slope K_m/V_{max} with an intercept on the 1/v axis of $1/V_{max}$ and an intercept on the 1/[S] axis of $-1/K_m$. These plots were used to calculate K_m values of the native and transgenic oxalate oxidases. K_m, the Michaelis constant is numerically equal to the substrate concentration at which the initial rate of reaction exhibits half maximal velocity (V_{max}), which is independent of enzyme concentration. V_{max} is the limiting value of the initial rate of reaction. The K_m value for a given oxalate oxidase is constant, which allows a comparison of oxalate oxidases isolated from different species and organs of the same species. The K_m value of an enzyme is often used to estimate the 'affinity' of an enzyme for its substrate, where an enzyme with a low K_m value has a 'higher affinity' for the substrate and can utilise lower levels of substrate. The four cereal oxalate oxidases (barley seedling, barley embryo, wheat embryo and barley root) had K_m values of about 220, 216, 206 and 192 μM, respectively. The enzymes

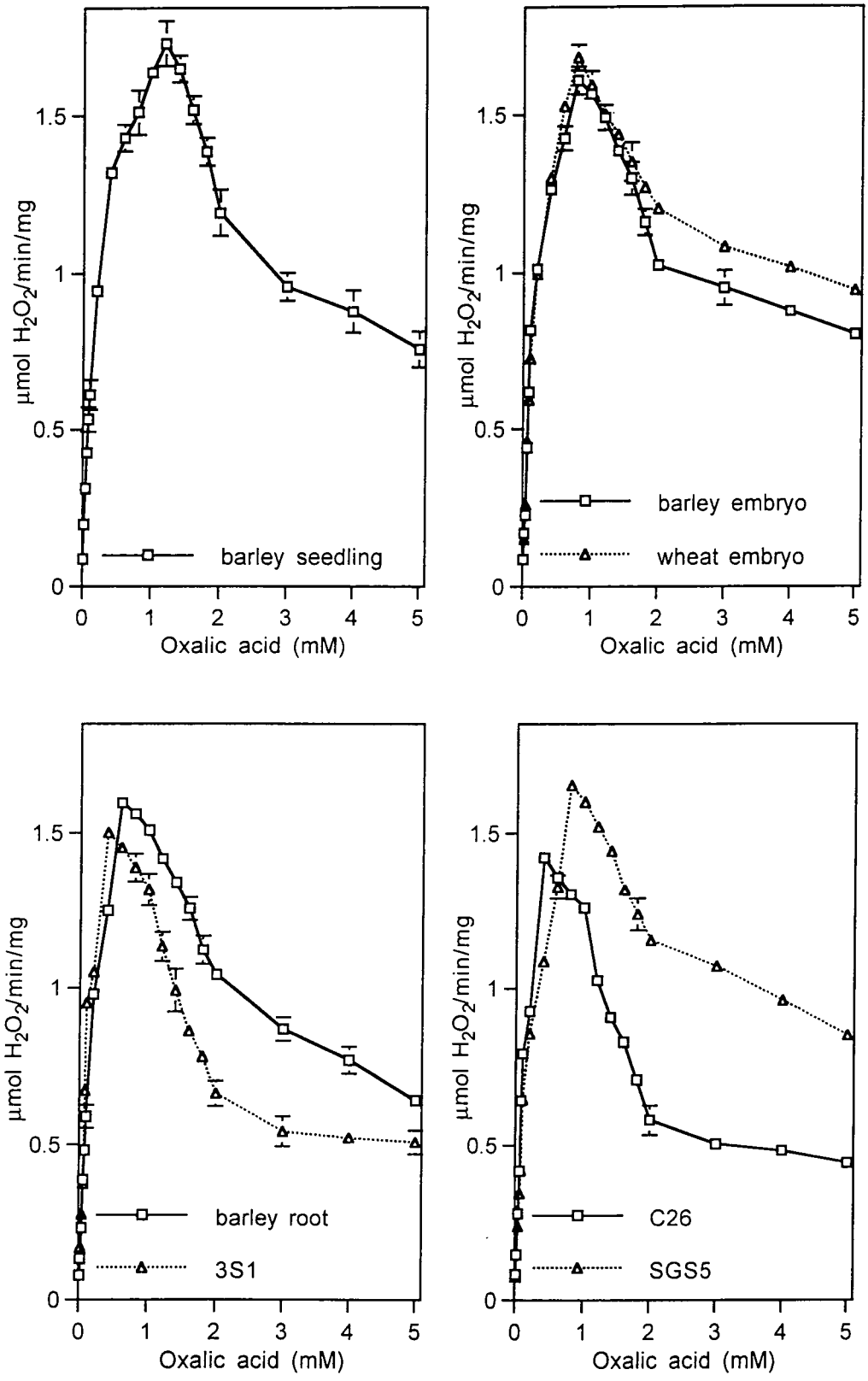


Figure 9.4. The effect of substrate concentration on oxalate oxidase activity. Native (barley seedling, root and embryo and wheat embryo) and transgenic (3S1 oilseed rape, C26 and SGS5 tobacco) oxalate oxidases were assayed using the standard microtitre plate enzyme assay protocol (60 min incubation), as described in section 3.11.4., except for substrate concentration which was altered as indicated. Error bars show mean ± standard errors.

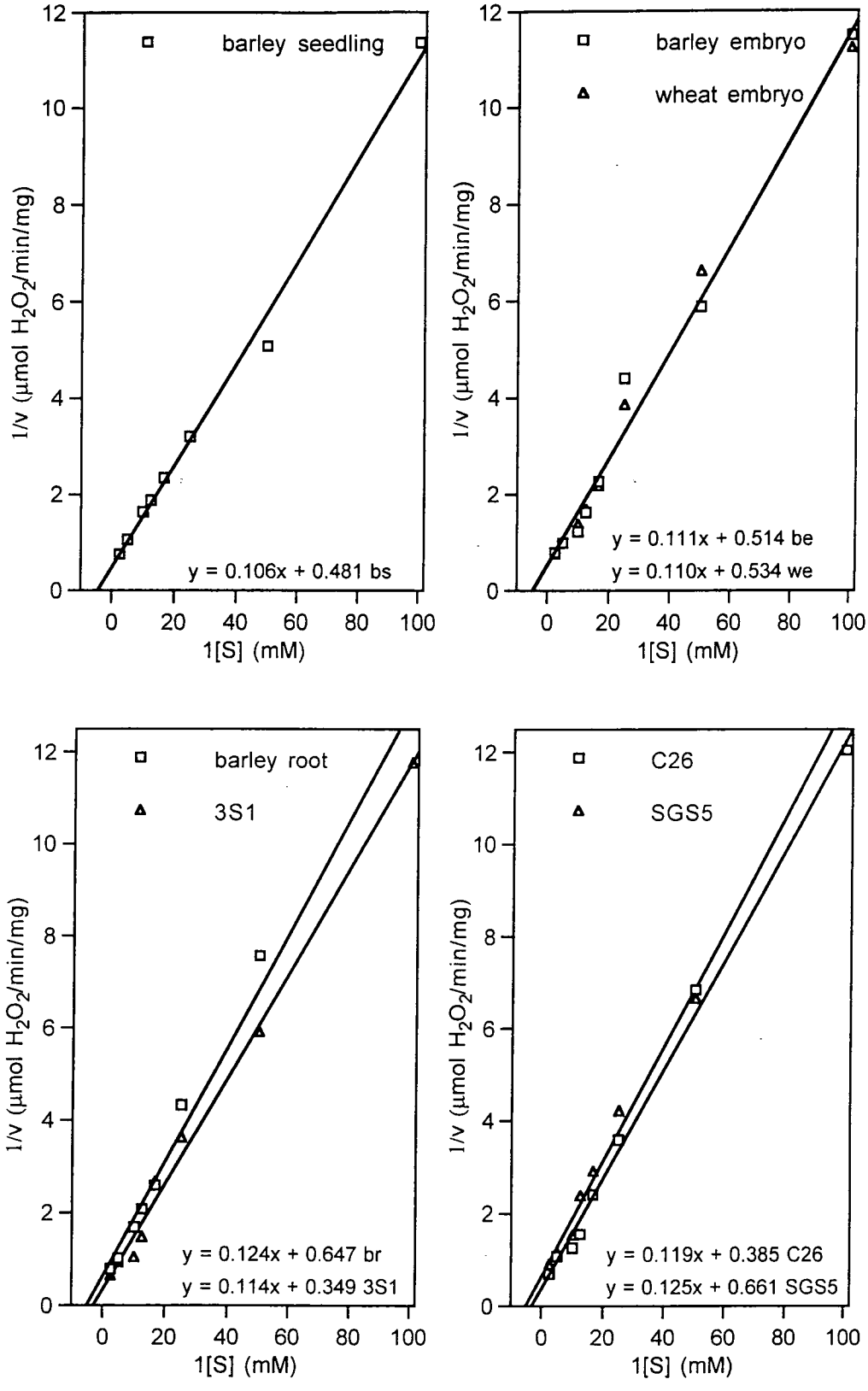


Figure 9.5. Lineweaver-Burk plots of the native and transgenic oxalate oxidases. The data was the same as that used for the substrate concentration graphs illustrated in Fig. 9.4.

had V_{max} values of 2.08, 1.95, 1.87 and 1.55 µmoles H_2O_2/min/mg, respectively. The transgenic proteins (3S1, C26 and SGS5) had K_m values of about 327, 309 and 189 µM respectively. These enzymes had V_{max} values of 2.87, 2.60 and 1.51 µmoles H_2O_2/min/mg, respectively. The native and transgenic SGS5 oxalate oxidases had similar K_m (about 209 µM) and V_{max} values (about 1.86 µmoles H_2O_2/min/mg), compared with transgenic 3S1 and C26 which had higher K_m (about 318 µM) and V_{max} values (about 2.74 µmoles H_2O_2/min/mg) than the native barley root oxalate oxidase. The native cereal and transgenic SGS5 tobacco oxalate oxidases had K_m values which were comparable with those reported for barley root oxalate oxidase (200 µM and 270 µM) [Schmitt 1991; Kotsira and Clonis 1997]. The native and SGS5 transgenic oxalate oxidases had a 'higher affinity' for oxalic acid and could utilise lower concentrations of substrate compared with 3S1 and C26 transgenic oxalate oxidase because of their lower K_m values. For the native cereal oxalate oxidase the K_m values provides information regarding the approximate levels of substrate likely to be present in the wheat and barley embryos and roots. The native cereal oxalate oxidases had K_m values of about 200 µM it was therefore unlikely that the oxalic acid concentration in the embryos and roots exceeded 400 µM the theoretical V_{max} concentration, as there was no sense in maintaining oxalic acid at concentrations above this since the initial rate of reaction cannot exceed V_{max}. The higher K_m values of the transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidase may have resulted from subtle differences in the quaternary structure of the pentamers, the implications of which are discussed in chapter 11.

9.4. Effect of pH on oxalate oxidase activity.

Enzyme activity relies on the amino acids in the active site of the enzyme being in the correct ionised state, therefore enzyme activity is pH dependent. The correct state of ionisation of other ionisable amino acids of a protein is also important in maintaining protein conformation. The effect of pH on native and transgenic oxalate oxidase enzymes was examined over a pH range of 1.5-6.5 (Fig. 9.6.). This pH range was selected as preliminary experiments using commercial barley seeding oxalate oxidase (Fig. 9.6. barley seedling) had shown there to be complete loss of activity outside this pH range. Barley seedling oxalate oxidase had a pH optimum of about 4.0 which exhibited only about 85% of the maximum activity at pH 3.5, the pH of the standard enzyme assay substrate buffer. Barley seedling oxalate oxidase exhibited activity over a pH range of 2.0-5.5, but was essentially inactive at the extremes of the pH range. Barley embryo and wheat embryo oxalate oxidases had very similar pH profiles and both of these proteins exhibited pH optimums of about 4.0. These enzymes exhibited about 86 and 96% of the maximum activity at pH 3.5 with broad activity over the pH range of 2.0-6.0. Barley root and transgenic SGS5 tobacco oxalate oxidases also had similar pH profiles with broader optimum pH's of about 4.0-4.5, exhibiting activity over a pH range of 2.0-6.0 with 65 and 70% of the maximum activity at pH 3.5, respectively. Schmitt [1991] and Kotsira and Clonis [1997] reported pH optima for barley root oxalate oxidase of about 3.5-3.8. Both transgenic 3S1 oilseed rape and C26 tobacco proteins had lower pH optimums compared with the corresponding native oxalate oxidases. Due to this phenomenon

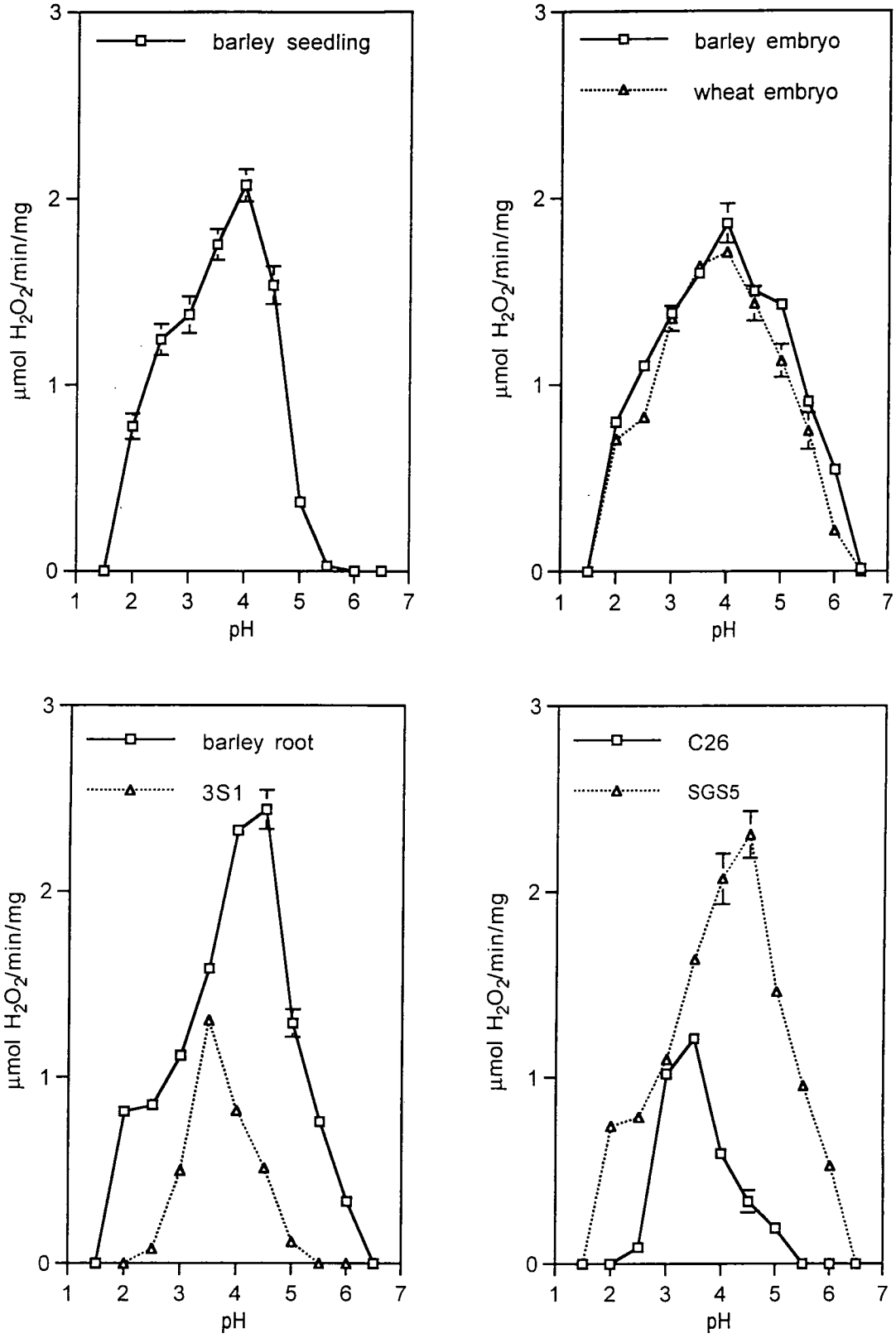


Figure 9.6. The effect of pH on native and transgenic oxalate oxidase activity. Native (barley seedling, root and embryo and wheat embryo) and transgenic (3S1 oilseed rape, C26 and SGS5 tobacco) oxalate oxidases were assayed using the standard microtitre plate enzyme assay protocol (60 min incubation), as described in section 3.11.4, except for pH which was altered as indicated. Error bars show mean ± standard errors.

these proteins exhibited 100% of their activity when incubated in the standard assay substrate solution (pH 3.5). The most noticeable difference was the observed shift in the pH activity range of these two transgenic proteins (pH 2.0-5.0) which were narrower (pH 2.5-5.0) compared with that of the native cereal and transgenic SGS5 enzymes (pH 2.0-6.0). The fact that all of these enzymes had bell shaped enzyme activity profiles with very narrow pH optima suggested that the amino acids present in the active site had similar pK values (this value represents the specific pH for a given amino acid residue). The loss of activity observed at the pH extremes tested (i.e. < 2.0 and > 6.5) presumably resulted from changes in the structural stability of the enzymes, which may be irreversible, resulting from the unfolding of the protein. These results suggest that the transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidase were less stable than the native enzymes which retained structural stability and activity over a broader pH range.

9.5. Effect of temperature on oxalate oxidase activity.

The effect of heat treatment on the stability and activity of the native and transgenic oxalate oxidases was studied using pre-heat treatment of the proteins for 30 min at increasing temperatures (0-90 °C). Fig. 9.7. demonstrates that all of the native cereal and transgenic SGS5 tobacco oxalate oxidases exhibited thermal stability up to about 60 °C with no significant decline in enzyme activity. This suggested that the proteins were stable and retained their active pentameric configuration up to this temperature. Generally at 70 °C the enzymes began to show loss of activity, presumably resulting from the dissociation of some of the pentameric structure. This was shown to be the case for the barley root enzyme where both pentamer and monomer were detected on a western blot of heat treated samples (Fig. 8.22). Differential scanning calorimetry demonstrated complete protein dissociation at about 82 °C which was in agreement with the enzyme assay data shown in Fig. 9.7. where complete loss of activity occurred between 80-90 °C for the barley root enzyme. The levels of activity at 70 °C ranged between 70 and 90% of the maximum activity for the native and transgenic SGS5 oxalate oxidases. Fig. 9.7. also illustrates the lower thermal stability of transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases. Both of these proteins exhibited reasonably stable activity when pre-incubated at 0-30 °C for 30 min. However, activity began to decline following pre-incubation at a temperature as low as 40 °C, with complete loss of activity at 50 °C and above. This corresponds to a difference in the temperature of inactivation of about 40 °C between the native and transgenic SGS5 oxalate oxidases and the transgenic 3S1 and C26 enzymes.

The long term stability of the native and transgenic oxalate oxidases was also examined by maintaining the enzymes (in water with no added chemicals) at 4, -20 and -80 °C for 9 months. The activities of the stored enzymes were then compared with the initial enzyme activities at time zero. Fig. 9.8. illustrates the effect of low temperature storage on the stability and activity of the oxalate oxidases. All of the native cereal oxalate oxidases exhibited activity in the range of about 63-76% when stored at 4, -20 or -80 °C. Transgenic SGS5 tobacco oxalate oxidase showed the greatest stability, exhibiting about 80% activity when stored at -20

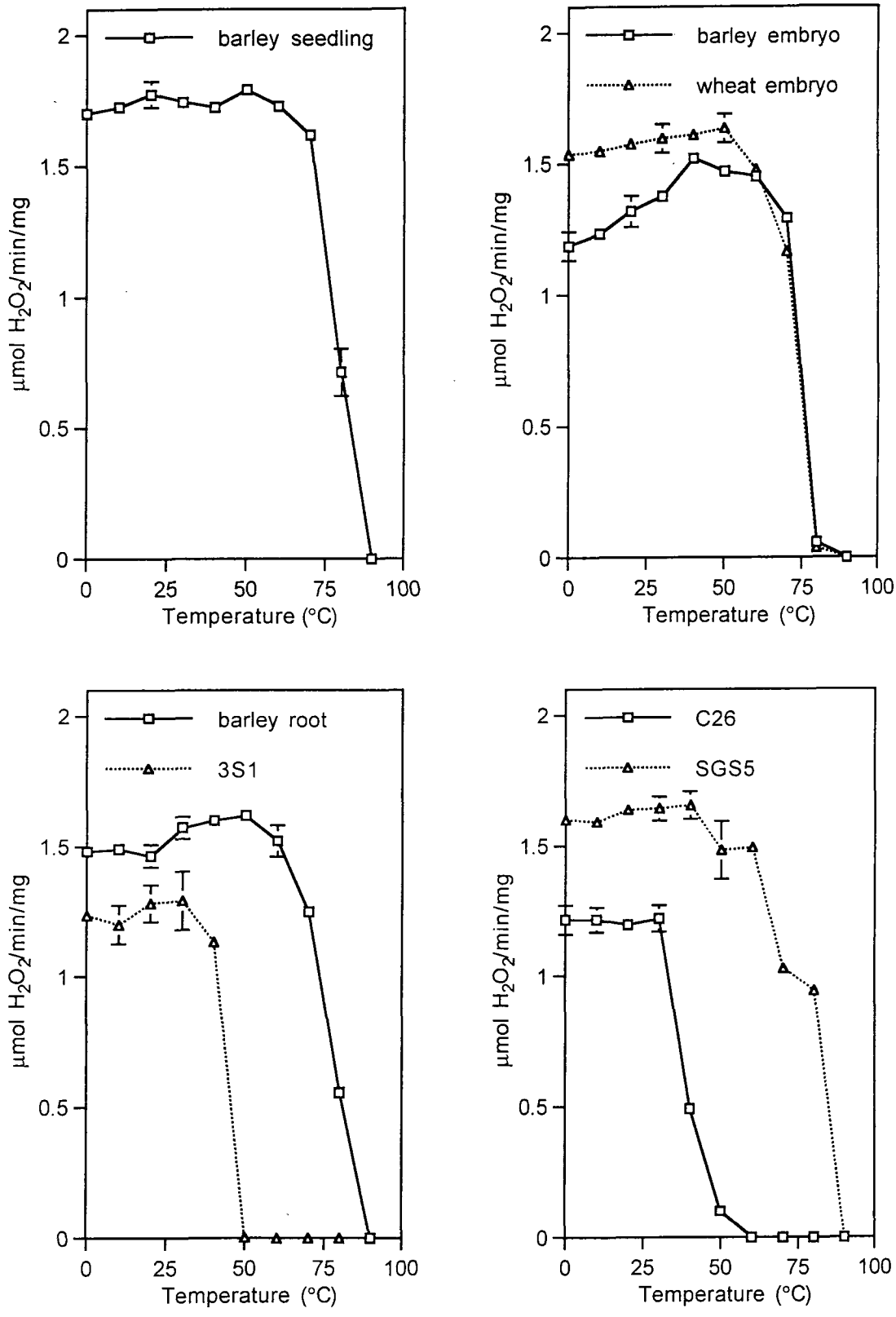


Figure 9.7. The effect of heat treatment on native and transgenic oxalate oxidase activity.
Native (barley seedling, root and embryo and wheat embryo) and transgenic (3S1 oilseed rape, C26 and SGS5 tobacco) oxalate oxidases were pre-incubated for 30 min at the temperatures indicated and then they were assayed using the standard microtitre plate enzyme assay protocol (37°C for 60 min incubation), as described in section 3.11.4. Error bars show mean ± standard errors.

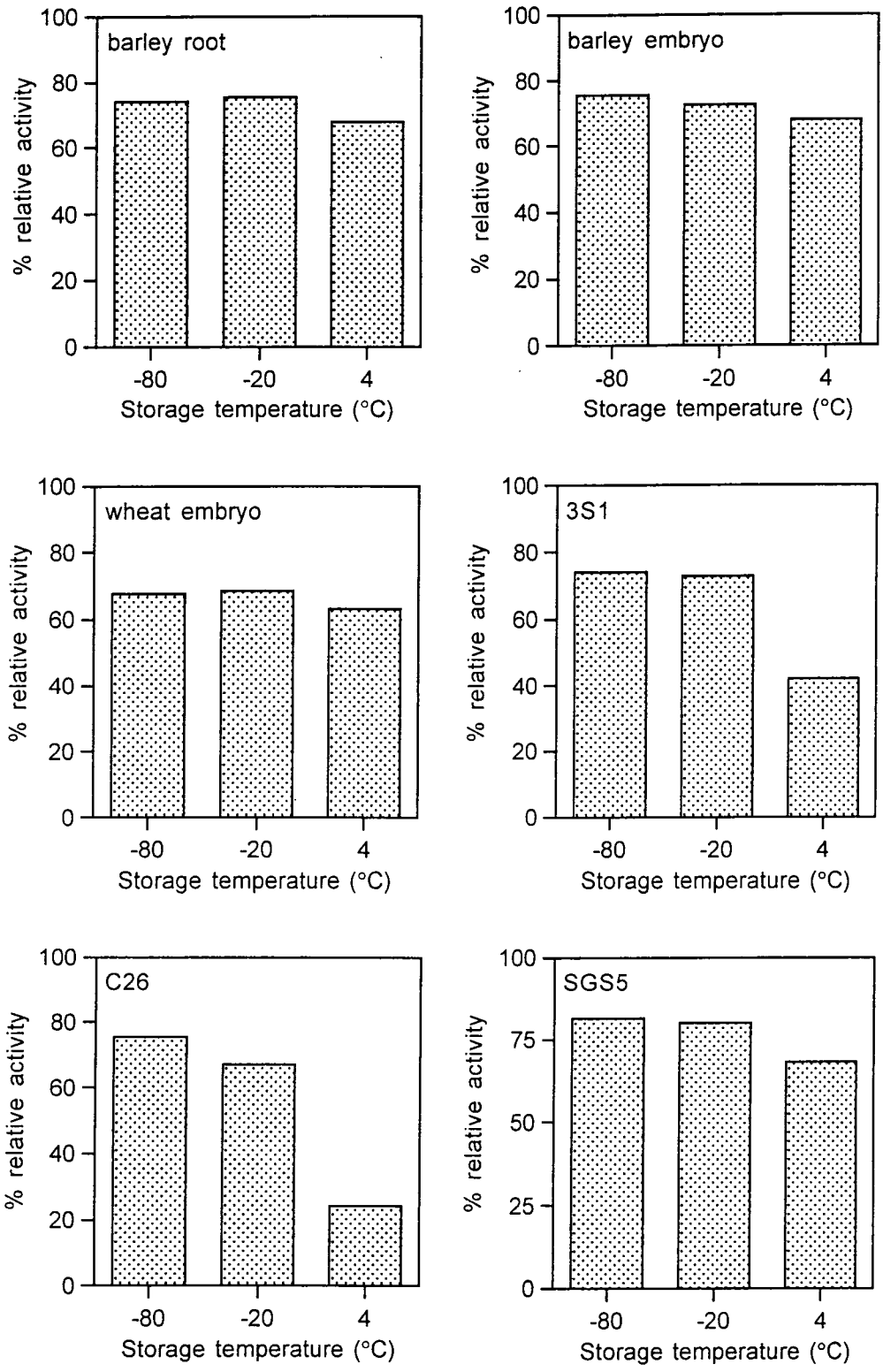


Figure 9.8. Protein stability and enzyme activity following long term storage at low temperatures.
Partially purified native (barley root and embryo and wheat embryo) and transgenic (3S1 oilseed rape, C26 and SGS5 tobacco) oxalate oxidases were incubated for 9 months at the temperatures indicated and were then assayed using the standard microtitre plate enzyme assay protocol (60 min incubation), as described in section 3.11.4. The values expressed represent the enzyme activity of the proteins after incubation as a percentage of the activity at time zero, which was taken as 100% activity.

or -80 °C and about 68% activity following storage at 4 °C. Transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases exhibited enzyme activity of about 67-75% when stored at -20 or -80 °C. However, enzyme activity was significantly lower (42.1 and 24.2%) when the 3S1 and C26 enzymes were stored in solution at 4 °C, compared with the native and SGS5 transgenic oxalate oxidases. The native cereal and transgenic SGS5 oxalate oxidases exhibited a loss of activity of up to 32% when stored for 9 months, regardless of temperature. All of the proteins appeared to be less stable at 4 °C than at -20 or -80 °C, this was particularly evident with the transgenic 3S1 and C26 oxalate oxidases.

9.6. Oxalate oxidase substrate specificity

A fixed concentration (1 mM) of several different dicarboxylic acids (succinic, citric, maleic, malic, ascorbic and oxaloacetic acids) were substituted for oxalic acid as potential alternative substrates in the oxalate oxidase assay. The results of this experiment clearly illustrated that all of the native and transgenic oxalate oxidases showed absolute specificity for oxalic acid and no activity was detected in the presence of any of the other dicarboxylic acids tested (results not shown).

9.7. The effect of NaCl on oxalate oxidase activity.

Sodium chloride was one of the major components of many of the extraction buffers used throughout this project for the isolation of oxalate oxidases. The presence of salt in the extraction buffers was found to be essential to achieve optimum oxalate oxidase recovery particularly from the leaf extracellular spaces. Salt was also a necessary component in the gel filtration column chromatography buffer to prevent protein-column matrix interactions a problem first encountered with the superose 12 column matrix which bound a proportion of the enzyme at concentrations of 50 mM NaCl. Fig. 9.9. showed the effect of increasing NaCl concentration in the assay (100, 200 and 400 mM) on the activity of the native and transgenic oxalate oxidases. HRPO activity did not appear to be affected by NaCl concentrations in this range, which meant that any decline in activity relative to untreated enzyme resulted only from a direct inhibition of the oxalate oxidase enzymes. Similar levels of enzyme inhibition were observed for all of the native cereal and transgenic SGS5 tobacco oxalate oxidases. As NaCl concentration increased so too did the degree of enzyme inhibition, ranging from about 55-65% relative activity at 100 mM NaCl to 25-40% relative activity at 400 mM NaCl. Transgenic 3S1 oilseed rape oxalate oxidase exhibited very significant inhibition at NaCl concentrations above 100 mM (less than 10% relative activity). The activity of transgenic C26 tobacco oxalate oxidases was almost completely inhibited by 100 mM NaCl. The sodium and chloride ions may have interfered with ionisable groups on the surface of the protein which help to maintain the configuration of the active oxalate oxidase, slight changes in the structure of the protein may have been sufficient to cause loss of activity. Lane *et al.* [1986] had previously shown that wheat embryo oxalate oxidase was structurally stable and did not dissociate in a saturated solution of NaCl. The charged ions may have interacted with ionised groups in the active site resulting in loss of activity. Previous experiments demonstrated that removal of NaCl from the

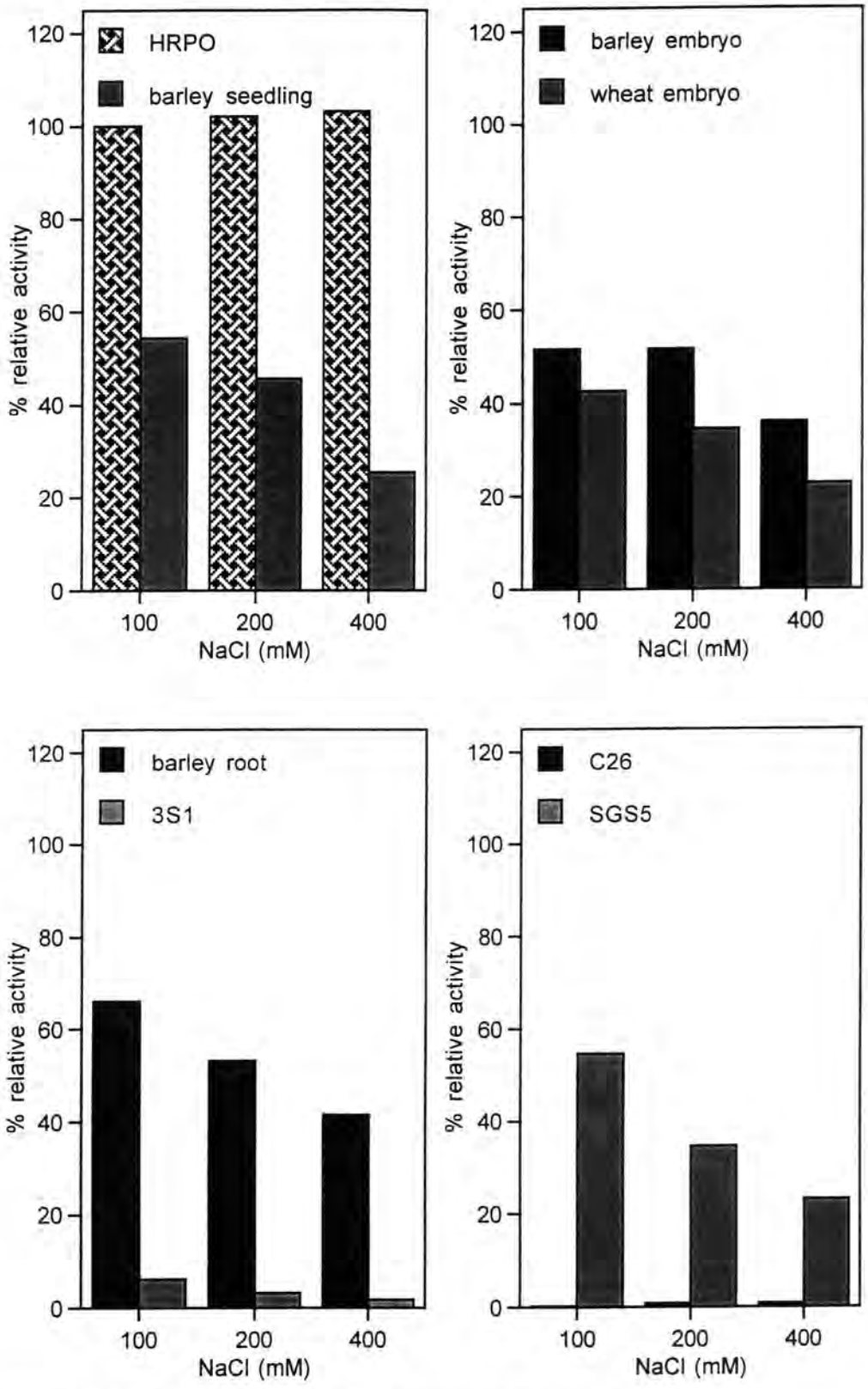


Figure 9.9. The effect of sodium chloride on oxalate oxidase activity.
Native (barley root and embryo and wheat embryo) and transgenic (3S1 oilseed rape, C26 and SGS5 tobacco) oxalate oxidases were assayed using the standard microtitre plate enzyme assay protocol (60 min incubation), as described in section 3.11.4., with the addition of NaCl as indicated. The values expressed represent the enzyme activity of the proteins incubated with NaCl as a percentage of the activity of the proteins incubated in the absence of NaCl, which was taken as 100% activity.

protein samples (by dialysis or dot blotting onto a nitrocellulose membrane) resulted in the recovery of enzyme activity for all of the native and transgenic proteins, which showed that this inhibition was reversible. Inhibition of oxalate oxidase by chloride ions was also reported by Suzuki and Meeuse [1965] the effect of which was reversed by dialysis. Kotsira and Clonis [1997] demonstrated a drop in the activity content and the specific activity of oxalate oxidase and a reduced rate of seedling growth in barley grown in NaCl compared with water.

9.8. Effect of SDS on the stability and activity of oxalate oxidase.

The instability of the transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases in the presence of SDS was previously illustrated when these proteins were analysed on SDS-PAGE gels (Fig. 8.10.). In these cases the pentamers were converted to the monomer in the presence of 1% (w/v) SDS. Whereas, the native cereal and transgenic SGS5 tobacco oxalate oxidases were stable at this concentration of SDS in the absence of heat, retaining their pentameric configuration and enzyme activity (Fig. 8.8. and 8.9.). In order to be able to determine the effect of a range of SDS concentrations (0-5% (w/v)) on enzyme activity an immobilised enzyme assay was carried out. HRPO was unstable in SDS containing solutions and so immobilised enzyme assays were carried out, which prevented the HRPO coming into contact with SDS. The immobilised oxalate oxidases were incubated in SDS containing succinic acid buffer (pH 3.5) for 60 min at room temperature. The nitrocellulose disks were then washed to remove the SDS and immobilised enzyme assays were performed as described in section 3.11.3. Fig. 9.10. shows the effect of increasing concentrations of SDS on the enzyme activity of immobilised oxalate oxidases. Under these conditions all the oxalate oxidase enzymes appeared to lose some activity when exposed to SDS. In the presence of 0.1% (w/v) SDS barley embryo, root and seedling, wheat embryo and transgenic SGS5 tobacco oxalate oxidases lost between 7-21% of the activity of the untreated enzymes. At 0.25% (w/v) SDS the activity for all of these protein was approximately equal to or less than half that of the enzymes incubated in 0.1% (w/v) SDS. Above 0.1% (w/v) SDS the transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidase proteins lost over 90% of their activity. The native cereal and transgenic SGS5 tobacco oxalate oxidases lost about 95% of their activity at a concentration of 5% (w/v) SDS. The low levels of activity retained by the enzymes incubated in 5% (w/v) SDS probably resulted from the stabilising effect of the enzyme being bound to the nitrocellulose membrane, which may have protected some of the enzyme from the dissociating effect of SDS.

The experiments described in this chapter further highlighted the instability of transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases compared with the native cereal proteins, as well as illustrating yet again that transgenic SGS5 tobacco oxalate oxidase exhibited properties comparable with the native cereal proteins. The relevance of the instability of the transgenic 3S1 and C26 oxalate oxidase will be discussed in chapter 11.

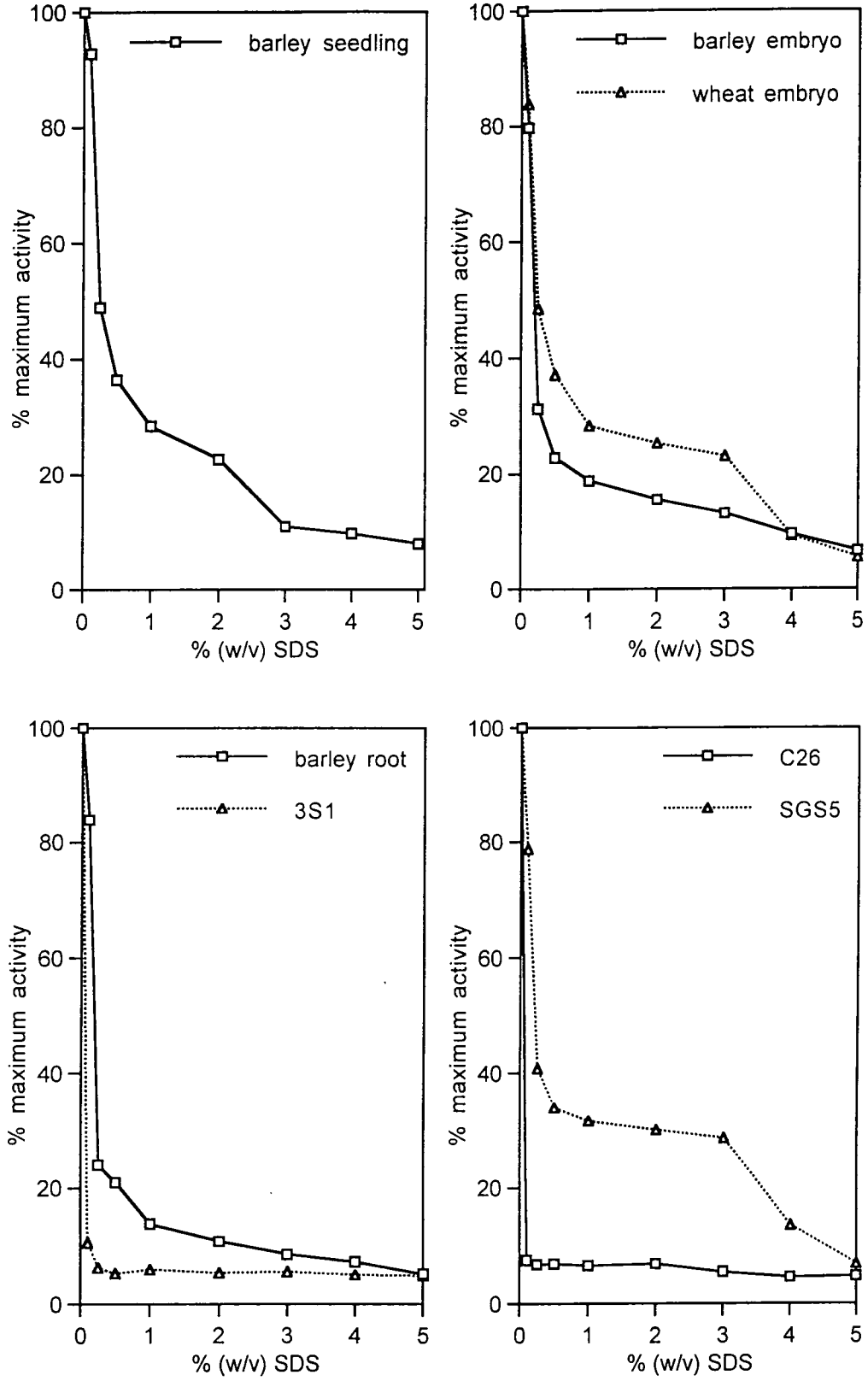


Figure 9.10. The effect of SDS on oxalate oxidase activity.
Native (barley root and embryo and wheat embryo) and transgenic (3S1 oilseed rape, C26 and SGS5 tobacco) oxalate oxidases were dot blotted onto a nitrocellulose membrane. The immobilised enzyme disks were incubated with SDS at the concentrations indicated for 60 min at room temperature. The nitrocellulose disks were washed in six changes of distilled water and each disk was then separately assayed at 550 nm, using the standard cuvette immobilised enzyme assay protocol (60 min incubation) as described in section 3.11.3. The values expressed represent the enzyme activity of the proteins after treatment with SDS as a percentage of the activity of the proteins treated with no SDS, which was taken as 100% activity.

10. RESULTS AND DISCUSSION.

Germin-like proteins in oilseed rape and tobacco.

This addendum to the results and discussion chapters is presented as a short description of research undertaken at the laboratories of Zeneca Seeds (Jealotts Hill Research Station, Berkshire, U.K.) as part of the requirement of the CASE award. The aim of the work was to attempt to PCR, amplify, clone and sequence germin-like proteins from oilseed rape. Work on the identification of proteins in oilseed rape and tobacco with amino acid sequence homology to wheat germin was undertaken in the laboratories at the Department of Biological Sciences, University of Durham, U.K. Some of the N-terminal protein sequence data discussed in this chapter was obtained in collaboration with Debbie Alexander a third year undergraduate project student at the University of Durham. Some of the cloning results were obtained at Zeneca Seeds by Amy Walker an undergraduate placement student from the University of Leeds.

10.1. Germin-like proteins in plants.

In the last few years more researchers have become involved with germin-like proteins often because they have identified protein in the course of their work which show DNA or protein sequence homology to wheat germin. When germin was first discovered by Thompson *et al.* [1980] as a dot on two-dimensional gels of extracts from germinating wheat embryos, the protein was thought to be very rare. However, in the intervening years a whole family of proteins with sequence homology to wheat germin were discovered and in 1993, Lane *et al.* demonstrated that wheat germin was in fact an oxalate oxidase which exhibited about 96% amino acid sequence homology with barley root oxalate oxidase. The term germin-like protein is still used because many of the discovered proteins which exhibited homology to germin exhibit no oxalate oxidase activity and are of unknown function. The germin-like protein sequences reported were derived from a wide variety of plant species, including *A. thaliana, B. napus, Sinapis alba* and barley. Information regarding the properties of some of these identified proteins were unknown as the sequences were experimentally determined from translated cDNA's. These sequences were identified by searching databases with the wheat germin DNA and protein sequences, including EMBL, GenBank and NCBI databases. With the advent some years ago of more easily accessible databases many more proteins were identified with homology to oxalate oxidase, including legumin-like and vicilin-like seed storage proteins [Bäumlein *et al.* 1995; Shutov *et al.* 1995; Braun *et al.* 1996].

The majority of the germin-like proteins were identified as a result of the random screening of cDNA libraries or as expressed sequence tags (ESTs). Whereas, other germin-like proteins were more fully characterised with regards to their, localisation, physical and kinetic properties [Heintzen *et al.* 1994; Domon *et al.* 1995; Dumas *et al.* 1995; Zhang *et al.* 1995; Ono *et al.* 1996]. Protein sequence data was not available for some of the proteins that were shown to exhibit oxalate oxidase activity [Dumas *et al.* 1995; Zhang *et al* 1995] or which

cross-reacted with anti-wheat germin antibodies [Domon *et al.* 1995; Zhang *et al.* 1995] or anti-barley oxalate oxidase antibodies [Dumas *et al.* 1995].

The functions and roles of some of these proteins were more understood than others, including involvement in stress responses [Michalowski and Bohnert 1992; Hurkman *et al.* 1989 1991 1994; Hurkman and Tanaka 1996] and pathogen resistance [Dumas *et al.* 1995; Hurkman and Tanaka 1995; Zhang *et al.* 1995]. Although these proteins all share varying levels of amino acid sequence homology to germin very few were shown to exhibit oxalate oxidase enzyme activity.

10.2.The identification of germin-like proteins in oilseed rape and tobacco.

In early experiments carried out during this study it was assumed that the additional protein bands which cross-reacted with anti-oxalate oxidase antibodies on western blots of oilseed rape and tobacco protein extracts were the result of non-specific binding, an observation also made by Thompson *et al.* [1995]. However, it was more likely that the antibodies were actually cross-reacting with germin-like proteins in these two plant species (Fig. 5.10. and Fig. 8.17.). In 1994 Heintzen *et al.* published the results of their work describing the presence of a mRNA in the leaves of *S. alba,* which appeared to be regulated by circadian oscillations. Translation of the gene transcript showed that the resulting protein shared 32% sequence identity to the wheat germin precursor and 36% sequence identity to the mature protein [Heintzen *et al.* 1994]. Antiserum raised against the identified *S. alba* germin-like protein (*Sa*glp) [Heintzen *et al.* 1994] was obtained from this group (courtesy of Dorothee Staiger) to see if it would cross-react with the native barley root and 3S1 transgenic oilseed rape oxalate oxidases.

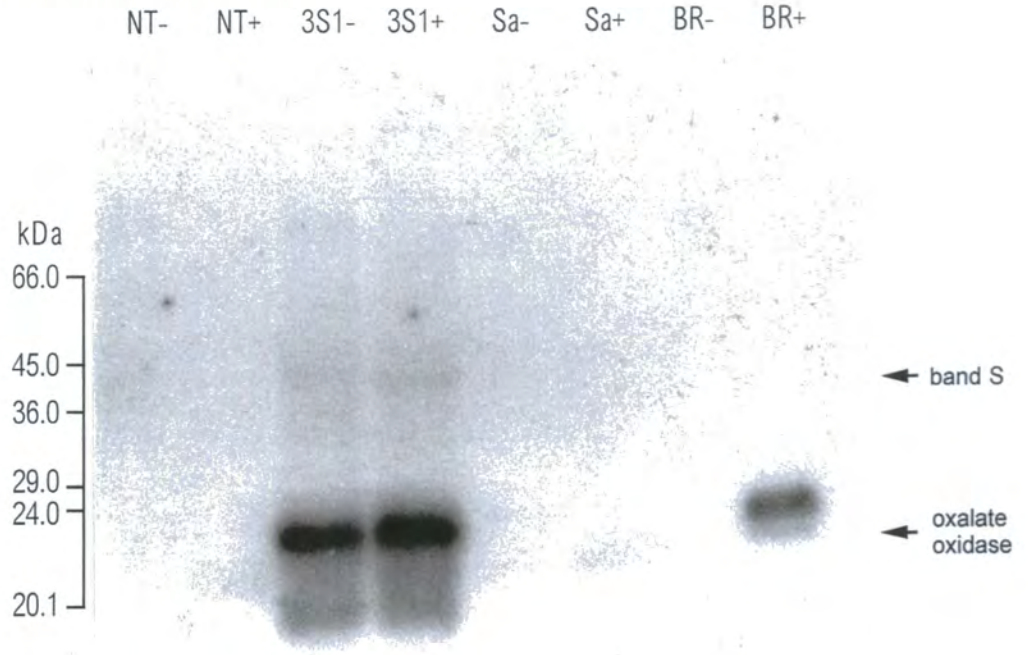
Fig. 10.1. shows two identical western blots of SDS-PAGE separated INF proteins from non-transgenic, transgenic 3S1 oilseed rape and *S. alba* isolated with infiltration buffer (200 mM NaCl, 50 mM Na_2HPO_4 pH 7.5), which were screened with either barley root oxalate oxidase or *Sa*glp antibodies. The oxalate oxidase antibodies strongly reacted with transgenic 3S1 oxalate oxidase in heat and non-heat treated INF samples (Fig. 10.1. 3S1- and 3S1+). The anti-oxalate oxidase antibodies did not cross-react with any bands in the non-transgenic oilseed rape INF (Fig. 10.1. NT- and NT+). No cross-reactivity with other proteins was observed in the barley root samples (Fig. 10.1. BR- and BR+). As was often the case with the antiserum raised against the monomeric (heat treated) oxalate oxidase subunits, the pentameric barley root oxalate oxidase (about 110-120 kDa) in non-heat treated samples reacted poorly with the oxalate oxidase antibodies (Fig. 10.1. BR-, there was no clearly visible band corresponding to the oligomer), compared with the monomeric subunits in the heat treated samples (Fig 10.1. BR+). There was no cross-reaction of the anti-oxalate oxidase antibodies with proteins in the *S. alba* INF (Fig. 10.1. Sa- and Sa+). A protein with a molecular weight of about 40 kDa in the 3S1 oilseed rape INF cross-reacted with the oxalate oxidase antibodies (Fig. 10.1. 3S1- and 3S1+, band S).

*Sa*glp antibodies strongly cross-reacted to proteins in both non-transgenic and transgenic 3S1 oilseed rape and in *S. alba* INF. Proteins with molecular weights of about 105-

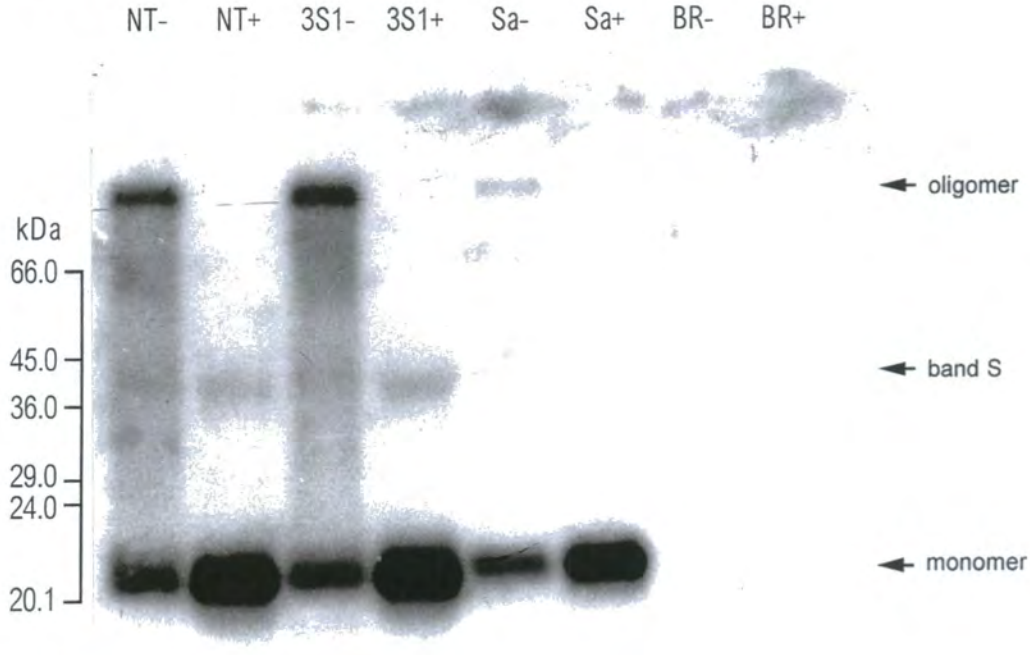
Figure 10.1. Detection of oxalate oxidase and germin-like proteins

Barley root oxalate oxidase antiserum (1:10,000 dilution) or *Sa*GIp antiserum were used as primary antibodies (1:250) and ^{125}I-conjugated donkey anti-rabbit antibodies as secondary antibodies for the detection of oxalate oxidase and germin-like proteins, respectively. Approximately 20 µg of extracellular proteins were loaded onto the gels. Lanes 1 and 2, non-transgenic oilseed rape leaf INF; lanes 3 and 4, transgenic 3S1 oilseed rape leaf INF; lanes 5 and 6, *S. alba* leaf INF; lanes 7 and 8, commercial barley seedling oxalate oxidase. Without (1, 3, 5 and 7) or with (2, 4, 6 and 8) heat treatment (2 min at 100 °C). Arrows indicate the monomers and oligomers of the germin-like proteins and the 40 kDa band S.

Anti-oxalate oxidase



Anti-SaGLP



110 kDa were detected in non-heat treated samples of the three INFs (Fig. 10.1. NT-, 3S1- and Sa-), as well as a lower molecular weight proteins of about 21 kDa (Fig 10.1. NT+, 3S1+ and Sa+). Heat treatment of the INFs before separation on SDS-PAGE gels led to the complete dissociation of the oligomer (about 105-110 kDa) to the monomer (about 21 kDa) (Fig. 10.1. NT-, 3S1- and Sa-). The oligomeric germin-like protein in 3S1 oilseed rape INF was also clearly visible on the periodic acid silver stained gel as a band with a slightly lower molecular weight than the pentameric oxalate oxidases (Fig. 8.17. 3S1). An additional band of about 40 kDa also reacted with the *Sa*gIp antibodies in the heat treated oilseed rape samples and to a lesser extent in the *S. alba* samples (Fig 10.1. NT-, 3S1- and Sa-, band S). It did not appear that the *Sa*gIp antibodies reacted with the oxalate oxidase protein in transgenic 3S1 oilseed rape, as no band corresponding to oxalate oxidase in the 22-23 kDa region of the gel was present (Fig. 10.1. 3S1- and 3S1+). *Sa*gIp antibodies did not cross-react with either the oligomer or monomer of barley root oxalate oxidase (Fig 10.1. BR- and BR+), however a faint band of about 40 kDa was present in both the non-heat and heat treated samples (Fig. 10.1. BR- and BR+, band S). Thus the germin-like proteins and the transgenic 3S1 and barley root oxalate oxidases were antigenically unique despite the observed amino acid sequence similarities.

The results of the western blots in Fig 10.1. demonstrated that there was no cross-reactivity between the oxalate oxidase antibodies and *Sa*gIp (Fig. 10.1. Sa- and Sa+) and apparently no cross-reactivity at all between native cereal or transgenic oilseed rape oxalate oxidase and anti-*Sa*gIp antibodies (BR- & BR+ and 3S1- & 3S1+, respectively). However, Fig. 10.1. demonstrated the presence of germin-like proteins in oilseed rape which cross-reacted with the anti-*Sa*gIp antibodies. The germin-like protein identified in oilseed rape (Fig 10.1. 3S1-), like the barley root oxalate oxidase was apparently oligomeric, probably a pentamer (about 105-110 kDa) and partially stable to SDS (1% (w/v)) dissociation. The oligomer was fully converted to the monomer (about 21 kDa) by brief heat treatment (2 min, 100 °C) (Fig. 10.1. 3S1+). The size of the oilseed rape germin-like protein (OSRgIp) was similar to that of the *Sa*gIp identified by Heintzen *et al.* [1994] and in this study (Fig.10.1. Sa- and Sa+). OSRgIp and *Sa*gIp were isolated in the INF of leaves by vacuum infiltration, demonstrating the extracellular nature of these proteins. A band with a molecular weight of about 40 kDa was also identified in tobacco INF and leaf total protein extracts which cross-reacted with anti-oxalate oxidase antibodies (Fig. 5.10. TOB, TE and INF). Barley, oilseed rape and tobacco all appeared to be expressing a 40 kDa protein which cross-reacted with the oxalate oxidase and *Sa*gIp antibodies, which suggests that this is a protein common to all of these plant species and presumably possesses common epitopes which cross-react with the antibodies.

Numerous attempts were made to determine if OSRgIp exhibited oxalate oxidase enzyme activity using both solution assays (section 3.11.2. and 3.11.4.) or enzyme assays on electroblots (section 3.11.1.) of INF, however no oxalate oxidase enzyme activity was ever observed. Parallel experiments were performed on *S. alba* INF and again no enzyme activity was detected, a result confirmed by Dorthee Staiger [personal communication].

10.3. N-terminal protein sequencing of germin-like proteins from oilseed rape and tobacco.

The results described in this section were obtained in collaboration with Debbie Alexander. Following the discovery that oilseed rape contained a protein which cross-reacted with the *Sa*glp antibodies attempts were made to determine the N-terminal sequence of this protein. Oilseed rape INF was concentrated by freeze drying as described in section 3.9., separated on a 15% (w/v) SDS-PAGE gel and then electroblotted onto a ProBlott™ membrane for sequencing. A prominent band in the SDS-PAGE analysed oilseed rape INF with a molecular weight of about 21 kDa (OSRglp1, Fig. 10.1.) was excised and the N-terminal amino acid sequence residues were determined, as shown in Fig 10.2. A further two monomeric OSRglp's were also discovered by random sequencing of abundant proteins in the INF of oilseed rape leaves. OSRglp2 had a monomer molecular weight of about 22 kDa in heat treated samples (2 min at 100 °C), which were extracted from the extracellular spaces with water. OSRglp3 also had a monomer molecular weight of about 22 kDa in heat treated samples but was only isolated from the extracellular spaces with 400 mM NaCl buffer and not with water unlike OSRglp2. This suggested that OSRglp2 was freely soluble and that OSRglp3 was electrostatically bound to cell wall components and its isolation depended on the use of solutions of higher ionic strength. N-terminal sequencing of OSRlp2 and OSRglp3 confirmed that these two proteins had different N-terminal sequences (Fig. 10.2.). OSRglp2 corresponded to the protein on the western blot in Fig. 10.1. 3S1+. The oligomeric form of OSRglp2 which strongly reacted with to *Sa*glp antibodies (Fig. 10.1. 3S1-) was also sequenced as this protein was present in partially purified samples from non-heat treated transgenic 3S1 oilseed rape INF when separated on 12% (w/v) SDS-PAGE gels.

OSRglp1 exhibited 60% sequence identity to barley root and wheat germin over the first 10 amino acid residues. A search of the NCBI Entrez database revealed that the N-terminus of OSRglp2 exhibited 100% amino acid residue identity to the translated *Sa*glp sequence identified by Heintzen *et al.* [1994] and an identical sequence isolated by Membré *et al.* [1997] from *A. thaliana* (*At*Ger3). OSRglp3 was found to be identical over the 15 N-terminal amino acids sequenced, to a translated cDNA product isolated from etiolated oilseed rape seedlings by Membré *et al.* [1997] and an identical sequence isolated by Membré *et al.* [1997] from *A. thaliana* (*At*Ger2). Therefore It was highly probable that the full amino acid sequences had already been indirectly determined for these two new germin-like proteins discovered in oilseed rape. OSRglp2 and OSRglp3 exhibited 62% and 47% N-terminal sequence identity to barley root oxalate oxidase, respectively (Fig. 10.2.).

A protein isolated in the INF of non-transgenic tobacco leaves with a molecular weight of about 40 kDa was also shown to cross-react with oxalate oxidase antibodies (Fig. 5.10. TOB, INF). The N-terminus of this protein (*Nt*glp) was sequenced and found to exhibit 40% N-terminal sequence identity to barley root oxalate oxidase. No oxalate oxidase activity was detected for the *Nt*oxlp following immobilisation onto a nitrocellulose membrane and enzyme activity assays (section 3.11.1.). This protein was also extracellular in nature given the fact that it was isolated in the INF by vacuum infiltration.

Figure 10.2. N-terminal amino acid sequences of oxalate oxidase-like proteins from oilseed rape and tobacco.

N-terminal sequence alignment of germin-like proteins isolated from the INF of leaves of oilseed rape (*Bn*glp) and tobacco (*Nt*glp) by vacuum infiltration, separated on 12% (w/v) SDS-PAGE gels and sequenced from PVDF electroblots. Identical amino acid residues at the N-terminus between the germin-like proteins and wheat germin are shown in bold typescript. The percentages refer to N-terminal amino acid sequence identity to barley root oxalate oxidase [Lane *et al.* 1993].

OSRglp 1	S**DFCV**V**D**NT**G**	(60%)
OSRglp 2	SV**QDFCVAD**PK**G**P	(62%)
OSRglp 3	SV**QDFCVA**N**L**KRAET	(47%)
*Nt*glp	YPPATF**LQDF**	(40%)
brox[1]	SDPDPLQDFCVADLDGKAVS	
germin[2]	TDPDPLQDFCVADLDGKAVS	
*Sa*glp[3]	SVQDFCVADPKGP	
*Bn*glp[4]	SVQDFCVANLKRAET	
AtGer1[5]	SVQDFCVANLKRAET	
AtGer2[6]	YDPSPLQDFCVAIDDLKGVF	
AtGer3[7]	SVQDFCVADPKGP	

Barley root oxalate oxidase (brox) [Lane *et al.* 1993][1]

Wheat germin (gf-2.8) [Dratewka-Kos *et al.* 1989][2]

Sinapis alba-germin like protein (*Sa*glp) [Heintzen *et al.* 1994][3]

Brassica napus-germin like protein (*Bn*glp) [Membré *et al.* 1997][4]

Arabidopsis thaliana-germin like protein 1 (AtGer1) [Membré *et al.* 1997][5]

Arabidopsis thaliana-germin like protein 2 (AtGer2) [Membré *et al.* 1997][6]

Arabidopsis thaliana-germin like protein 3 (AtGer3) [Membré *et al.* 1997][7]

10.4. Cloning of the oilseed rape germin-like protein 1 (OSRglp1) gene.

Two partial PCR clones of OSRglp's (GerPCR1 GerPCR2) were identified by Amy Walker at Zeneca Seeds using degenerate oligonucleotide primers based on regions of known DNA sequence for wheat germin [Dratewka-Kos *et al.* 1989] and *Sa*glp [Heintzen *et al.* 1994]. Alignment of these translated DNA sequences showed that GerPCR1 and 2 exhibited 46% and 42% identity to barley root oxalate oxidase, respectively. GerPCR1 and GerPCR2 exhibited 91% amino acid sequence identity, the main residue differences occurred in the C-terminal regions of the polypeptides (see Appendix 1).

Neither of these two partial clones shared significant amino acid sequence homology with either the *Bn*glp isolated by Membré *et al.* [1998] or with *Sa*glp [Heintzen *et al.* 1994]. The protein sequencing performed in Durham showed that the N-terminal sequences of OSRglp2 and OSRglp3 shared 100% sequence homology to already identified DNA / protein sequences, *Sa*glp [Heintzen *et al.* 1994] and *Bn*glp [Sáez-Vásquez, unpublished], respectively. These results suggested that oilseed rape probably contains at least 5 genes which encode proteins with varying degrees of sequence homology to barley root oxalate oxidase (OSRglp1, OSRglp2, OSRglp3, GerPCR1 and GerPCR2). The functions of these proteins were unknown.

Several attempts were made to clone the gene sequence encoding OSRglp1 using a 5' degenerate primer prepared from the N-terminal sequence of OSRglp1 (See section 3.33. table 3.5. C1), 3' degenerate primers corresponding to GerPCR 1 and GerPCR2 sequences were prepared (see section 3.33. Table 3.5. C2 and C3 corresponding to GerPCR1 and 2, respectively) which were identified and sequenced. The main objective of this exercise was to try to determine if one of the partial PCR clones (GerPCR1 or 2) formed part of the gene which encoded OSRglp1.

A combination of oligonucleotide C1 with either C2 or C3 was expected to generated a DNA fragment of about 300 bp. The PCR appeared to amplify sequences from the oilseed rape genomic DNA which were of the correct size (about 300 bp), which may have corresponded to the OSRglp1 gene fragment. However, the PCR fragments of interest were not the major DNA components in the final PCR mix. Various attempts were made with different method to improve the yield, isolate and clone the PCR DNA fragments. Unfortunately it was not possible to successfully clone the PCR products resulting from the various oligonucleotide combinations.

11. GENERAL DISCUSSION.

The results in this work describe the analysis and comparison of native cereal (barley root, barley embryo and wheat embryo) and transgenic oxalate oxidases (3S1 oilseed rape, C26 and SGS5 tobacco). The work examined the basic expression and localisation of the transgenic oxalate oxidases with the majority of the work concentrating on direct comparison of the structure, stability and enzyme kinetics of the native and transgenic oxalate oxidases. In an attempt to answer the objectives laid out in section 1.14. various strategies were employed to study the properties of the transgenic enzymes and to further our knowledge in this area of oxalate oxidase research. The following represents the major findings from this work and their relevance to the overall objective of producing genetically engineered plants with improved resistance to fungal pathogens.

11.1. Oxalate oxidases expressed in cereal embryos and roots are essentially identical.

Barley root and wheat embryo oxalate oxidases were included in this work as controls, since the genes expressed in the transgenic plants originated from these plant organs. Barley embryo oxalate oxidase was included to allow a direct comparison of the oxalate oxidases expressed in the same organs of these two cereal species. There are many reports in the literature regarding the purification, structure, stability and kinetics of cereal oxalate oxidases, but there are few studies which include multiple sources of the enzymes for direct comparison. The monomeric subunit of the cereal (barley and wheat) oxalate oxidases are generally reported to have a molecular weights of about 25-26 kDa, as determined by gel filtration chromatography and SDS-PAGE analysis [Grzelczak *et al.* 1985; Lane *et al.* 1986; McCubbins *et al.* 1987; Schmitt 1991; Dumas *et al.* 1993; Kotsira and Clonis 1997]. However, there are widely differing reports regarding the size of the oligomer and the level of aggregation of the monomeric subunits. Some reports show the wheat embryo and barley root / seedling oxalate oxidases to be pentameric with molecular weights of about 125 kDa [Grzelczak *et al.* 1985; Lane *et al.* 1986; McCubbins *et al.* 1987; Kotsira and Clonis 1997]. Whereas, others have shown barley seedling oxalate oxidase to be tetrameric with a molecular weight of about 100 kDa [Dumas *et al.* 1993]. In this study the cereal oxalate oxidases were shown to have monomer molecular weights of about 22.6-22.8 kDa, as confirmed using the highly resolving technique of MALDI-ToF mass spectrometry, which would form pentamers with estimated molecular weights of about 114-115 kDa (section 8.2.2.). Mass spectrometry has not previously been used to determine the molecular mass of oxalate oxidase and so these estimates are likely to be the most accurate size measurements of the monomers to date. Gel filtration chromatography and SDS-PAGE analysis provided only a rough estimation of monomer and pentamer molecular weights (i.e. about 110-120 kDa, section 8.2.1.). The values provided in this study (22.6-22.8 kDa) are several kilodaltons lower than previously reported, however they correspond very well with the molecular weights calculated for the apoprotein from the amino acid sequence (about 20 kDa) [McCubbins *et al.* 1987] and the

additional oligosaccharide chain(s) (about 2-3 kDa) [Jaikaran *et al.* 1990], which suggests a monomer molecular weight of about 22.5 kDa.

There is now some debate as to whether oxalate oxidase is in fact pentameric. Gane *et al.* [in press] have put forward a new model for the three dimensional structure of oxalate oxidase. They have suggested that the protein may be a dimer of trimers, based on the crystallographic structure of vicilin. This is plausible since vicilin and oxalate oxidase share statistically significant amino acid sequence homology [Baulein *et al.* 1995]. The evidence presented in this thesis concures with that presented by other researches in this field suggesting that the protein is pentameric. However, further analysis of the oligomeric form of the protein is required to determine once and for all how many subunits aggregate to form the oligomer. This is at present underway in several laboratories, in the form of x-ray crystallography, so the structure of oxalate oxidase should be fully characterised in the near future.

Cross-linking of the oxalate oxidases with DMS and detection by western blotting with anti-oxalate oxidase antibodies confirmed that the cereal enzymes were pentameric (section 8.3.). The pentamers were shown to be stable in the presence of 1% (v/v) β-mecaptoethanol, which suggests that the subunits were not maintained in the pentameric configuration by disulphide bonds. The oxalate oxidase polypeptide contains two cysteine residues which probably helps maintain the integrity and stability of the subunits through an internal disulphide bond [Kotsira and Clonis 1997]. The subunits of most oligomers are held together by non-covalent interactions (i.e. Van der Waals forces, hydrogen bond, hydrophobic interactions and ionic interactions) [Hurtley and Helenius 1989].

CD spectroscopy performed in this study showed that the barley root oxalate oxidase had a higher α-helix content and a lower β-sheet content as determined using the selcon method of Sreerama and Woody [1993], compared with the calculated secondary structure contents of wheat embryo oxalate oxidase [McCubbins *et al.* 1987] for the reasons discussed in chapter 8.

In this study the cereal oxalate oxidase pentamers were almost completely stable when analysed by SDS-PAGE in the presence of 1% (w/v) SDS, only low levels of dissociation to the monomers were observed (section 8.2.1). The cereal oxalate oxidases retained over 80% of their activity in the presence of 0.1% (w/v) SDS and the secondary structure of barley root oxalate oxidase was largely unaffected by SDS treatment (0-0.2% (w/v)), as judged by a lack of significant alteration in the secondary structure content of the native protein (section 8.1.).

SDS-PAGE analysis, enzyme assay stained electroblots and western blotting confirmed the existence of both the G and G' isoforms in barley roots, barley embryos and wheat embryos and that both isoforms exhibit oxalate oxidase activity (section 8.2.1.). Lane *et al.* [1986] showed that the G' isoform of oxalate oxidase was usually present at lower levels in wheat embryos compared with G, this would also appear to be the case for the barley oxalate oxidase isoforms (section 8.2.1.). The only difference observed between the embryo and root oxalate oxidases in this study was the existence of an additional lower molecular weight

isoform (X) present in barley roots but not in barley or wheat embryos (section 8.2.1.). This additional protein was shown to be an oxalate oxidase which exhibited enzyme activity and was immunologically related to G and G' (i.e. the protein cross-reacted with anti-oxalate oxidase antibodies). All of the mature cereal oxalate oxidases isoforms (G, G' and X) had the same N-terminal sequences (chapter 6, SDPDPLQDFCV), which where also identical to barley seedling oxalate oxidase [Lane *et al.* 1993]. The N-terminal sequences differed from the reported N-terminal sequence for wheat embryo oxalate oxidase by a single residue (serine in place of threonine at position 1 of the mature protein) [Lane *et al.* 1991].

The cereal oxalate oxidases all bound to a ConA affinity column suggesting the presence of mannose, glucose and/or α-N-acetylglucosamine as terminal residues of these proteins (section 8.4.2.). Tunicamycin treatment of barley seedlings and isolated barley and wheat embryos led to the generation of unglycosylated oxalate oxidase subunits with molecular weights of about 20-21 kDa, as determined from SDS-PAGE analysis (section 8.4.3.). This indicated the presence of an oligosaccharide side chain(s) of about 2-3 kDa attached to the oxalate oxidase apoprotein, which was in agreement with the results of Jaikaran *et al.* [1990] for wheat embryo oxalate oxidase. Furthermore tunicamycin treatment led to the formation of pentamers with different ratios of glycosylated and unglycosylated subunits. Many researchers have concluded that the carbohydrate moieties of glycoproteins do not play any significant role in the secretion of proteins at the plasma membrane [Darnell 1990]. However, many proteins will not fold into their correct oligomeric structure if one or more of the subunits has not been correctly glycosylated, these abnormal oligomers may be selectively retained in the rough ER by association with ER resident binding proteins [Darnell 1990]. In this study glycosylation did not appear to be a necessary requirement for oligomerisation, stability or enzyme activity of the any cereal oxalate oxidases, which was in agreement with the results of Jaikaran *et al.* [1990].

Barley root oxalate oxidase has a half life of about 20 sec at 100 °C the same as wheat embryo oxalate oxidase [Lane *et al.* 1992] and the protein undergoes irreversible dissociation / denaturation at a temperature of about 82 °C (section 8.5.1.). The cereal oxalate oxidases exhibit 71-85% of their activity at 70 °C, confirming the results of Sugiura *et al.* [1979] for barley seedling oxalate oxidase (section 9.5.). CD studies indicated that heat treatment of barley root oxalate oxidase (30 min at 60 and 80 °C) resulted in a decline in the levels of α-helix and an increase in aperiodic content (i.e. β-turn and random coil) compared with the non-heat treated protein (section 8.1.). These results contradict those of McCubbins *et al.* [1987] which showed no change in the secondary structure of wheat embryo oxalate oxidase following heat treatment at 100 C for 2 min.

The use of proteolysis initially reported by Grzelczak and Lane [1984] as a means of purifying wheat embryo oxalate oxidase did not appear to have any detrimental effects on the structural integrity of any of the cereal oxalate oxidases. Barley root, barley embryo and wheat embryo oxalate oxidases were shown to be one of only several proteins in extracts from these organs which were resistance to pepsin treatment. The oxalate oxidases were structurally stable and retained their enzymically active pentameric configuration (section 8.5.3.).

The cereal oxalate oxidases have very similar kinetic properties, particularly the wheat and barley embryo enzymes which exhibited almost identical properties under the various treatments tested. The enzymes had K_m values of about 209 mM, which were comparable with previous reports for cereal oxalate oxidases [Schmitt 1991; Kotsira and Clonis 1997] (section 9.3.). The enzymes exhibited activity over a broad pH range (2.0-6.0) with pH optima of about 4.0-4.5, which was higher than that previously reported for barley root oxalate oxidase [Schmitt 1991; Kortisra and Clonis 1997] (section 9.4.). Kotisra and Clonis [1997] reported the inhibitory effect of growing barley in NaCl-containing media, this was reflected in reduced oxalate oxidase content and specific activity and a reduction in the actual growth of the seedlings. In this study NaCl was shown to have a marked effect on the activity of the isolated cereal oxalate oxidases (i.e. about 60-75% enzyme inhibition at 400 mM NaCl, section 9.7.). This effect is interesting since oxalate oxidase has been linked to growth and cell expansion in wheat embryos as a result of the hydrogen peroxide produced from the hydrolysis of oxalic acid [Lane *et al.* 1993]. Hydrogen peroxide is utilised by many peroxidases in plants, including lignin peroxidase, which mediates the cross-linking of carboxylated sugars in pectins and hemicellulosic glucuronogalactoarabinoxylans [Halliwell 1978; Darvill *et al.* 1980; Carpita and Gilbeaut 1988; Cassab and Varner 1988; Showalter 1993; Olsen and Varner 1993; Lane *et al.* 1994]. A reduction in oxalate oxidase content and specific activity in the NaCl-treated barley seedling may therefore have reduced the production of hydrogen peroxide resulting in a reduced rate of growth.

All the information presented in this thesis provides evidence which confirms that the oxalate oxidases expressed in barley and wheat embryos are very similar if not identical in terms of their structure, stability and enzyme kinetics. Oxalate oxidase isolated from 10 day old barley roots was shown to exhibit very similar structural and kinetic properties compared with the enzyme isolated from 3 day germinated barley embryos, the only difference being the appearance of the additional oxalate oxidase enzyme in roots. This additional isoform isolated in barley roots may have resulted from differential gene expression activated in the developing seedling or due to differential glycosylation of the oxalate oxidase apoprotein. The G and G' isoforms of oxalate oxidase are present in the roots, presumably resulting from the accumulation of this highly stable protein originally expressed in the germinating embryos and as a result of *de novo* synthesis in root cells. The staining of isolated 10 day old barley roots with oxalate oxidase enzyme assay substrate and developer solution resulted in the rapid and intense staining of the entire root, confirming the presence of oxalate oxidase on the root surfaces (results not shown). The accumulation of the G and G' oxalate oxidase isoforms in the barley seedlings may have resulted from the low turnover rate of the protein due to its highly stable structure, which is resistant to digestion by broad specificity proteases [Grzelczak and Lane 1984, 1985].

The fact that the additional oxalate oxidase isoform (x) was not detected in extracts of 3 day germinated barley embryos isolated from the same grains sown to generate root material suggests that either the gene which encodes this protein was not activated or that the protein had not accumulated to sufficient levels to be detected by western blotting and enzyme

assays. It would appear from this work that the G and G' isoforms of oxalate oxidase have undergone very similar if not identical processing in the ER and trans-Golgi network in barley and wheat (i.e. signal peptide cleavage, folding, glycosylation and oligomerisation) to form very similar if not identical enzymes.

11.2. Oxalate oxidase is processed in transgenic SGS5 tobacco to give an enzyme nearly identical to the native wheat embryo oxalate oxidase.

Transgenic SGS5 tobacco plants expressing the CaMV 35S-gf-2.8 (wheat embryo oxalate oxidase) gene, which contained the native gf-2.8 signal peptide were used to study the heterologously expressed oxalate oxidase. Molecular weight size determination by MALDI-ToF mass spectrometry confirmed that transgenic SGS5 oxalate oxidase monomers had molecular weights of about 22.8-22.9 kDa, which in turn would generate pentamers with molecular weights of about 114-115 kDa (section 8.2.2.). Gel filtration chromatography and SDS-PAGE analysis showed that the pentamers had molecular weights of between 110 and 120 kDa (section 8.2.1.). Transgenic SGS5 tobacco was shown to express two active wheat oxalate oxidase isoforms comparable with the G and G' isoforms of native wheat embryo oxalate oxidase. The SGS5 oxalate oxidase isoforms were cross-linked with DMS to confirm that the subunits also aggregated in the transgenic plants to form pentamers (section 8.3.).

This study confirmed that both of the transgenic oxalate oxidase isoforms had N-terminal amino acid sequences identical to those of the wheat embryo G and G' isoforms (chapter 6), confirming that the foreign wheat gf-2.8 signal peptide was efficiently recognised by the signal recognition particles (SRPs) in the transgenic tobacco. The G and G' isoforms did not result from the differential removal of the signal peptide. SRPs are responsible for directing nascent polypeptides towards docking proteins in the membrane of the rough ER [Vitale *et al.* 1993]. Membrane-associated signal peptidase in the ER of dicotyledonous tobacco cells was able to recognise the 'foreign', monocotyledonous gf-2.8 signal peptide and cleave it from the mature protein sequence to produce a transgenic oxalate oxidase with an N-terminal sequence identical to the native protein. Both isoforms exhibited structural stability towards SDS, retaining their pentameric structure and enzyme activity on electroblots of SDS-PAGE separated proteins. Like wheat embryo oxalate oxidase the transgenic enzyme retained 80% of its activity in the presence of 0.1% (w/v) SDS (section 9.8.).

SGS5 oxalate oxidase was also shown in this study to be glycosylated, as it bound to ConA sepharose and was eluted with α-mannopyranoside (section 8.4.2.). The SGS5 pentamers exhibited the same thermostability as the native cereal oxalate oxidases with a dissociation / denaturation temperature of about 80 °C, as well as retaining about 80% of its activity when pre-incubated at 70 °C for 30 min, like the cereal oxalate oxidases (section 9.5.). The SGS5 oxalate oxidase isoforms were also resistant to pepsin digestion, which is one of the unusual characteristics reported for cereal oxalate oxidases [Grzelczak and Lane 1984,1985] (section 8.5.3.).

Transgenic SGS5 tobacco oxalate oxidase also exhibited very similar kinetic properties compared with the native cereal oxalate oxidases, with a K_m of about 192 mM (c.f.

209 mM) and a pH optimum of between 4.0 and 4.5. (c.f. 4.0-4.5) The transgenic enzyme showed similar levels of NaCl inhibition observed with wheat embryo oxalate oxidase.

Berna and Bernier [1997] also reported the presence of the two pentameric oxalate oxidase isoforms (G and G') in transgenic tobacco expressing the intact gf-2.8 gene and commented that the enzyme appeared to have the same regulatory and biochemical properties as the native wheat embryo oxalate oxidase. The protein was resistant to dissociation in the presence of 0.1% (w/v) SDS and it retained full enzyme activity after heating at 70 °C for 10 min. Differences in the ratio of G to G' in the transgenic tobacco similar to wheat were observed for the transgenic protein (i.e. a lower ratio of G compared with G'). Berna and Bernier [1997] confirmed that the G and G' isoforms of oxalate oxidase were synthesised from the same transgene (gf-2.8) in transgenic tobacco and that the presence of the two isoforms resulted from differential glycosylation of the apoprotein in tobacco, to give proteins very similar if not identical to native wheat embryo oxalate oxidase isoforms. The transgenic SGS5 tobacco plants were also able to produce the G and G' isoforms, these plants were expressing the CaMV 35S-gf-2.8 gene compared with the intact gf-2.8 gene expressed by Berna and Bernier [1997] in their transgenic tobacco.

Oxalate oxidase produced by transgenic SGS5 tobacco was essentially the same if not identical to the native wheat embryo oxalate oxidase in terms of its structural and kinetic properties, correct processing, folding, glycosylation and subunit aggregation. The fact that the transgenic oxalate oxidase was identical to the native protein suggests that the processing mechanisms in monocotyledons (i.e. wheat and barley) and dicotyledons (i.e. tobacco) are essentially the same, an observation also made by Berna and Bernier [1997]. The only observed difference involved the expression of similar levels of the G and G' isoforms in the transgenic plants compared with wheat embryos where the G isoform of oxalate oxidase was always present at about twice the level of G'. Presumably this resulted from slight differences in the efficiencies or levels of the processing mechanisms in tobacco which may have led to the different degrees of processing and removal of the anntenary N-acetylglucosamine residues from G to give equal levels of G' in the transgenic tobacco plants.

11.3. Transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases exhibit structural instability and altered kinetic properties.

Transgenic 3S1 oilseed rape and C26 tobacco plants were expressing the CaMV 35S-extensin-barley root oxalate oxidase chimeric gene. The native barley root oxalate oxidase signal peptide was replaced in this construct with the extensin signal peptide from *N. plumbaginifolia*. In this study CD spectroscopy demonstrated that 3S1 oxalate oxidase had a lower α-helix content compared with the native barley root protein, which suggests that there were differences in the secondary structure of the native and transgenic proteins (section 8.1.). Gel filtration column chromatography confirmed the existence of the transgenic 3S1 oilseed rape and C26 tobacco oxalate oxidases as enzymatically active pentamers in the transgenic plants with molecular weights of about 110-120 kDa (section 8.2.1.). However, the 3S1 and C26 enzymes were only detected as monomers in non-heat treated samples

following separation by SDS-PAGE compared with the cereal and transgenic SGS5 oxalate oxidases which retained their pentameric structure and activity under the same conditions (Section 8.2.1.). The 3S1 and C26 oxalate oxidases were shown to have very similar subunit molecular weights (about 22.7-22.8 kDa) compared with the native barley root protein, generating pentamers of about 114-115 kDa (section 8.2.2.). CD spectroscopy showed that the treatment of 3S1 and C26 oxalate oxidases with 0.05% (w/v) SDS did not have any significant effect on the secondary structure of the transgenic proteins compared with protein in phosphate buffer (section 8.1.). However the proteins precipitated out of solution in the presence of 0.1% (w/v) SDS and it was shown that these two transgenic enzymes lost over 90% of their activity in the presence of 0.1% (w/v) SDS, which presumably resulted from a loss of structural stability and dissociation of the active pentamer (section 9.8.). Cross-linking of the 3S1 and C26 subunits with DMS followed by SDS-PAGE analysis and western blotting confirm that the two proteins existed as pentamers in the transgenic plants with molecular weights of about 110-120 kDa (section 8.3.).

Analysis of the proteins on ConA sepharose provided evidence to show that the 3S1 and C26 transgenic oxalate oxidase were glycosylated, since they both bound the lectin and were eluted from the column by α-mannopyranoside (section 8.4.2.). SDS-PAGE analysis of extracts of 3S1 oilseed rape seedlings grown in the presence of tunicamycin also showed that the transgenic protein possessed an oligosaccharide side chain(s) with a molecular weight of about 2-3 kDa, similar to the molecular weight for the oligosaccharide side chain(s) of the native barley root protein (section 8.4.3.). Unlike the cereals and transgenic SGS5 tobacco which expressed the two oxalate oxidase isoforms the transgenic 3S1 oilseed rape and C26 tobacco plants only appeared to express single proteins. The doublet of oxalate oxidase isoforms was not observed at any time during this study in these two transgenic lines, which suggests that somehow the processing of the nascent proteins was affected resulting in the production of only a single form of the protein. The only difference between the cereal G and G' isoforms is in their level of glycosylation, which suggests that the 3S1 and C26 oxalate oxidases did not undergo the same type of glycosylation as the native proteins. The 3S1 and C26 proteins may have by-passed a stage in the glycosylation process possibly the removal of the antennary N-acetylglucosamines. From the monomer molecular weights determined by mass spectrometry it is not really possible to determine which of the two isoforms (G or G') these proteins may represent. It is possible that they may not even exhibit the same pattern of glycosylation as the native proteins.

Unlike the native cereal and transgenic SGS5 oxalate oxidases which exhibited stability and enzymatic activity following pepsin treatment the transgenic 3S1 and C26 enzymes were unstable and were completely degraded (section 8.5.3.). The transgenic 3S1 and C26 oxalate oxidase were also shown to be very unstable above a temperature of about 40 °C, with complete loss of activity above 50 °C compared with the highly stable barley root oxalate oxidase, which retained activity up to about 80 °C (section 9.5.). During purification of the 3S1 and C26 oxalate oxidases the heat treatment step (80 °C for 3 min), often used as a means of removing contaminating proteins was omitted, as this would have led to the

denatuation of these thermolabile proteins, compared with the native oxalate oxidases which would have retained their active structure following this heat treatment [Chiriboga 1966; Sugiura *et al.* 1979; Pietta *et al.* 1982].

Transgenic 3S1 and C26 oxalate oxidases also exhibited considerably different kinetic properties compared with the native barley root enzyme. The transgenic proteins had higher K_m values (about 318 mM, c.f. 209 mM, section 9.3.) and a lower pH optimum compared with the native cereal enzyme (c.f. 4.0-4.5, section 9.4.), which effectively meant that these transgenic enzymes had a lower 'affinity' for the substrate (oxalic acid) and were therefore 'less efficient' than the native cereal and transgenic SGS5 enzymes (K_m about 209 mM). Interestingly the 3S1 and C26 enzymes exhibited almost complete inhibition of their activity in the presence of NaCl (100-400 mM), which may have resulted from changes in the structure of the protein due to interaction of charged side chains with the sodium and chloride ions (section 9.7.). This inhibition was reversed following dialysis against NaCl.

Experiments carried out on transgenic SGS5 tobacco oxalate oxidase had shown that expression of the gf-2.8 gene containing the native signal peptide resulted in the production of a transgenic protein with almost identical properties compared with the native wheat embryo oxalate oxidase. The only major difference between the gene expressed in the transgenic 3S1 and C26 plants was the substitution of the native signal peptide with the *N. plumbaginifolia* extensin signal peptide. The use of the extensin signal peptide was not expected to have any effect on the structure, stability or kinetics of the transgenic oxalate oxidase, as it was only used to ensure that the nascent protein was targeted to the rough ER and to facilitate passage through the ER membrane, where it would be cleaved by signal peptidase. However N-terminal sequencing of the 3S1 and C26 proteins showed that the transgenic proteins did not possess the expected N-terminal sequences identical to the native barley root oxalate oxidase (i.e. SETDPDPLQDFCV instead of SDPDPLQDFCV). The signal peptidase had not cleaved the extensin peptide at the correct position to give a protein with an N-terminal sequence identical to the native barley root oxalate oxidase.

Thompson *et al.* [1995] substituted the native barley root signal peptide with the signal peptide of extensin assuming that it would lead to ER transport and eventual secretion. However, DeLoose *et al.* [1991] showed that the *N. plumbaginifolia* extensin signal peptide was cotranslationally removed from the nascent polypeptide upon translocation through the rough ER membrane and that it played no role in directing the subsequent targeting of the mature protein to the extracellular compartment for cell wall integration. DeLoose *et al.* [1991] showed that it was the N-terminal portion of the mature extensin protein which mediated its transport and secretion from the cell. Secretory proteins are thought to contain regions on the surface of the protein which are recognised by a specific receptor in the trans-Golgi network which directs the protein to the correct subcellular localisation [Burgess and Kelly 1987].

Appendix 2 shows an alignment of several partial extensin sequences including the C-terminals of the signal peptides and the N-terminals of the mature extensin proteins. The sequences originated from related *Nicotiana* and *Solanum* species and from *B. napus*. The key feature in these sequences is the presence of serine as the N-terminal amino acid of the

Nicotiana and *Solanum* mature extensin proteins. Thompson *et al.* [1995] introduced a point mutation in the codon at the N-terminus of the mature oxalate oxidase polypeptide giving rise to threonine as the N-terminal residue as opposed to serine. This mutation was introduced because Lane *et al.* [1991] had shown the N-terminal residue of the mature wheat embryo oxalate oxidase polypeptide to be threonine and not serine. It was assumed that for the correct cleavage of the extensin signal peptide to take place the N-terminal residue of the mature oxalate oxidase polypeptide would need to be threonine. The alignment in appendix 2 also illustrates that the N-terminal residue in the mature *Brassica* extensin was threonine, so it was thought that the processing mechanisms in the transgenic oilseed rape plant would recognise the threonine as the N-terminal residue and therefore cleave the signal peptide at this amino acid residue. Presumably the signal peptide contains a code in its primary amino acid sequence which informs the signal peptidase in the rough ER to cleave the signal peptide. For example, the signal peptides of *Nicotiana* and *Solanum* presumably contain a code to ensure that signal peptidase cleaves the signal peptide to produce polypeptides with serine as their N-terminal residues. This could explain why the additional residues originating from the C-terminus of the extensin signal peptide were present on the N-terminus of the mature transgenic oxalate oxidase protein. Presumably threonine was not recognised by the signal peptidase in the rough ER of the transgenic 3S1 and C26 plants as the N-terminal residue of the mature oxalate oxidase polypeptide and so the signal peptide was cleaved at the nearest serine in the C-terminal sequence of the signal peptide giving rise to SETDPDP instead of the native SDPDP N-terminal sequence of oxalate oxidase.

Sequencing of the gene construct revealed no other mutations which would result in amino acid substitution. Therefore the only difference between the native and the transgenic oxalate oxidases expressed in 3S1 oilseed rape and C26 tobacco involved the presence of the additional amino acid residues (SE) and a substituted residue (S for T) on the N-terminus of the mature transgenic proteins. The differences observed between the kinetics of the native and transgenic enzymes was most likely due to the structural instability of the 3S1 and C26 pentameric oxalate oxidases resulted from the altered N-terminal sequence.

Manning [1993] reported that single amino acid substitutions (resulting from point mutations generated by site-directed mutagenesis) can greatly effect the conformational stability of a protein as well as its ability to aggregate to form oligomers. Many seed storage proteins are oligomeric and it has been demonstrated that by modifying the structure of their subunits it was possible to affect their assembly [Ceriotti *et al.* 1991; Dickinson *et al.* 1990], stability [Chrispeels 1991] and intracellular transport [Ceriotti *et al.* 1991]. Dickinson *et al.* [1987] used cell-free translation to demonstrated that the presence of a signal peptide on a synthetic mRNA's comprising either the complete soybean glycinin coding region or a derivative without the signal peptide led to inhibition of trimerisation and increased misfolding of monomers. Presumably the presence of the extra residues on the transgenic oxidase enzymes may be having a similar effect on the correct folding and aggregation. The lower levels of oxalate oxidase detected in the 3S1 and C26 plants compared with SGS5 tobacco may have resulted from the incorrect folding of some of the subunits, misassembly of the

pentamers and the subsequent retention and removal of the transgenic proteins in the ER. Association of misfolded and misassembled protein with binding protein in the ER often occurs when mutant or transgenic proteins are expressed, and when N-linked glycosylation is inhibited [Hurtley and Helenius 1989].For oligomers to become exported from the ER they must correctly aggregate. Non-aggregated and partially aggregated forms of the oligomer associate with the ER resident binding proteins and are retained and digested in the ER [Hurtley and Helenius 1989].

Oligomer stability may be enhanced by bound water molecules and cations are occasionally seen in the interface between proteins [Hurtley and Helenius 1989]. Stability of the monomeric subunits may be enhanced by glycoproteins which span the surface of two subunits. This would not seem to be the case for oxalate oxidase where pentamers composed of five unglycosylated monomer were completely stable and active. Misfolded proteins are generally excessively sensitive to proteases [Hurtley and Helenius 1989]. Thus the 3S1 and C26 oxalate oxidases were completely degraded by pepsin which suggests that these proteins have aggregated to form structurally unstable yet active oxalate oxidases.

As yet the crystalline structure of oxalate oxidase has not been determined and so we can only speculate as to the consequences of the additional residues on the N-terminals of the 3S1 and C26 transgenic oxalate oxidases. It is unknown if the N-terminus of the protein is buried within the folded structure of the protein, if it is on the surface of the folded protein or if it is involved in the interactions of the subunits to form the pentamer. When polypeptides undergo folding and assembly into oligomers in the ER the precise positioning of each amino acid and its side chain is critical to ensure correct bond formation occurs to maintain the correct protein structure. Most amino acids play an essential role in the protein. for example in enzymes the active site where catalysis takes place usually only contains one or two amino acid residues the exact positioning of which will influence activity. The amino acids in the mature protein sequence are precisely positioned in the secondary and tertiary structures, which help maintain the correct configuration of the active site. The smallest change in the primary structure of the polypeptide (i.e. changes in amino acids) can significantly effect the proteins ordered structure and stability and as a consequence the activity of enzymes.

If the N-terminus of the polypeptide is buried within the folded structure of the protein then presumably the presence of the SE amino acid residues will hinder the correct positioning of the N-terminus. The non-covalent forces which maintain the ordered structures of the folded protein require the amino acid side chains to be in very close proximity for bonds to form. Oxalate oxidases are very stable and resistant to treatments, such as SDS and proteolysis, which would normally result in the dissociation / degradation of the protein. The stability and activity of the native oxalate oxidases has been linked to its pentameric conformation, therefore a slight change in the structure of the subunits resulting from the introduction of additional amino acid residues could have serious consequences for the stability of the resulting oligomer, as seen with 3S1 and C26 oxalate oxidases. The stable aggregation of proteins is dependent on the overall surface structure of the monomeric units as well as the presence of key amino acid side chains at these surfaces. Subunit aggregation

can often be prevented if mutations occur at the sites of interaction between the monomeric units [Wu *et al.* 1983]. If the N-terminus of the protein is involved in subunit interaction to form the pentamer then the presence of additional residues could effect the assembly of the subunits to produce a stable protein. The stability of the 3S1 and C26 oxalate oxidase appears to have been compromised as a result of the additional amino acid residues.

The glutamic acid residues added onto the N-terminus of the polypeptide as a result of the incorrect cleavage of the signal peptide possesses a negatively charged (carboxyl) side group which can interact with other amino acid residues in the protein which carry positively charged (amino) side groups (i.e. lysine, arginine or histidine). Amino acids with ionised side chains tend to be found on the surface of proteins where they can undergo interactions [Price and Stevens 1989]. An interaction between the side group of glutamic acid and a positive charge elsewhere on the protein would presumably prevent the polypeptide from assuming its correct configuration. The charge on the side chain of glutamic acid is pH dependent which may result in the narrower pH range and reduced stability of the 3S1 and C26 enzymes compared with the native and SGS5 oxalate oxidases. It is possibly that a small change in the structure of the monomers subsequently affected the stability of the pentamer, which was reflected in the reduced structural stability and altered kinetic properties of the transgenic 3S1 and C26 oxalate oxidases compared with the native barley root enzyme.

The changes in the structure of the subunits and the subsequent effect on aggregation are most likely to be quite subtle, as proteins which are misfolded and misassembled are retained and degraded in the ER. It is unlikely that the 3S1 and C26 oxalate oxidases are being retained in the ER, since detection of 3S1 and C26 oxalate oxidases in the extracellular spaces of transgenic leaves proves that the enzymes were transported from the ER.

The fact that the SGS5 tobacco plants are expressing an oxalate oxidase which is virtually indistinguishable from the native wheat embryo protein shows that this strategy for producing transgenic plants expressing oxalate oxidase is a viable one. Genetic engineering has also been employed successfully to produce transgenic proteins in plants that are correctly processed and targeted, most of these are indistinguishable from the native proteins, for example barley lectin [Bednarek *et al.* 1990], *B. napus* oleosin [Batchelder *et al.* 1994], *Chlamydomonas reinhardtii* carbonic anhydrase [Roberts and Spalding 1995], kiwifruit protease actinidin [Paul *et al.* 1995], all of which were correctly expressed in transgenic tobacco. The results presented in this thesis show that by substituting the serine with a threonine residue at the N-terminus of the mature transgenic 3S1 and C26 oxalate oxidases instead of producing enzymes with the unusual stability properties characteristic of the native barley root oxalate oxidase the 3S1 and C26 plants are actually producing structurally unstable yet active oxalate oxidases. Had the serine not been substituted for threonine then the 3S1 and C26 oxalate oxidases would most probably have been correctly processed to produced a structurally stable enzyme with similar kinetic properties to the native enzyme as was the case for SGS5 oxalate oxidase in transgenic tobacco.

11.4. The relevance of expression levels and localisation of oxalate oxidase in the transgenic plants for effective fungal pathogen resistance.

In this study the transgenic oilseed rape and tobacco plants were shown to express high levels of oxalate oxidase, resulting from the expression of the introduced transgenes (0.84-2.7% of total protein). During the growth and development of the transgenic plants the highest levels of oxalate oxidase enzyme activity were detected in the mature flowering plants. High levels of oxalate oxidase activity were detected in SGS5 tobacco throughout growth and development compared with 3S1 oilseed rape and C26 tobacco where oxalate oxidase activity was almost undetectable in 4 week old plants. The highest levels of oxalate oxidase were identified in the leaves of the three transgenic plant lines followed by stems, petals and roots. However only about 7-12% of the transgenic proteins were located in the extracellular spaces, as determined by repeated vacuum infiltration of leaf pieces with a high ionic strength buffer (i.e. 400 mM NaCl) using immunoassays. The exact intracellular location of the other 88-93% of the transgenic protein is unknown.

The overall objective for producing transgenic oilseed rape expressing oxalate oxidase was to determine if the presence of this enzyme in the plants effectively reduces the rate of infection and spread of the fungal pathogen *S. sclerotiorum*. *S. sclerotiorum* generally infects plants via the germination of ascospores on dead or morbid cells, usually petals which then adhere to leaves and stems. The mycelia then pass into sound tissue and begin producing oxalic acid, which is the primary phytotoxin of this pathogen. The main role of oxalic acid is to reduce the pH in the extracellular spaces to about 4.0 which favours pectinolytic enzyme activity [Bateman and Millar 1966]. It was hoped that by producing transgenic oilseed rape expressing oxalate oxidase the rate of pathogen infection would be reduced such that the plants could set seed and so the infection would not significantly reduce the yield of the plants. The successful use of genetic engineering to produce transgenic plants expressing proteins with anti-fungal properties depends very heavily on the selection of highly expressing plants and also on the correct localisation of the transgenic protein not only in the main organs infected but also to the cellular compartments which come into contact with the pathogen. In the case of fungal pathogens it is important that the transgenic protein is localised to the extracellular spaces through which the mycelia pass as the fungal infection spreads through the plant tissues.

Leaves of non-transgenic and 3S1 transgenic oilseed rape were incubated in water and increasing concentrations of oxalic acid (25, 50 and 100 mM), to determine if the expression and accumulation of an active oxalate oxidase in the transgenic 3S1 leaves provided improved protection to the symptoms of exposure to oxalic acid. This experiment illustrated that the severity of symptoms were greatly reduced in 3S1 compared with non-transgenic leaves, which was particularly evident in leaves incubated in 100 mM oxalic acid, where the non-transgenic leaves were completely dehydrated but the transgenic leaves only showed wilting around the leaf edges. The leaves of the non-transgenic and transgenic plants were unaffected following incubation in water at pH 4.0, which confirmed that it was the presence of oxalic acid and not the reduced pH which was responsible for the observed wilting

and necrosis. Obviously the high levels of expression of active oxalate oxidase albeit of an unstable protein (0.84% of leaf total protein) in the transgenic leaves was responsible for a reduction in the severity of symptoms. Cotyledons of the 3S1 plants were also induced to form callus on media containing oxalic acid, whereas cotyledons of non-transgenic oilseed rape did not form callus and subsequently died [Jim Dunwell, personal communication]. This showed that the transgenic oilseed rape cells were producing an active oxalate oxidase which was capable of degrading exogenous oxalic acid in the media allowing cell division and growth.

Improved resistance in 3S1 oilseed rape to *S. sclerotiorum* infection was very much dependent on the expression and accumulation of oxalate oxidase in the main organs attacked by the pathogen (i.e. leaves and stems). Expression of a stable enzyme resistant to proteolysis is also a desirable characteristic particularly in fallen petals, which are usually the initial organ infected. The highest levels of oxalate oxidase were detected in the leaves onto which the infected petals usually land. Once the mycelium penetrates the sound leaf tissue it was hoped that the expression of high levels of active oxalate oxidase would significantly reduce the spread of infection as a result of hydrolysis of the secreted oxalic acid, thereby reducing the rate at which the pH of the infected tissue dropped. Once the mycelia have penetrated the leaf the infection spreads into the stems where new sclerotia form. When this occurs the upper parts of the plant wilt, preventing seed set and eventually the stem splits and the sclerotia return to the soil. If the infection passes into the main stem then the entire yield of that particular plant is lost. Therefore high levels of expression of oxalate oxidase is also necessary in the stems. In 3S1 oilseed rape root expression was very low but this was of no consequence since the roots are not attacked by *S. sclerotiorum.*

According to the vacuum infiltration experiments some of the oxalate oxidase proteins were expressed in the leaf extracellular spaces of the three transgenic lines. As was previously discussed the extensin signal peptide was not directly responsible for the secretion of the protein into the extracellular spaces. The signal responsible for cellular localisation is usually carried on the mature folded protein. Localisation of the transgenic oxalate oxidase in the extracellular spaces was very important as this is the major compartment through which the pathogen passes into the plant tissues. These results showed that the majority of the transgenic oxalate oxidase was most likely located within the cells and presumably plays no immediate role in pathogen resistance as it is only likely to come into contact with the pathogen once the cells have been damaged. It would appear that even this low level of total oxalate oxidase in the extracellular spaces was sufficient to reduce the spread of the pathogen in infected leaves.

Thompson *et al.* [in press] reported that the expression of oxalate oxidase in these transgenic plants did indeed provide a significant level of protection against *S. sclerotiorum* as determined from the lack of mycelial growth of the pathogen on transgenic leaves, compared with controls where the establishment and spread of infection is more rapid. This suggests that oxalate oxidase is being expressed in the leaves of the transgenic plants at a sufficient level to enable the hydrolysis of the oxalic acid produced by the pathogen. Without the drop in pH caused by the production of oxalic acid the lytic enzymes produced by the pathogen

cannot achieve their optimal activity, which will hinder the establishment and spread of infection in the transgenic plants .

Zeneca Seeds have performed field trials on several of the highest expressing transgenic oilseed rape lines [Thompson *et al.* in press] and have reported that all of the transgenic lines tested have reduced disease severity and that there were a lower number of infected plants compared with the control plants. These differences are statistically significant.

The production of hydrogen peroxide is one of the first responses to be initiated by the host plant following pathogen infection. The hydrolysis of oxalic acid by the oxalate oxidase expressed in the transgenic 3S1 oilseed rape will presumably increase the levels and rate of hydrogen peroxide produced by the host plant. A more rapid generation of hydrogen peroxide in the host plant would be advantageous as it would lead to a more rapid induction of other plant defence responses and it would provide a substrate to the various peroxidases (i.e. lignin peroxidase) in the extracellular spaces which would strengthen the cell walls in the direct vicinity of the infection. Hydrogen peroxide is also a defence factor in its own right the production of which initiates the oxidative burst [Wojtaszek 1997].

The two major kinetic parameters which are likely to influence the activity of the transgenic oxalate oxidase in infected plants are substrate concentration and pH in the infection sites. The enzyme kinetic experiments showed that 3S1 oxalate oxidase has a lower substrate optimum (0.4 mM oxalic acid) compared with the native cereal enzymes, which meant that the protein has a lower 'affinity' to the substrate and that it is not as efficient at utilising lower concentrations of oxalic acid compared with the native enzymes. 3S1 oxalate oxidase also exhibited strong substrate inhibition at a lower oxalic acid concentration (> 0.4 mM oxalic acid) compared with the native enzymes. 3S1 transgenic oxalate oxidase only exhibited about 60% of its activity at pH 4.0 the reported pH of infected tissue [Bateman and Millar 1966] all of the native oxalate oxidases had optimum activity at pH 4.0-4.5. As the pathogen secretes oxalic acid at the infection site substrate concentration will increase and pH will decrease. However, should localised oxalic acid concentration increases above 0.4 mM then presumably the transgenic oxalate oxidase will exhibit inhibition resulting in a sharp decline in activity, which will allow the pathogen to penetrate further into the hosts tissues. Bateman and Millar [1966] showed that the localised pH around the site of infection only goes down to pH 4.0 if this is the case then the transgenic oxalate oxidase will only be exhibiting a fraction of its optimum activity. Had the SGS5 transgenic oxalate oxidase with almost identical kinetic properties to the native cereal oxalate oxidase been expressed in oilseed rape presumably the level of resistance would be increased compared with 3S1 oxalate oxidase expressing plants. SGS5 oxalate oxidase exhibited kinetic properties with higher affinity towards oxalic acid, higher optimum substrate concentration and a pH optimum of 4.0-4.5, which are more favourable characteristics as far as degradation of pathogen produced oxalic acid is concerned. The general instability of the transgenic protein presumably also has an effect on the hydrolysis of oxalic acid in infected plants.

Even though the transgenic 3S1 oilseed rape plants were expressing a structurally unstable enzyme with altered kinetic properties compared with the native barley root oxalate

oxidase, it still provided improved protection against *S sclerotiorum* fungal pathogen infection. Had the plants been expressing an oxalate oxidase identical to the native oxalate oxidase then it is possible that the level of resistance may be enhanced compared with the 3S1 plants. There is now strong evidence that barley and wheat express oxalate oxidase as a result of fungal pathogen infection, which shows that in the native plant environment this enzyme has evolved a natural role in pathogen resistance against oxalic acid producing fungal pathogens, such as powdery mildew [Dumas *et al.* 1995; Zhang *et al.* 1995; Hurkman and Tanaka 1995].

11.5. Future work.

One of the most important questions still to be addressed involves the determination of the 3-dimensional structure of oxalate oxidase, to analyse how the subunits interact to form the stable pentamers, which regions of the polypeptide form the active sites and what role if any the N-terminal sequences of the polypeptides may play in maintaining the pentamer This would be of great interest for determining the possible effects of the addition of the SE amino acid residues on the N-terminals of the 3S1 and C26 oxalate oxidases.

More detailed immunocytochemistry is required to determine the accurate cellular and subcellular localisation of the native oxalate oxidases. Lane *et al.* [1992] performed some basic immunocytochemistry on sections of wheat embryos which illustrated the association of oxalate oxidase with the cell walls and extracellular spaces. Subcellular fractionation and vacuum infiltration are the main techniques used to determine the cellular distribution of oxalate oxidase [Lane *et al.* 1986, 1992, Berna and Bernier 1997], which demonstrated that about 40% of the protein was associated with the pellet fraction and the other 60% with the soluble fraction. The intracellular localisation of this 60% of oxalate oxidase has yet to be determined, which would require the use of transmission microscopy of immunogold labelled sections reacted with anti-oxalate oxidase antibodies.

If the use of oxalate oxidase as a means of improving resistance to oxalic acid producing fungal pathogen is to be pursued it would seem logical to produce transgenic oilseed rape expressing the CaMV 35S-gf-2.8 gene. In transgenic SGS5 tobacco the expression of this transgene produced an oxalate oxidase identical to the native wheat embryo enzyme in terms of its stability and kinetics, which may provide greater protection against infection compared with the unstable oxalate oxidase expressed in 3S1 oilseed rape.

11.6. Concluding remarks.

This work demonstrated that it is possible to produce a genetically engineered plants expressing an oxalate oxidase which is essentially identical (i.e. structure, stability and kinetics) to the enzyme isolated from the native plant species (i.e. SGS5 tobacco). However it also highlighted the consequences of the expression of mutated proteins in transgenic plants (i.e. 3S1 oilseed rape and C26 tobacco), where the presence of the extra amino acid residues originating from the signal peptide on the N-terminus of the oxalate oxidase polypeptides compromised the structural stability and kinetics of the transgenic enzymes. The results presented in this thesis showed that the expression of oxalate oxidase in transgenic oilseed

rape significantly improved their ability to withstand treatment with oxalic acid. Thompson *et al.* [in press] had demonstrated the improved resistance of these oxalate oxidase expressing plants to fungal infection, as a result of the hydrolysis of the oxalic acid secreted by the pathogen. However, it is likely that the plants may have exhibited even greater resistance to *S. sclerotiorum* infection had the plants been expressing a stable oxalate oxidase with enzyme kinetics comparable with the native barley root oxalate oxidase.

12. APPENDICES.

Appendix 1.

Germin-like protein DNA sequences isolated from oilseed rape.

These DNA sequences were isolated by Amy Walker, an industrial placement undergraduate student from the University of Leeds, who performed PCR and cloning work of germin-like protein gene sequences from oilseed rape. The percentages refer to sequence identity to barley root oxalate oxidase (brox) [Lane *et al.* 1993].

brox GTNPPHIHPRATEIGMVMKGELLVGILGSLDSGNKLYSRVVRA

GerPCR1 **PPH**M**HPRATEI**LI**V**QQ**G**T**LLVG**FVS**S**NQD**GN**R**L**FAKTLNV

GerPCR2 RQS**PPH**M**HPRATEI**LV**V**QQ**G**T**LLVG**FVS**S**NQD**GN**R**L**FAKTLNV

brox GETFVIPRGLMHFQFNVGKTEAYMVVSFN

GerPCR1 **G**DV**FV**F**P**E**GL**I**HFQ**LRHPGL**E**RRTA**V**EL (46%)

GerPCR2 **G**DV**FV**F**P**E**G**FL**HFQ**LKASGL**E**RRTA**V**ELP (42%)

Appendix 2.

The signal peptides and mature N-terminal sequences of extensins.

An alignment of the C-terminal sequences of the extensin signal peptides (in italics) and the N-terminal sequences of the extensin mature polypeptides. The N-terminal amino acid residues of the mature extensin polypeptide are in bold.

amino acid sequence	plant species	accession number
*SLSLASE***S**SANYGYSSPPPPKKPYHPS	*Nicotiana plumbaginifolia*	170233
*SLSLASE***S**SANYGYSSPPPPKKPYHPS	*Nicotiana tabacum*	1161293
*SLSLASE***S**SANYGYSSPPPPVHVYPSP	*Solanum tuberosum*	629719
*SLGFVSE***T**TANYYYSSPPPPVKHYTPP	*Brassica napus*	512381
*SLGFVSE***T**TANYYYSSPPPPVKHYTPP	*Brassica napus*	99806

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