Identification and characterization of glucosyltransferases involved in the biosynthesis of cyanogenic glucosides in *Sorghum bicolor*

by

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A thesis submitted for the degree of Doctor of Philosophy

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TABLE OF CONTENTS

ABSTRACT

Many plants are capable of accumulating cyanogenic glucosides, which upon breakdown will liberate hydrogen cyanide. The biosynthetic pathway of the cyanogenic glucoside dhurrin has been established in etiolated seedlings of *Sorghum bicolor*, and is catalyzed by two membrane-bound multi-functional cytochrome P450s and a soluble glucosyltransferase.

A glucosyltransferase that glucosylates *p*-hydroxymandelonitrile, *Sorghum bicolor p*-hydroxy-(*S*)-mandelonitrile-*O*-β-glucosyltransferase (sbHMNGT), the aglycone precursor of dhurrin, was isolated from *S. bicolor* and the corresponding cDNA cloned. Recombinant sbHMNGT was then functionally expressed in *Escherichia coli*, isolated, and characterized with respect to substrate specificity. The quantitative and qualitative substrate specificity suggested that the isolated cDNA clone encodes *p*-hydroxymandelonitrile-glucosyltransferase. sbHMNGT did not exhibit exclusive specificity for *p*-hydroxymandelonitrile, as it was also able to glucosylate *p*-hydroxybenzyl alcohol and the monoterpenoid geraniol at a relatively high rate. The isolation of sbHMNGT and it's corresponding cDNA completes the identification and isolation of all enzymes believed necessary for the conversion of tyrosine to dhurrin in *S. bicolor*.

This was confirmed by reconstituting the entire cyanogenic glucoside biosynthetic pathway *in vitro* and in transgenic *Arabidopsis thaliana*. Transgenic *A. thaliana* functionally expressing two cytochrome P450s (CYP79A1 and CYP71E1) and sbHMNGT were able to accumulate dhurrin to similar levels as that found in young seedlings of *S. bicolor*, whilst transgenic *A. thaliana* expressing the two cytochrome P450s, but lacking sbHMNGT, only accumulated break-down metabolites of *p*-hydroxymandelonitrile. This verified the importance of sbHMNGT for the accumulation of a stable cyanogenic glucoside, since wild-type *A. thaliana* is unable to glucosylate *p*-hydroxymandelonitrile at the cyanohydrin function. Although dhurrin-accumulating *A. thaliana* cultivars were cyanogenic, the relative rate cyanogenic glucoside hydrolysis was not as efficient as that of *Sorghum,* most likely due to an absence of hydrolyzing enzymes with high activity towards dhurrin. Transgenic *A. thaliana* synthesizing dhurrin, with or without the insertion of a dhurrin-hydrolyzing β-

glucosidase encoded gene, can now be used in conjunction with wild-type *A. thaliana* to test the ecological significance of cyanogenic glucoside accumulation and cyanogenesis.

Finally, a cyanogenic glucoside glucosyltransferase, that may or may not be involved in the mobilization of dhurrin, was described and partially purified for the first time.

STATEMENT OF AUTHORSHIP

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

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Patrik R. Jones

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ABBREVIATIONS

A	adenine
ab	absorption
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	degrees Celsius
cDNA	complementary DNA to an RNA
Ci	Curie
cm	centimetre
conc.	concentration
C-terminal	carboxy-terminal
cv	cultivar
CYP71E1	*Sorghum bicolor* cytochrome P450 catalyzing the conversion of *p*-hydroxyphenylacetaldoxime to *p*-hydroxymandelonitrile
CYP79A1	*Sorghum bicolor* cytochrome P450 catalyzing the conversion of L-tyrosine to *p*-hydroxyphenylacetaldoxime
Da	dalton
f.w.	fresh weight
dGTP	2'-deoxyguanine 5'-triphosphate
dhurrin	*p*-hydroxy-(*S*)-mandelonitrile-*O*-β-D-glucopyranoside
DNA	deoxyribonucleic acid
dUTP	2'-deoxyuridine 5'-triphosphate
DTT	1,4-dithithreitol
EDTA	ethylenediamine tetra-acetic acid
g	gravity
G	guanine
Gbq	gigabecquerel
GFP	green fluorescent protein
GT	glycosyltransferase
hr	hours
HPLC	high performance liquid chromatography
I	inosine
IAA	indole-3-acetic acid
IPTG	isopropyl-1–thio-β-D-galactopyranoside
kb	kilobase
kDa	kilodalton
l	litre
L.	Linnaeus
LB	Luria-Bertani
LC	liquid chromatography
LSC	liquid scintillation counting
M	molar
meGT	*Manihot esculenta* glycosyltransferase
min	minute
mm	millimetre
MQ	milli-Q
µl	microlitre

ml	millilitre
mM	millimolar
MOPS	3-(N-morpholino)propane sulfonic acid
M_r	relative molecular mass
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
m/z	mass to charge ratio
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
nkatals	nanokatals
nm	nanometre
NMR	nuclear magnetic resonance
N-terminal	amino-terminal
ntGT	*Nicotiana tabacum* glucosyltransferase
OD	optical density
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
Ponceau S	3-hydroxy-4-[2-sulfo-4-(sulfophenylazo)phenylazo]-2,7-naphthalenedisulfonic acid
prunasin	hydroxy-(*R*)-mandelonitrile-*O*-β-D-glucopyranoside
psGT	*Pisum sativum* glucuronosyltransferase
PSPG	plant secondary product glycosyltransferase
PVPP	polyvinylpolypyrrolidone
pPZP111-GT	pPZP111-glucosyltransferase cDNA construct
pPZP221-GT	pPZP221-glucosyltransferase cDNA construct
P450	cytochrome P450
R_f	the ratio of the distance traveled by a compound to that of the solvent front
RIC	reconstructed ion chromatogram
RNA	ribonucleic acid
RT	retention time
rpm	revolutions per minute
s	second
SA	salicylic acid (2-hydroxybenzoic acid)
sambunigrin	hydroxy-(*S*)-mandelonitrile-*O*-β-D-glucopyranoside
sbHMNGT	*Sorghum bicolor p*-hydroxy-(*S*)-mandelonitrile-*O*-β-glucosyltransferase
sbHMNGT	cDNA encoding for sbHMNGT
SDS	sodium dodecyl sulfate
silwet L-77	84% (v/v) polyalkyleneoxide modified heptamethyltrisiloxane 16% (v/v) allyloxypolyethyleneglycol methyl ether
SSC (20x)	3 M NaCl, 0.3 M Na_3citrate buffer
T	thymine
TAE (50x)	2 M tris-HCl buffer, 1 M acetic acid, 50 mM EDTA, pH 8
TCA	trichloroacetic acid
TE	10 mM tris-HCl buffer, pH 8, 1 mM EDTA
TLC	Thin layer chromatography

Tricine	N-tris-(hydroxymethyl)-methylglycine
Tris	tris(hydroxymethyl)aminomethane
Tween-20	polyoxyethylene-sorbitan monolaurate
UDP	uridine diphosphate
UV	ultraviolet
vol	volume
V_{max}	maximal velocity
v/v	volume/volume
vvUFGT	*Vitis vinifera* UDP-glucose:flavonoid-glucosyltransferase
w/v	weight/volume
w/w	weight/weight
zmIAAGT	*Zea mays* indole acetic acid-glucosyltransferase
zmUFGT	*Zea mays* UDP-glucose:flavonoid-glucosyltransferase
µl	microlitre
1x	Transgenic plant expressing sbHMNGT
2x	Transgenic plant expressing CYP71E1 and CYP79A1
3x	Transgenic plant expressing CYP71E1, CYP79A1 and sbHMNGT

One and three letter codes for amino acids

A	Ala	alanine	L	Leu	leucine
R	Arg	arginine	K	Lys	lysine
N	Asn	asparagine	M	Met	methionine
D	Asp	aspartic acid	F	Phe	phenylalanine
C	Cys	cysteine	P	Pro	proline
E	Glu	glutamic acid	S	Ser	serine
Q	Gln	glutamine	T	Thr	threonine
G	Gly	glycine	W	Trp	tryptophan
H	His	histidine	Y	Tyr	tyrosine
I	Ile	isoleucine	V	Val	valine

PUBLICATIONS

Parts of the work described in this thesis have been published or submitted for publication in the following:

Experimental

Jones PR, Møller BL, and Høj PB (1999) The UDP-glucose:*p*-hydroxymandelonitrile-*O*-glucosyltransferase which catalyzes the last step in synthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor*: isolation, cloning, heterologous expression, and substrate specificity. J Biol Chem 274: 35483-35491

Tattersall DB, **Jones PR**, Bak S, Høj PB, Møller BL. Metabolic engineering of a complete cyanogenic glucoside pathway into *Arabidopsis*. Manuscript in preparation.

Reviews

Jones PR, Andersen MD, Nielsen JS, Høj PB, Møller BL. (2000) The biosynthesis, degradation, transport and possible function of cyanogenic glucosides. In: Romeo JT, Ibrahim R, Varin L, De Luca V (eds), Evolution of metabolic pathways. Permagon, Amsterdam, Vol 34, pp 191-247

Vogt T, **Jones PR** (2000). Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. Trends Plant Sci 5: 380-386

Jones PR, Vogt T (2001). Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. Planta. In Press.

Chapter 1

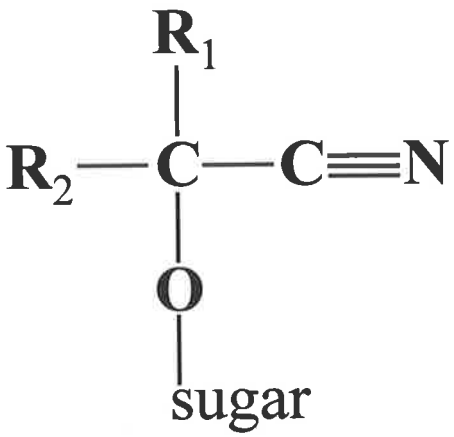
General Introduction

1.1 Introduction

Plants accumulate a large array of differently structured, low molecular weight, carbon-containing compounds that collectively are classified as secondary plant metabolites (Hartmann, 1996). Typically, several hundred seondary metabolites of great structural diversity can exist in any plant species at greatly varying levels of concentration (Halkier and Møller, 1989; Bar-Peled *et al.*, 1991; Sefton *et al.*, 1993; Sefton *et al.*, 1994; Hartmann, 1996) Prior to the 20th century it was commonly believed that these compounds played no pivotal role in the biology of plants and most likely constituted metabolic waste-products (Hartmann, 1996). Today it is widely recognized that secondary metabolites play an integral role in determining the fitness of plants (Williams *et al.*, 1989; Bennett and Wallsgrove, 1994; Hartmann, 1996), although it has been suggested that only a minor proportion of all natural plant products are bioactive (Firn and Jones, 1991).

One major class of secondary plant metabolites is cyanogenic glucosides. The ability of living organisms to produce hydrogen cyanide from constitutive precursors, such as cyanogenic glucosides, is characterized as cyanogenesis. This trait is common among many plant species including ferns, gymnosperms as well as monocotyledonous and dicotyledonous angiosperms (Seigler and Brinker, 1993; Lechtenberg and Nahrstedt, 1999; Møller and Seigler, 1998). Most cyanogenic precursors have been found to be cyanogenic glucosides, although other forms, *e.g.* cyanogenic lipids, are known to exist (Seigler and Brinker, 1993). All known cyanogenic glucosides are *O*-β-glycosides of α-hydroxynitriles (Figure 1.1) and are with few exceptions synthesized from six different parent amino acids (Lechtenberg and Nahrstedt, 1999; Møller and Seigler, 1998). Several agriculturally important crop plants are known to be cyanogenic, including cassava, sorghum, barley, bamboo, lima beans, flax, cherry, almond and macadamia (Jones, 1998). When cyanogenic plants are macerated the cyanogenic glucosides are broken down by endogenous β-glucosidases and α-hydroxynitrilases, resulting in the release of cyanide, which has well known toxicological properties (Solomonsen, 1981). It has therefore been suggested that cyanogenic glucosides and their breakdown products may play a role in plant defense (Nahrstedt, 1985; Hruska, 1998; Patton *et al.*, 1997; Jones, 1998). Unfortunately, foods containing cyanogenic glucosides have

A



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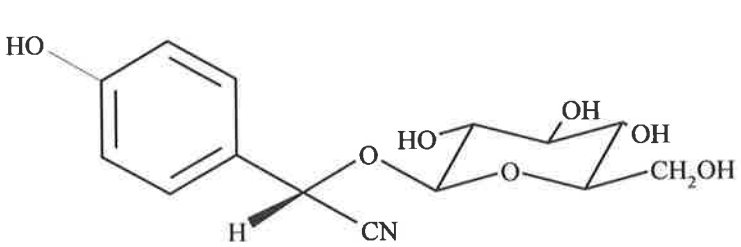


Figure 1.1 The general structure of cyanogenic glucosides. (A) The general structure of cyanogenic glucosides (Lechtenberg and Nahrstedt, 1999; Møller and Seigler, 1998). (B) The cyanogenic glucoside dhurrin.

reduced nutritional value and may pose a health hazard if not properly processed prior to consumption (Rosling, 1988; Jones, 1998). It is therefore desirable to engineer acyanogenic varieties of cyanogenic food crops, for example cassava (*Manihot esculenta* L.), given that traditional breeding methods so far have not achieved this aim (Dixon *et al.*, 1994). Conversely, it may be worthwhile to introduce the capability of cyanogenic glucoside accumulation into particular acyanogenic crop tissues in order to improve their pest or pathogen resistance.

To be able to modulate the accumulation of cyanogenic glucosides in a predetermined fashion it is necessary to first study the processes responsible for their biosynthesis and degradation. The biosynthetic pathway of the cyanogenic glucoside dhurrin in *Sorghum bicolor* is catalyzed by only three enzymes (Figure 1.2). The first two enzymes are membrane-bound multifunctional cytochrome P450s that synthesize the cyanohydrin *p*-hydroxymandelonitrile from the parent amino acid tyrosine (Sibbesen *et al.*, 1994; Sibbesen *et al.*, 1995; Halkier *et al.*, 1995; Koch *et al.*, 1995; Kahn *et al.*, 1997; Bak *et al.*, 1998). The cyanohydrin is finally converted to a stable storage form by a soluble glucosyltransferase. Whilst the two cytochrome P450s had been characterized at the start of this study, the glucosyltransferase(s) remained elusive. The main objective of this study was therefore to obtain and characterize the responsible protein(s), and corresponding gene sequence(s), which catalyze the transfer of a glucose-moeity from UDP-glucose to *p*-hydroxymandelonitrile. In addition to this, cyanogenic glucosides are also found in a di-glucosidic form that has been suggested to constitute a transport form within certain cyanogenic plants (Selmar *et al.*, 1988). Cyanogenic glucoside glucosyltransferases, the enzymes which catalyze the production of cyanogenic diglucosides, are yet to be described and isolated. A minor study was therefore also initiated, in order to obtain some preliminary information regarding such glucosyltransferases in *S. bicolor*.

Henceforth, the main focus of this introductory review is two-fold; (1) the biology of cyanogenic glucosides and (2) the biology of β-glucosyltransferases involved in plant secondary metabolism. The metabolism of cyanogenic glucosides, with a focus on *S. bicolor*, will be briefly covered first. This will be followed by selected topics on plant β-glucosyltransferases, pertaining to the current study, with a focus on the biological

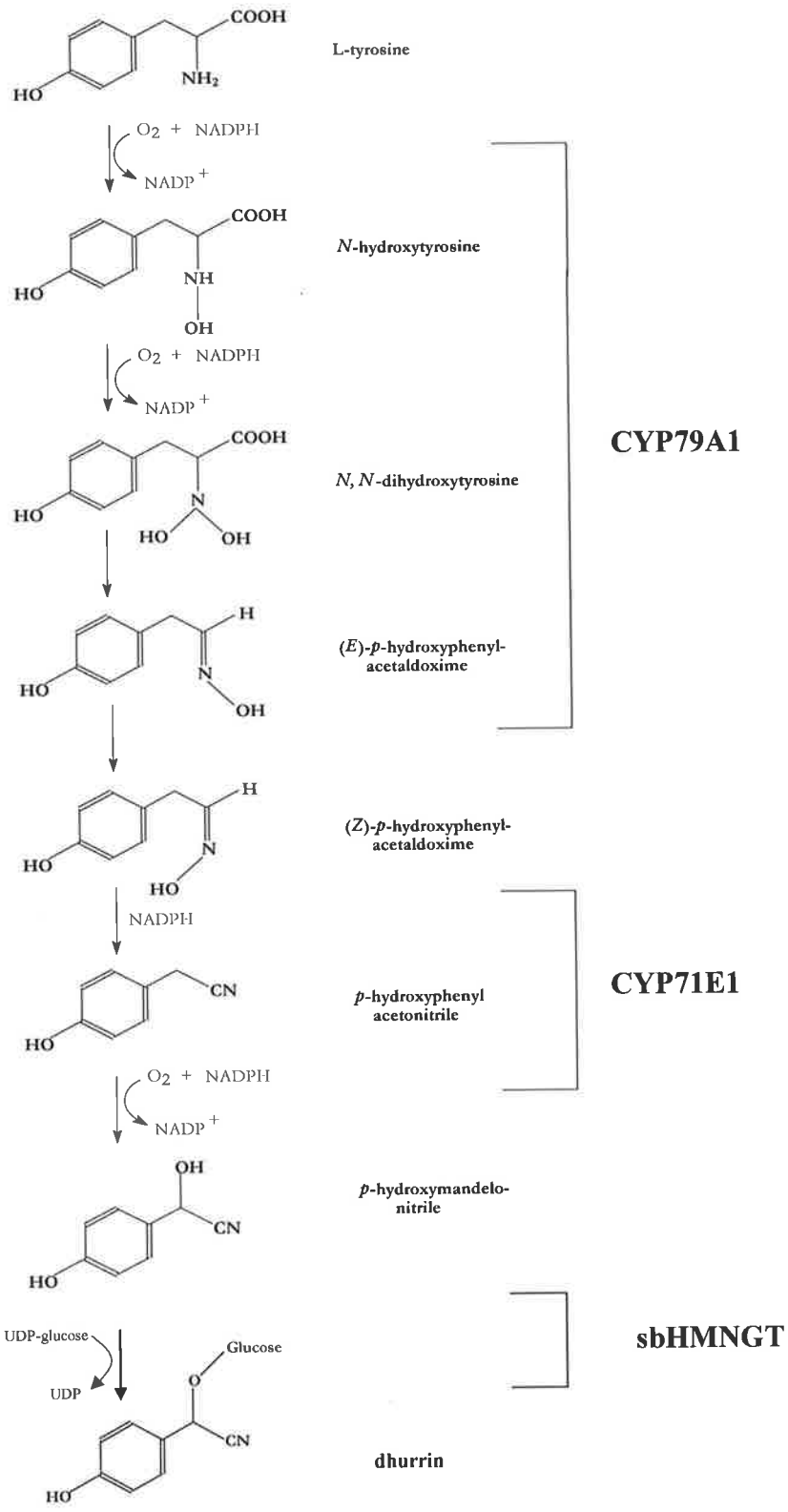


Figure 1.2 The biosynthetic pathway of the cyanogenic glucoside dhurrin in *S. bicolor*. The entire pathway, starting from L-tyrosine and ending with the cyanogenic glucoside dhurrin, is catalyzed by three enzymes; multi-functional CYP79A1, multi-functional CYP71E1 and sbHMNGT (Møller and Seigler, 1998). The two cytochrome P450s receive reducing equivalents in the form of NADPH from a NADPH-cytochrome P450 oxidoreductase (Halkier, 1996).

roles of glycosylation and biochemical features of glycosyltransferases. These subjects have been extensively reviewed recently (Møller and Seigler, 1998; Li *et al.*, 2000; Jones and Vogt, 2000; Jones *et al.*, 2000; Vogt and Jones, 2000).

1.2 Cyanogenic glucosides

1.2.1 The biosynthesis of cyanogenic glucosides

The first studies into the biochemistry of cyanogenic glucoside biosynthesis were conducted by Eric Conn and co-workers (Hahlbrock *et al.*, 1968; Conn, 1969), and the complete pathway sequence in *S. bicolor* (Figure 1.2) was not elucidated until 20 years later (Halkier *et al.*, 1991). Only recently were the two microsomal cytochrome P450s from *S. bicolor* finally isolated and characterized (Sibbesen *et al.*, 1994; Halkier *et al.*, 1995; Koch *et al.*, 1995; Kahn *et al.*, 1997; Bak *et al.*, 1998). In *Sorghum*, CYP79A1 subjects the amino acid L-tyrosine to two *N*-hydroxylations, followed by a dehydration and a decarboxylation, resulting in the formation of (*Z*)-*p*-hydroxyphenylacetaldoxime. The oxime is subsequently converted to the cyanohydrin, *p*-hydroxy-(*S*)-mandelonitrile through a dehydration and a C-hydroxylation reaction by CYP71E1 (Figure 1.2). The *S*-enantiomer of the cyanohydrin is then glucosylated at the cyanohydrin function by a soluble UDP-glucose:*p*-hydroxy-(*S*)-mandelonitrile-*O*-β-glucosyltransferase (sbHMNGT; Reay and Conn, 1974). The first enzyme of the dhurrin-synthesizing pathway, CYP79A1, has been found to be rate-limiting *in vitro* (Halkier *et al.*, 1991) and to exhibit stricter substrate specificity than the down-stream enzymes (Reay and Conn, 1974; Kahn *et al.*, 1999). Cytochrome P450s that catalyze the synthesis of oximes in cassava (*Manihot esculenta*; Andersen *et al.*, 2000) and *Triglochin maritima* (Nielsen and Møller, 1999; Nielsen and Møller, 2000) have also been characterized. Furthermore, microsomal preparations which catalyze cyanohydrin formation from amino acids *in vitro* have also been obtained from several other cyanogenic species (Collinge and Hughes, 1982; Cutler *et al.*, 1985; Hösel *et al.*, 1985). Although the studies carried out using these additional *in vitro* systems have not been as thorough as those carried out in *Sorghum*, the data supports the assumption that the cyanogenic glucosides present in each of these plant species are synthesized by the basic scheme

outlined for *Sorghum* (Figure 1.2). The intracellular localization of the pathway enzymes has only been investigated using classical biochemical techniques such as co-purification. Whilst the microsomal cytochrome P450s co-purify with the endoplasmic reticulum in *Sorghum* (Saunders *et al.*, 1977b), a significant proportion of the soluble glucosyltransferase activity (>41%) was judged to be associated with a plastid fraction, as shown by separation by sucrose density gradients (Würtele *et al.*, 1982).

1.2.2 The accumulation and degradation of cyanogenic glucosides

In *Sorghum* there are two cyanogenic glucosides, dhurrin and dhurrin-6'-glucoside (Selmar *et al.*, 1996). Whilst the seeds contain very low amounts of cyanogenic glucosides (Erb *et al.*, 1981), dhurrin may constitute up to 30% (w/w) dry weight of the shoot apex in young seedlings (Halkier and Møller, 1989). Despite reports that no dhurrin is present nor synthesized in roots of young *Sorghum* seedlings (Halkier and Møller, 1989), other researchers have found evidence for such accumulation in root tissue (Adewusi, 1990; Arias *et al.*, 1983). The concentration of dhurrin and dhurrin-6'-glucoside in shoots of *Sorghum* seedlings has been estimated to reach approximately 6 mg per gram of plant material (f.w.) and 0.05 - 0.3 mg per gram of plant material (f.w.), respectively (Halkier *et al.*, 1989; Selmar *et al.*, 1996). The concentration of dhurrin peaks during the first days after germination and declines thereafter, although the total amount of dhurrin per shoot increases steadily throughout the growing season (Halkier and Møller, 1989; Loyd and Gray, 1970). A common trend amongst most cyanogenic plant species is that young plants contain the highest concentrations of cyanogenic glucosides (Frehner *et al.*, 1990; Swain and Poulton, 1994). Dhurrin is most actively turned over during darkness in young *Sorghum* seedlings with reported metabolic half-lives of 10 hours (Bough and Gander, 1971; Adewusi, 1990).

The catabolic pathway of dhurrin in *Sorghum* is catalyzed by two enzymes, dhurrin-specific β-glucosidases of which there are two known isoforms (Hösel *et al.*, 1987; Cicek and Esen, 1998) and an α-hydroxynitrilase (Wajant and Mundry, 1993; Wajant *et al.*, 1994) (illustrated in Figure 1.3). Similar pathways have been proposed for other cyanogenic plants (Selmar *et al.*, 1988; Swain *et al.*, 1992; Hughes and Hughes, 1994; Hu and Poulton, 1997; Trummler and Wajant, 1997). Both cyanogenic glucosides and catabolic enzymes are constitutive in most plants (Frehner *et al.*, 1990; Wajant *et al.*,

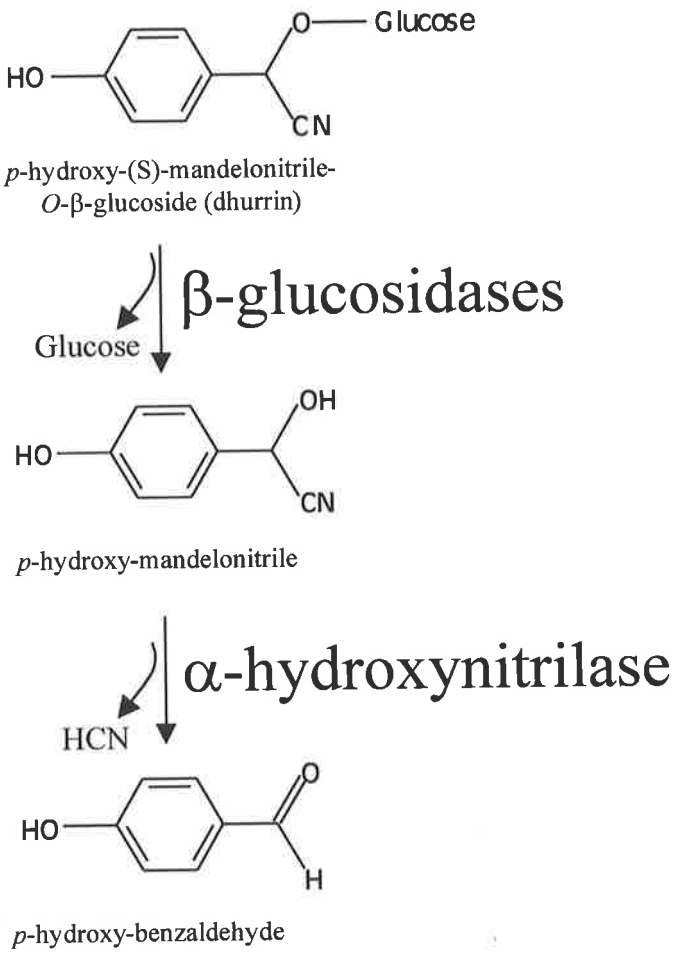


Figure 1.3 The catabolic pathway of the cyanogenic glucoside dhurrin in *S. bicolor*. The catabolism of the cyanogenic glucoside dhurrin in *S. bicolor* is catalyzed by specific β-glucosidases (Hösel *et al.*, 1987; Cicek and Esen, 1998) and an α-hydroxynitrilase (Wajant and Mundry, 1993; Wajant *et al.*, 1994).

1994; Osbourn, 1996; Cicek and Esen, 1998), necessitating some form of spatial differentiation between substrates and enzymes *in vivo*, in order to avoid large-scale internal cyanogenesis. Cyanogenic glucosides, including dhurrin, accumulate in vacuoles of certain tissues (Saunders *et al.*, 1977a; Saunders and Conn, 1978; Gruhnert *et al.*, 1994). The cellular and intracellular localization of the catabolic enzymes has been investigated in several cyanogenic plants (Kojima *et al.*, 1979; Thayer and Conn, 1981; Wajant *et al.*, 1994; Kakes, 1985; Frehner and Conn, 1987; Swain *et al.*, 1992), and have been found to vary between plants. Generally speaking, β-glucosidases with cyanogenic glucoside hydrolyzing capabilities have been found to be localized in non-cytoplasmic compartments (Thayer and Conn, 1981; Kakes, 1985; Frehner and Conn, 1987; Swain and Poulton, 1992; Gruhnert *et al.*, 1994). α-Hydroxynitrilases have not been studied extensively except for *S. bicolor* and *P. serotina* (Swain and Poulton, 1992). Early reports suggested that dhurrin accumulates in different tissues of *Sorghum* than the α-hydroxynitrilase (Kojima *et al.*, 1979; Thayer and Conn, 1981). However, subsequent investigations using immunolocalization suggested that both the substrate precursor (dhurrin) and catabolic enzyme were present in the same tissues (Wajant *et al.*, 1994). In conclusion, these data suggest that in most studied cases, plants avoid internal cyanogenesis by spatial separation between glucosides and catabolic enzymes, as a result of differential cellular and/or intracellular localization. Evidence for temporal separation between catabolic enzymes and substrates has also been presented (Swain and Poulton, 1994).

1.2.3 Biological roles of cyanogenic glucosides

Hydrogen cyanide is volatile and CN^- has a high affinity for metal groups (Hall and Rumack, 1986). The toxicity of cyanide is particularly attributed to the inhibition of metallo-proteins such as cytochrome oxidase A3, a key-enzyme of the mitochondrial respiratory pathway of most organisms (Solomonsen, 1981). Given the toxicity of the cyanogenic glucoside end-products and their method of release, it is possible that plant cyanogenesis has evolved in response to certain herbivore and pathogen pressures. Numerous ecological studies have favored this argument (Ferreira *et al.*, 1997; Woodhead and Bernays, 1977; Hruska, 1985). However, most of the evidence has been circumstantial (Narhstedt, 1985; Hruska, 1988). Cyanogenesis is widespread in nature and co-evolution of cyanogenic plants and their pests have provided ample time for the

development of pests able to metabolize cyanide into non-toxic constituents. For example, it has been documented that the cyanogenic rubber tree has reduced pathogen defense abilities towards the fungus *Microcyclus ulei* due to the presence of cyanogenic glucosides (Lieberei *et al.*, 1989). This effect has been attributed to the inhibition of *de novo* biosynthesis of the major phytoalexin scopoletin by cyanide released upon infection (Lieberei *et al.*, 1996). Similarly, a positive correlation between cyanogenic glucoside content in barley (*Hordeum vulgare*) and the sporulation efficiency of powdery mildew has been reported (Pourmohseni *et al.*, 1991; Pourmohseni *et al.*, 1993). Hence, the effect of particular natural products will most likely be different on different organisms.

Alternative "roles" for cyanogenic glucosides have been proposed. Weston *et al.* (1989) provided evidence for the allelopathic potential of cyanogenic *Sorghum* hybrids towards several weed and vegetable species. The observed effect was attributed to dhurrin and *p*-hydroxybenzaldehyde, a breakdown product of dhurrin. However, studies by Forney and Foy (1985) showed that the most phytotoxic compounds isolated from *Sorghum* rhizospheres were anthocyanins. It has also been suggested that cyanogenic glucosides may constitute mobilizable nitrogen-storage forms (Clegg *et al.*, 1979; Selmar *et al.*, 1988). In such a scenario, nitrogen would be "recycled" by breakdown of the glucoside, followed by the conversion of cyanide to asparagine by β-cyanoalanine synthase (Blumentahl-Goldschmidt *et al.*, 1963). The concept of cyanogenic glucoside mobilization is introduced and discussed further in chapter 5.

1.3 Glycosyltransferases in secondary plant metabolism

1.3.1 Introduction

Plants are capable of synthesizing several thousand different small molecular weight compounds (Wink 1999), defined as secondary plant metabolites. Part of this diversity arises from decoration with glycosyl-, carboxyl-, methyl- and hydroxyl-groups by glycosyltransferases (Vogt and Jones, 2000), acyltransferases (Fujiwara *et al.*, 1997), methyltransferases (Ibrahim *et al.*, 1998) and dioxygenases and cytochrome P450s (Bolwell *et al.*, 1994; De Carolis and De Luca, 1994; Schuler 1996), respectively. The conjugation of endogenous and exogenous organic molecules with sugars is one such important tool that appears to be employed by all plants investigated so far (Pflugmacher and Sandermann, 1998). The addition of a carbohydrate-moiety to endogenous and exogenous organic molecules has a wide range of effects including increased water solubility, improved chemical stability, reduced chemical reactivity and altered biological activity. Secondary plant metabolites are glycosylated to O (OH- and COOH-), N, S and C atoms by glycosyltransferses using nucleotide-activated sugars as donor substrates (Figure 1.4). Hydroxylated molecules are the most common acceptors, whilst UDP-glucose is the most common donor. A broad range of different carbohydrate moieties can be added employing a large range of nucleotide-sugars, independently (monoglycosides), in parallel or in chains (di-, tri-glycosides,..) (Figure 1.4). This gives rise to a broad spectrum of glycosidic structures for any given aglycone. For example, out of a total of 5,000 different flavonoids, 300 different glycosides of one single flavonol, quercetin, have already been identified (Harborne and Baxter 1999).

Individual plants accumulate a large range of glycosides. For example, berries of grapevine (*Vitis vinifera*) contain more than 200 different aglycones conjugated to glucose (Sefton *et al.* 1993; Sefton *et al.* 1994). Foreign compounds originating from other organisms and man-made chemicals, defined as xenobiotics, are also glycosylated by plants (Pflugmacher and Sandermann 1998). Overall, the complement of all glycosylating activities in any given individual plant must therefore be regarded as broad. However, the number of expressed glycosyltransferases involved in secondary plant metabolism and the substrate specificities of these enzymes remain largely unknown. Results from the *Arabidopsis* genomic sequencing project indicate that more

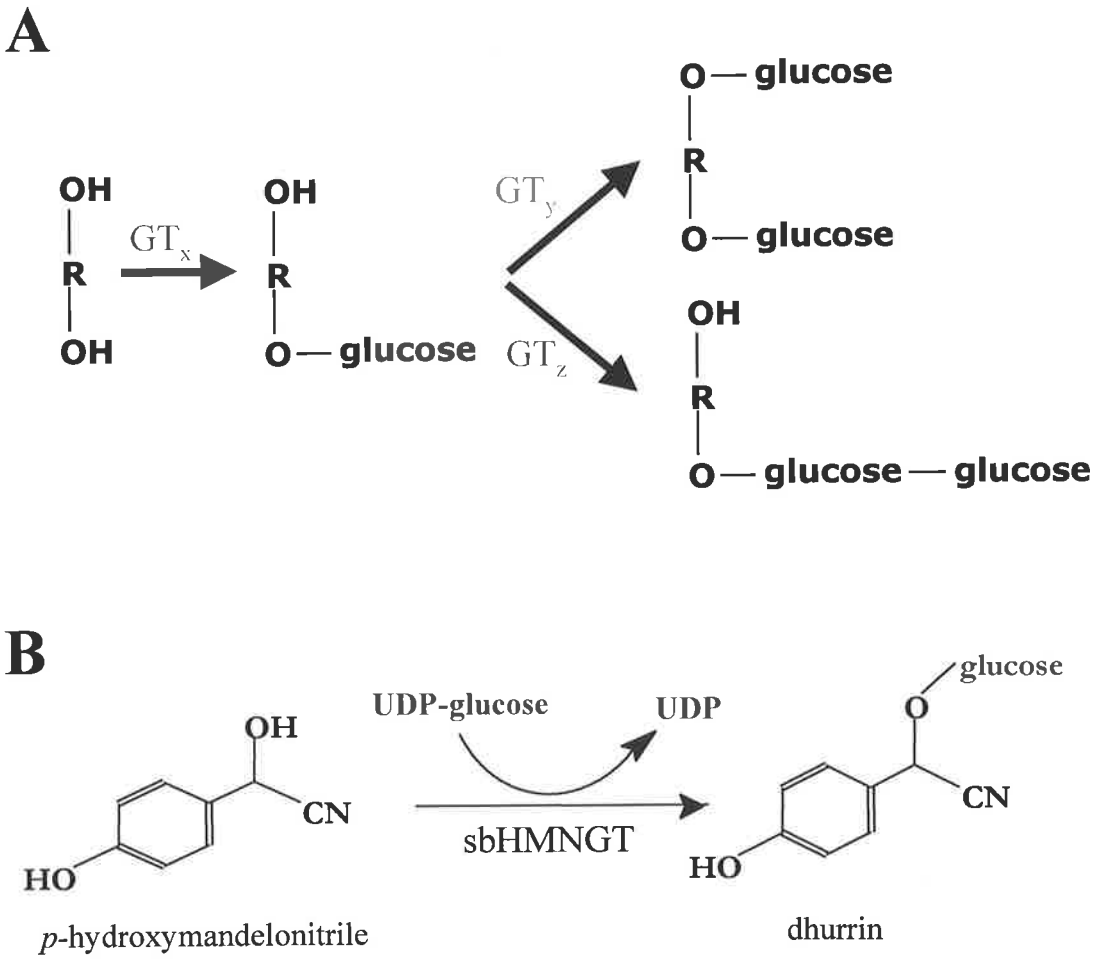


Figure 1.4 The transfer of sugars by plant secondary metabolism glycosyltransferases. (A) The formation of mono and diglycosides by glycosyltransferases (GT). GT_x catalyzes the synthesis of a monoglycoside of R (the aglycone). GT_y and GT_z, can then synthesize two forms of diglycosides. For illustrative purposes only, the glycosyl-units have been depicted as glucosyl units. (B) An example of a reaction catalyzed by a glycosyltransferase. *p*-Hydroxymandelonitrile is conjugated with glucose at the cyanohydrin-function by sbHMNGT, resulting in the formation of the cyanogenic glucoside dhurrin.

than 100 putative glycosyltransferases are present in the genome (Li *et al.*, 2000). It is possible that a proportion of these are not expressed or involved in primary metabolism rather than secondary metabolism. It is important to note, however, that the border between primary and secondary metabolism is not clearly marked and many compounds defined as secondary metabolites, like sterols or hormones play a crucial and conserved role in most, if not all plant species (Croteau *et al.*, 2000).

1.3.2 Glucosylation as a chemically modifying 'safety net'

Glycosylation converts reactive and toxic aglycones into stable and non-reactive storage forms, thereby limiting their interaction with other cellular components (Figure 1.5) (Sandermann, 1994; Kreuz *et al.*, 1996). Sugars are highly polar and the addition of carbohydrate moieties to hydrophobic substrates will serve to increase the water solubility of the resultant glycoside. Thus, the main chemical roles for glycosylation are (1) stabilization, (2) detoxification (reduced reactivity), and (3) solubilisation (increased polarity). Combined, glycosylation enables plants to store large amounts of otherwise toxic constituents (Halkier *et al.*, 1989; Sandermann 1994; Kreuz *et al.*, 1996; Moehs *et al.*, 1997).

Molecules derived from exogenous and endogenous sources are glycosylated by plants. Endogenous aglycones can originate from both biosynthetic as well as degradative or turnover metabolism (Sandermann, 1994; Bak *et al.*, 1999; Walter *et al.*, 2000). Plants have a glycosylating capacity for most endogenous compounds that have exerted selective pressure in the evolution of glycosyltransferase substrate specificity. However, plants also have the capacity to glycosylate novel compounds which have not exerted any prior evolutionary selective pressure, derived either from exogenous (Sandermann 1994; Pflugmacher and Sandermann, 1998; Lamoureux *et al.*, 1991; Leah *et al.*, 1992; Tanaka *et al.*, 1990) or endogenous sources (Li *et al.*, 1997b; Bak *et al.*, 1999).Anthocyanidins (anthocyanin aglycones), cyanohydrins (cyanogenic glucoside aglycones) and thiohydroximates (glucosinolate aglycones) are all examples of anabolic aglycones, which do not normally accumulate in plants. The thiohydroximates (Halkier and Du 1997) and the cyanohydrins (Møller and Seigler, 1998) will without conjugation break down to smaller substituents. Glycosylation stabilizes these molecules and thereby preserves their structural integrity to allow their accumulation. The

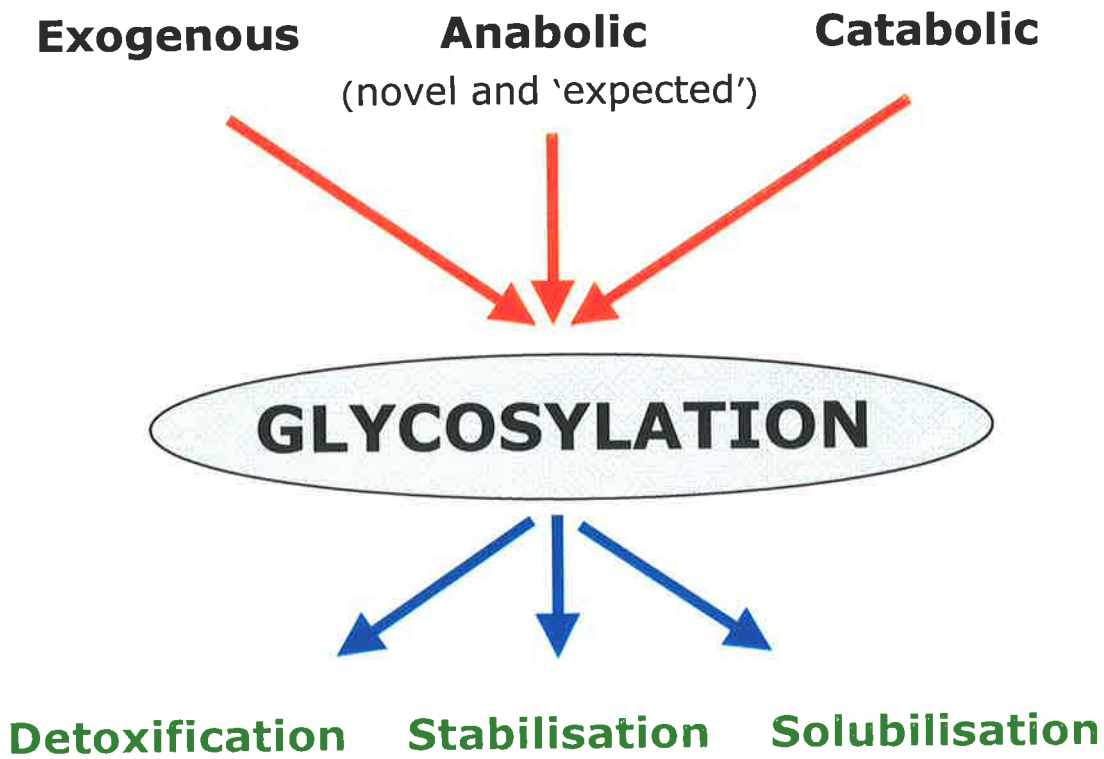


Figure 1.5 Graphic illustration of glycosylation as a chemically modifying 'safety net'. Aglycones commonly originate from anabolic, catabolic or exogenous sources. Plants can glycosylate aglycones that they have previously been exposed to as well as certain novel compounds that they have never been exposed to. The roles of glycosylation as a chemically modifying 'safety net' include detoxification, stabilisation and solubilisation (Jones and Vogt, 2000).

anthocyanidins on the other hand will autocatalytically convert to colourless flavylium cations at either vacuolar or cytosolic pH (Brouillard 1982; Goto 1987). Studies with grapevine mutants void of berry anthocyanins have shown that the absence of vvUFGT (anthocyanidin-glucosyltransferase; Ford *et al.*, 1998) results in anthocyanin-less berries (Boss *et al.*, 1996). Hence, glycosylation at the C-3, C-5 or C-7 position of anthocyanidins will inhibit autoconversion into colourless forms, which are substrates for further catabolism (Calderon *et al.*, 1992). The position of the glucosyl-moeity is also important, and this is highlighted by the greater stability of 2-*O*-glucosides of L-ascorbic acid and dhurrin, compared with 6-*O*-glucosides of L-ascorbic acid and gluco-*p*-hydroxymandelonitrile, respectively (Yamamoto *et al.*, 1990; Abrol *et al.*, 1966). *In vivo,* the catabolism of cyanohydrins, flavonols and anthocyanidins, but not of their corresponding glucosides, is facilitated by catabolic enzymes (Wajant *et al.*, 1994; Calderon *et al.*, 1992, Morales *et al.,* 1993). The apparent improved stability of glycosides may therefore in some cases be more related to their lack of acceptance by catabolic enzymes rather than inherent aglycone instability. The reduced toxicity of certain glycosides compared to their respective aglycones is illustrated by the potato alkaloid solanidine. Moehs *et al.* (1997) utilized the differential toxicity of solanidine and solanidine-glucoside to isolate a cDNA encoding for potato solanidine-glucosyltransferase. A positive selection system was developed in which a yeast expression library, carrying a potato cDNA library, was streaked onto a solanidine-containing medium. Only those yeasts cells that were carrying the solanidine-glucosyltransferase-encoding cDNA were able to grow in the presence of solanidine.

1.3.3 Biochemical features of glycosyltransferases

Early studies into the enzymatic properties of plant natural product glycosyltransferases were carried out using enzyme preparations purified from native plant material (Hösel, 1981). The complete isolation of glycosyltransferases has often proved to be a difficult task due to the minute amounts and labile nature of plant glycosyltransferases (Bar-Peled *et al.*, 1991; Reed *et al.*, 1993; Guo and Poulton, 1994; Vogt *et al.*, 1997). Despite this, several successful purification protocols have been developed for a range of glycosyltransferases (Kowalczyk and Bandurski, 1991; Bar-Peled *et al.*, 1991; Reed *et al.*, 1993; Hasegawa *et al.*, 1997; Li *et al.*, 1997a; Kuai *et al.*, 1997; Vogt *et al.*, 1997; Lee and Raskin, 1999; Miller *et al.*, 1999; Kita *et al.*, 2000). Purification has commonly

been achieved by a combination of ion exchange and affinity chromatography. Of the latter, "pseudo" affinity dye ligand chromatography has been employed as a particularly successful key step in combination with substrate elution (Bar-Peled *et al.*, 1991; Li *et al.*, 1997a; Kuai *et al.*, 1997; Vogt *et al.*, 1997; Lee and Raskin, 1999). These studies have shown that glycosyltransferases involved in secondary plant metabolism typically are monomeric enzymes with a molecular mass between 45 kDa and 60 kDa *in vitro*. They exhibit neutral to weakly basic pH-optima (pH 7.5 - 9.0), have isoelectric points in the range of 4.5 - 5.5, do not require additional co-factors such as metal ions, and are prone to a loss of activity in the absence of reductive conditions. In contrast to mammalian glucuronosyltransferases (Meech and Mackenzie 1997), most plant glycosyltransferases are soluble and are therefore thought to be cytosolic, with only few exceptions (Leah *et al.*, 1982; Warnecke and Heinz, 1994; Vogt and Taylor, 1995).

The first sequence of a gene encoding a glycosyltransferase involved in the synthesis of plant natural products was that of the maize (*Zea mays*) *Bronze-1* locus. This locus encodes a glycosyltransferase thought to be involved in the conjugation of anthocyanidins and flavonols *in planta* (Fedoroff *et al.*, 1984; Ford *et al.*, 1998). Several hundred different known and putative plant glycosyltransferase-encoding gene and cDNA sequences have since then become available in international data banks. Over one hundred of these sequences have been derived from the *Arabidopsis* genomic sequencing project (Li *et al.*, 2000). Known and putative plant secondary metabolism glycosyltransferases in general exhibit a very low degree of overall similarity, with the exception of the C-terminal region which contains a postulated UDP-sugar binding motif, defined as a **p**lant **s**econdary **p**roduct **g**lycosyltransferase sequence motif (**pspg**-motif, Hughes and Hughes, 1994, Figure 1.6). Based on this observation the C-terminal region is thought to encode the UDP-sugar binding domain, whereas the N-terminal end of the protein may be responsible for binding the divergent and structurally dissimilar substrate aglycones (Ritter *et al.*, 1992; Hughes and Hughes, 1994). Part of the PSPG-motif is also conserved in some inverting (β) glycosyltransferases from non-plant eukaryotes, prokaryotes and archaebacteria (Kapitonov and Yu, 1999), , although absent from many other inverting (β) and non-inverting (α) glycosyltransferases (Breton *et al.*, 1998; Imberty *et al.*, 1999; Stasinopoulos *et al.*, 1999). At the start of this study (July, 1997), the products of only three glycosyltransferase-encoding genes had been

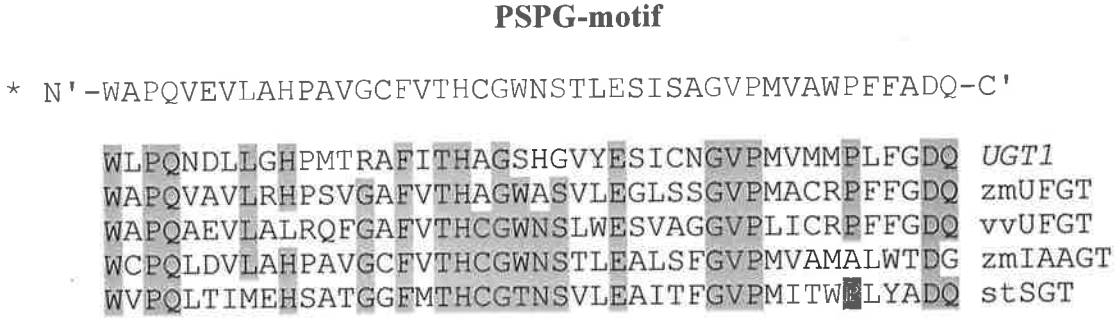


Figure 1.6 The PSPG-motif consensus sequence of plant secondary metabolite glycosyltransferases. The PSPG-consensus sequence (*) (Hughes and Hughes, 1994). Below the PSPG-motif are relevant segments of five glycosyltransferase-encoding gene sequences. These include four plant glucosyltransferases; zmUFGT (*Z. mays* flavonoid-glucosyltransferase, *Bz-Mc-2 allele*: X13501, vvUFGT (*V. vinifera* anthocyanidin glucosyltransferase: AF000371), zmIAAGT (*Zea mays* indole acetic acid glucosyltransferase: L43847), stSGT (*Solanum tuberosum* solanidine-glucosyltransferase: U82367), and an exon which encodes the carboxy-terminal end of six xenobiotic glucuronosyltransferases of *Homo sapiens* (*UGT1*; Ritter *et al.*, 1992). Residues that share identity with the PSPG-motif sequence are highlighted in grey. Highly conserved amino acids in the PSPG-motif are highlighted in red. N' = N-terminal end. C' = C-terminal end.

functionally verified by heterologous expression. These gene products were an indole acetic acid glucosyltransferase from maize (Szerszen *et al.*, 1994), an anthocyanidin-glucosyltransferase from *Gentiana triflora* (Tanaka *et al.*, 1996) and a solanidine-glucosyltransferase from potato (Moehs *et al.*, 1997). Since then, a larger range of glycosyltransferase-encoding cDNAs have been functionally expressed (Table 1.1a) (Gong *et al.*, 1997; Warnecke *et al.*, 1997; Ford *et al.*, 1998; Fraissinet-Tachet *et al.*, 1998; Lee and Raskin, 1999; Martin *et al.*, 1999; Miller *et al.*, 1999; Vogt *et al.*, 1999; Yamazaki *et al.*, 1999; Hirotani *et al.*, 2000; Jackson *et al.*, 2000; Lim *et al.*, 2000; Kita *et al.*, 2000). With the availability of isolated recombinant glycosyltransferases it has become possible to investigate the *in vitro* substrate specificity of the enzymes.

1.3.3.1 The substrate specificity of glycosyltransferases

Is there one glycosyltransferase which glycosylates all aglycones or one glycosyltransferase for each and every substrate? The answer is most likely somewhere in between, although we cannot exclude that there may be a great variation in the range of substrates that different glycosyltransferases are able to accept. It has been suggested that glycosyltransferases are highly regiospecific (or selective) rather than substrate-specific (Vogt *et al.*, 1997). In other words, acceptance is strictly based on a certain substructure, with little regard for the entire structure of the acceptor molecule. This is illustrated by the acceptance, by several flavonoid-3-*O*- and flavonoid-5-*O*-glucosyltransferases, of flavonoids with differently decorated A- and B-rings (Vogt *et al.*, 1997; Ford *et al.*, 1998; Miller *et al.*, 1999; Vogt *et al.*, 1999; Yamazaki *et al.*, 1999). However, there are exceptions to this rule. For example, Marcinek *et al.* (2000) purified an indoxyl-glycosyltransferase 863-fold from *Baphicacanthus cusia* and found that it accepted 4-OH, 5-OH, 6-OH and 7-OH-indole. Similarly, two carboxyl-glucosyltransferases have been identified, which also glycosylate other hydroxyl-groups, that are not part of the carboxyl-groups, of the carboxyl-carrying substrates (Fraissinet-Tachet *et al.*, 1998; Lee and Raskin, 1999). The availability of *in vitro* substrate specificity profiles for five different isolated recombinant glycosyltransferases allows us to make some preliminary conclusions regarding the substrate specificity of glycosyltransferases in general (Table 1.1b). The anthocyanidin-3-*O*-glycosyltransferase from grapevine and flavonol-3-*O*-glucosyltransferase from *Petunia* both exhibit exclusive specificity for their putative *in vivo* substrates, flavonoids and

Table 1.1 Recombinant secondary plant product glycosyltransferases and the substrate specificity profiles of five recombinant glycosyltransferases. (A) A list of the believed main acceptor substrate of glycosyltransferases expressed in heterologous hosts, and the native plant source of the glycosyltransferase-encoding sequences. (B) The substrate specificity of five recombinant glycosyltransferases. The profiles display relative V_{max}-values of a range of tested substrates compared with the preferred substrate of each glycosyltransferase (100%) as determined in order by the following papers: Ford *et al.* (1998), Miller *et al.* (1999), Vogt *et al.* (1999), Fraissinet-Tachet *et al.* (1998) and Lee and Raskin (1999). The substrates are: (1) cyanidin, (2) delphinidin, (3) peonidin, (4) pelargonidin, (5) malvidin, (6) quercetin, (7) kaempferol, (8) myricetin, (9) fisetin, (10) galangin, (11) iso-rhamnetin, (12) rhamnetin, (13) betanidin, (14) luteolin, (15) esculetin, (16) scopoletin, (17) *p*-coumaric acid, (18) caffeic acid, (19) *O*-coumaric acid, (20) *p*-hydroxybenzoic acid, (21) cinnamic acid, (22) umbelliferone, (23) coniferyl alcohol, (24) ferulic acid, (25) *m*-hydroxybenzoic acid, (26) benozoic acid, (27) salicylic acid. Note that ntGT2 accepted all 15 of 15 tested substrates, however, only the ten most prefferable substrates were included in the figure.

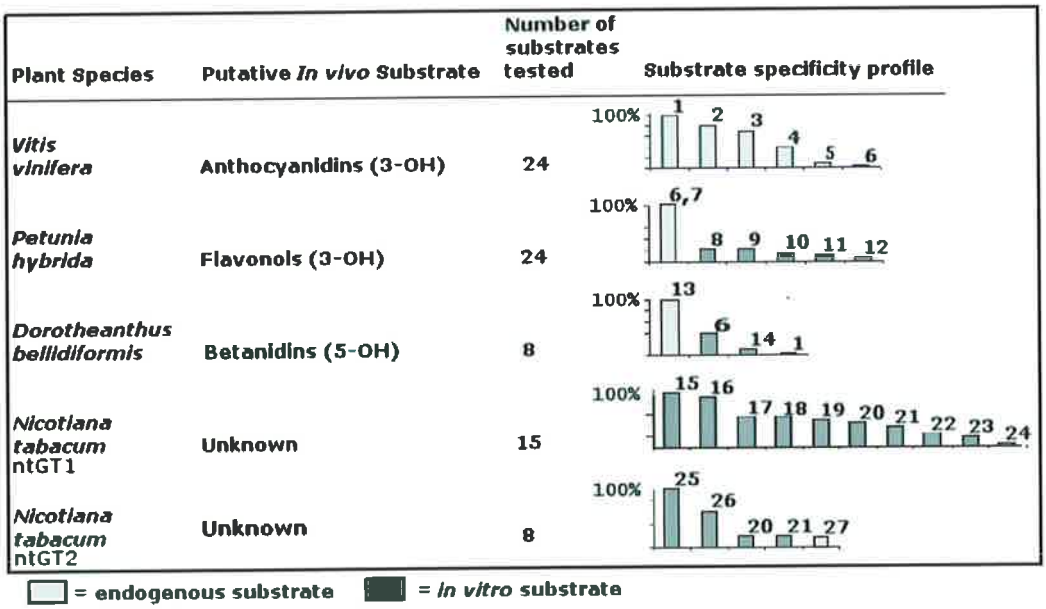
A

Recombinant natural product glycosyltransferases

Main acceptor substrate	Native plant	Reference
Anthocyanidins	*Gentiana triflora*	Tanaka *et al.* (1996)
Anthocyanidins	*Perilla frutescen*	Gong *et al.* (1997)
Anthocyanidins	*Vitis vinifera*	Ford *et al.* (1998)
Anthocyanins	*Perilla frutescens*	Yamazaki *et al.* (1999)
Flavonols	*Petunia hybrida*	Miller *et al.* (1999)
Flavonols	*Scutellaria baicalensis*	Hirotani *et al.* (2000)
Betanidins	*Dorotheanthus bellidiformis*	Vogt *et al.* (1999)
Phenylpropanoids?	*Nicotiana tabacum*	Fraissinet-T. *et al.* (1998)
Phenylpropanoids?	*Nicotiana tabacum*	Lee and Raskin (1999)
Phenylpropanoids?*	*Arabidopsis thaliana*	Lim *et al.* (2000)
Steroidal glycoalkaloids	*Solanum tuberosum*	Moehs *et al.* (1997)
Sterols	*Avena sativa*	Warnecke *et al.* (1997)
Limonoids	*Citrus unshiu*	Kita *et al.* (2000)
Indole acetic acid	*Zea mays*	Szerszen *et al.* (1994)
Indole acetic acid	*Arabidopsis thaliana*	Jackson *et al.* (2000)
Zeatin	*Phaseolus lunatus*	Martin *et al.* (1999)

* Five glycosyltransferases with activities towards various phenylpropanoids were described.

B



flavonols, respectively (Ford *et al.*, 1998). The glycosyltransferase from *Dorotheanthus*, however, also accepts substrates which are not thought to be conjugated *in vivo*, esculetin and luteolin, although at lower maximal rates compared with the believed endogenous substrate, betanidin (Vogt *et al.*, 1999). Unfortunately, it is not clear what the native substrate of the salicylic acid (SA)-inducible ntGT1 (profile 4, Table 1.1b; Fraissinet-Tachet *et al.*, 1998) or the second SA-inducible ntGT2 (profile 5, Table 1.1b; Lee and Raskin 1999) are. The glucosyltransferase-encoding cDNAs were both isolated from *N. tabacum* and the encoded glycosyltransferases glucosylate both the hydroxyl- and carboxyl-groups of SA, as well as a range of other compounds, suggesting a rather broad substrate specificity. Interestingly, SA is only weakly accepted by either of the two enzymes and their *in vivo* substrates may therefore be compounds other than salicylic acid. According to these substrate specificity profiles we can group these five glycosyltransferases into broad (*N. tabacum*: Fraissinet-Tachet *et al.*, 1998; Lee and Raskin 1999), intermediate (*Dorotheanthus*: Vogt *et al.*, 1999) and narrow (*Vitis*: Ford *et al.*, 1998 and *Petunia*: Miller *et al.*, 1999) substrate specificity. However, a more exhaustive range of glycosyltransferases and substrates needs to be tested in order to confirm this preliminary classification. The range of substrates that any glycosyltransferase is exposed to *in vivo* may also be restricted. The *in vivo* substrate specificity of any enzyme may therefore differ from the reported *in vitro* substrate specificity. Two mechanisms for such a restriction are transcriptional control (1.3.3.2) and the co-localization of glycosyltransferases with other enzymes of natural product biosynthetic pathways (1.3.3.3).

1.3.3.2 Transcriptional control of glycosyltransferase synthesis

There are several ways in which plants can regulate the temporal and spatial distribution of plant glycosylation. For example, enzymes involved in xenobiotic metabolism in the liver of mammals, including glucuronosyltransferases, are known to be induced by xenobiotic agents (Tephly and Burchell 1990). A possible analogy to this may be found in plants, where several enzymes thought to be involved in xenobiotic metabolism are known to be inducible (Farago *et al.*, 1994; Robineau *et al.*, 1998). Several putative and verified glycosyltransferase-encoding genes have been found to be induced by methyl jasmonate (Imanishi *et al.*, 1998), SA (Horvath and Chua, 1996; Fraissinet-Tachet *et al.* 1998; Lee and Raskin, 1999) and wounding (O'Donnell *et al.*, 1998). The induction was

both rapid and transient in several of these cases (Horvath and Chua 1996; O'Donnell *et al.*, 1998; Fraissinet-Tachet *et al.*, 1998; Lee and Raskin, 1999). Several plants show differential glycosylation patterns depending on whether they are grown in light or dark (Leah *et al.*, 1992; Bandurski *et al.*, 1977) and it has been demonstrated that the glycosyltransferase-encoding mRNA levels also are modulated by light conditions (Gong *et al.*, 1997; Yamazaki *et al.*, 1999). Glycosyltransferases are also known to be developmentally regulated (Sparvoli *et al.*, 1994; Boss *et al.*, 1996; Miller *et al.*, 1999; Woo *et al.*, 1999; Kita *et al.*, 2000) and restricted in tissue localization (Schmid *et al.*, 1982; Vogt and Taylor 1995; Boss *et al.*, 1996; Gong *et al.*, 1997; Ford and Høj 1998; Martin *et al.*, 1999; Miller *et al.*, 1999).

1.3.3.3 The co-localization of glycosyltransferases with secondary metabolic pathways

Organisms apparently contain non-covalent complexes of enzymes ("metabolons") involved in the same metabolic pathways. This has been observed for both primary and secondary metabolic pathways, *e.g.* the phenylpropanoid pathway (Srere, 1987; Winkel-Shirley, 1999). Several metabolic pathways with labile aglycones, such as the biosynthetic pathways of cyanogenic glucosides, glucosinolates and anthocyanins, may benefit from a co-localization of glycosyltransferases with upstream enzymes. Clustering of glycosyltransferases together with cytochrome P450s in the metabolism of xenobiotic substances may also be beneficial, particularly in circumstances where the hydroxylated intermediates are more toxic than their precursors (Plewa and Wagner 1993). The notion of metabolon-complex formation has been supported by experiments which demonstrate that certain intermediates of several biosynthetic pathways are channelled, *eg.* do not readily exchange with exogenously added intermediates (Møller and Conn, 1980; Winkel-Shirley, 1999). It has recently been verified that a number of soluble and microsomal enzymes of the phenylpropanoid biosynthetic pathway interact physically *in vivo* and *in vitro* (Rasmussen and Dixon, 1999; Burbulis and Winkel-Shirley, 1999). However, to date there are no reports that substantiates the involvement of a plant natural product glycosyltransferase in a "metabolon"-complex. As an alternative to metabolon-complexes, certain enzymes or pathways may be localized to specialized compartments such as vesicles, which could also explain the observed chanelling of intermediates.

Many secondary plant metabolism biosynthetic pathways involve one or more membrane-bound enzymes, such as cytochrome P450s (Hrazdina and Jensen, 1992; Winkel-Shirley, 1999). If soluble glycosyltransferases of such pathways are physically associated with such upstream enzymes *in vivo*, then we can expect that glycosyltransferases reside in close proximity to membranes such as the endoplasmic reticulum or other specialized compartments such as vesicles. Unfortunately, the intracellular localization of glycosyltransferases has not been investigated thoroughly and opinions vary. Two studies made with cellular fractionation have found glycosyltransferase activities to be cytosolic (Blume *et al.*, 1979; Yazaki *et al.*, 1995). A third study by Anhalt and Weissenböck (1992) found that mono- and di-glycosyltransferase activities of luteolin were cytosolic, whilst a third tri-glycosyltransferase was found to be vacuolar. As mentioned above, a portion of the *p*-hydroxymandelonitrile-glucosyltransferase activity of *S. bicolor* (>41%) was found to be associated with a plastid fraction (Wurtele *et al.*, 1982). Immunolocalization has been employed in two instances. Schmid *et al.* (1982) showed a cytosolic localization of a coniferyl-glycosyltransferase in spruce seedlings, whilst Latchinian-Sadek and Ibrahim (1991) presented evidence for a vesicle-like and cytoplasmic membrane localization of a flavonol B-ring specific glycosyltransferase in *Chrysoplenium americanum*. Ibrahim (1992) subsequently suggested that these results were indicative of a very loose association between this glycosyltransferase and the endoplasmic reticulum of *C. americanum*.

It has been suggested earlier that intermediates of the cyanogenic glucoside biosynthetic pathway are highly channelled in microsomal preparations of *S. bicolor* (Møller and Conn, 1980) and *Triglochin maritima* (Cutler *et al.*, 1981), except for the intermediates *p*-hydroxyphenylacetaldoxime and *p*-hydroxymandelonitrile. This channelling may now be explained by the fact that both CYP79A1 and CYP71E1 are multi-functional enzymes that retain their intermediates until fully metabolized. Nevertheless, it is still pertinent to ask if the entire pathway is channelled *in vivo* since α-hydroxynitrilases which catalyze the breakdown of the aglycone precursor of dhurrin, *p*-hydroxymandelonitrile, are present in the cytosol of biosynthetic tissue in *Sorghum* (Wajant *et al.*, 1994). Furthermore, freely available *p*-hydroxymandelonitrile forms a chemical equilibrium in solution with it's degradation products, CN^- and *p*-

hydroxybenzaldehyde, at cellular pH (Reay and Conn, 1974). Since freely available *p*-hydroxybenzaldehyde can be glucosylated or metabolized to *p*-hydroxybenzoic acid *in planta* (Reay and Conn, 1974; Bak *et al.*, 2000), one would therefore expect that the biosynthesis of dhurrin would generate appreciable amounts of *p*-hydroxybenzaldehyde metabolites and cyanide in the absence of a physical association between sbHMNGT and CYP71E1 (illustrated in Figure 1.7). Nevertheless, the relative efficiency of dhurrin synthesis in *S. bicolor* remains unknown, since the accumulation of tyrosine-derived *p*-hydroxybenzaldehyde metabolites in *S. bicolor* has not been investigated to date. However, attempts by Møller and Conn (1980) to cross-link the glucosyltransferase with the microsomal component in an active state proved unsuccessful.

1.3.4 Glucosyltransferases for cyanogenic glucosides

Knowledge about glucosyltransferases involved in the biosynthesis of cyanogenic glucosides was at the start of this study restricted to the partial purification and characterization of crude enzyme preparations from *Linum usitatissimum* (Hahlbrock and Conn, 1970), *S. bicolor* (Reay and Conn, 1974), *T. maritima* (Hösel and Nahrstedt, 1980), *P. serotina* (Poulton and Shin, 1983) and *M. esculenta* (Mederacke *et al.*, 1995; Mederacke *et al.*, 1996). Evidence for more than one cyanohydrin-glucosyltransferase was presented by Mederacke *et al.* (1996). All of the described glucosyltransferase preparations were soluble and utilized UDP-glucose as the donor substrate. Interestingly, all enzyme preparations were specific for only one stereoisomer when tested with a racemic mixture of cyanohydrins, except for the enzyme preparation from *L. usitatissimum* (Hahlbrock and Conn, 1970). However, only the *R*-form of lotaustralin accumulates in this plant, suggesting that the glucosyltransferase(s) only is(are) presented with the *R*-form of the cyanohydrin, or that only one *R*-specific glucosyltransferase has access to the cyanohydrin substrate (Zilg and Conn, 1974). Since freely available (*R*)-2-hydroxy-2-methylbutyronitrile will racemize at cytosolic pH (Tapper and Butler, 1971), this supports a putative co-localization of the cyanohydrin-glucosyltransferase(s) with the microsomal cyanohydrin-synthesizing component of *L. usitatissimum*. Hughes and Hughes (1994) screened a cassava seedling cotyledon cDNA library using an anthocyanidin-glucosyltransferase-encoding cDNA from *A. majus* (Martin *et al.*, 1991; Schwinn *et al.*, 1997) as a probe, resulting in the isolation of six different putative glycosyltransferase-encoding cDNAs. No reports of

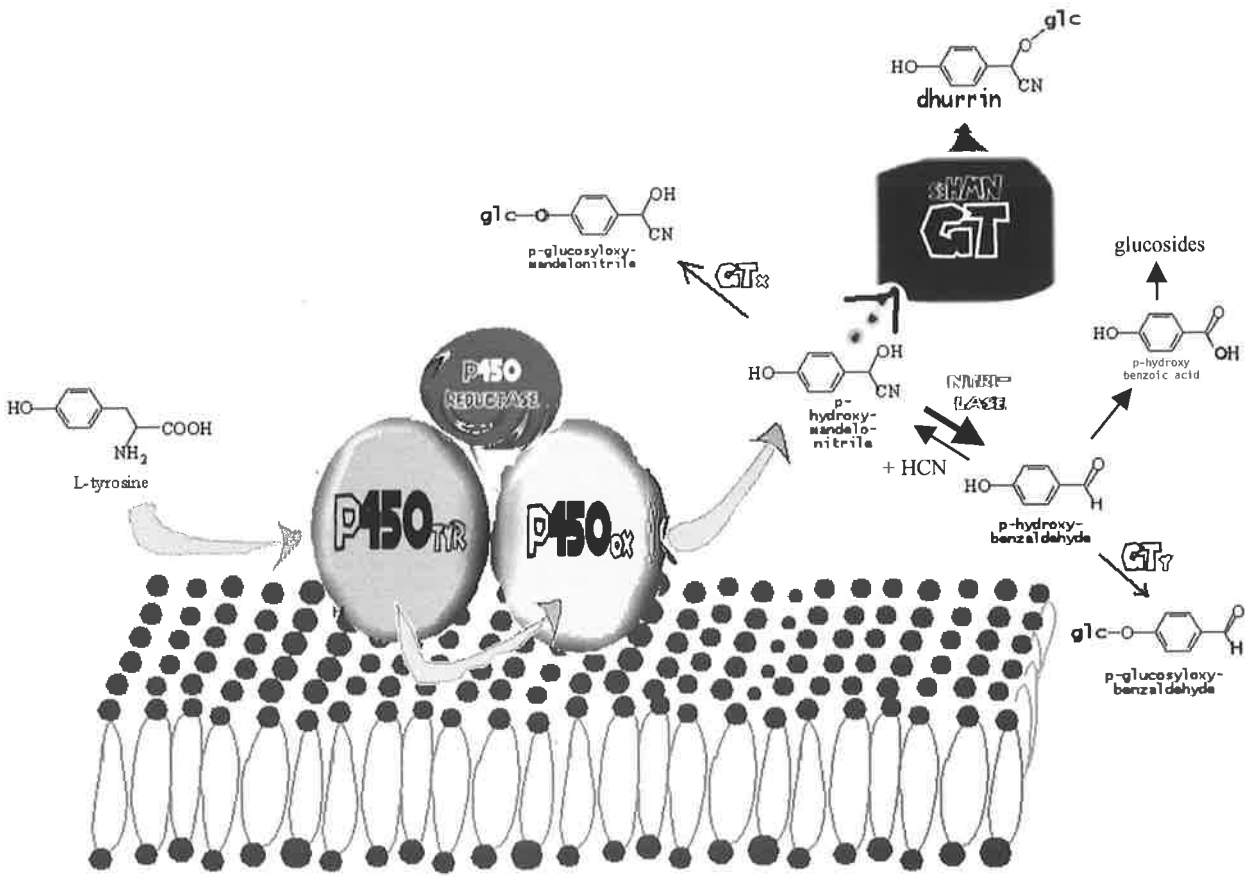


Figure 1.7 A graphical illustration of the possible fate of *p*-hydroxymandelonitrile during the biosynthesis of dhurrin in *S. bicolor*. The biosynthesis of dhurrin in *S. bicolor* is catalyzed by three enzymes. Microsomal cytochrome P450s convert tyrosine into *p*-hydroxymandelonitrile, which subsequently is glycosylated by sbHMNGT, yielding dhurrin (Møller and Seigler, 1998). *p*-Hydroxymandelonitrile may potentially be converted into other end-products if sbHMNGT is not co-located or compartmentalized together with CYP71E1. For example, *p*-Hydroxymandelonitrile may be broken down to *p*-hydroxybenzaldehyde, autocatalytically or catalyzed by α-hydroxynitrilase (Reay and Conn, 1974; Wajant and Mundry, 1993). *p*-Hydroxybenzaldehyde can be metabolized by glycosyltransferases (GT_y; Reay and Conn, 1974), or converted to *p*-hydroxybenzoic acid, followed by glycosylation (GT_y; Bak *et al.*, 2000). *p*-Hydroxymandelonitrile can also be glycosylated at the aromatic ring (GT_x; Reay and Conn, 1974).

attempts to identify these sequences through transformation studies or functional expression in a heterologous organism have appeared to date. However, it is possible that one of these cDNAs encodes an acetone cyanohydrin glucosyltransferase as a tissue actively synthesizing cyanogenic glucosides was chosen for cDNA library construction.

Whilst cyanogenic diglucosides have been reported from several plants (Selmar *et al.*, 1996; Lechtenberg and Nahrstedt, 1999), to date, there are no reports which describe the existence of a cyanogenic glucoside glucosyltransferase. Poulton and Shin (1983) attempted, but were unable to detect cyanogenic di-glucoside forming activities in the amygdalin(di-glucoside)-accumulating plant, *P. serotina*.

Thus, in conclusion, at the start of this study, knowledge about glucosyltransferases involved in the biosynthesis of cyanogenic glucosides was strictly limited to biochemical experiments performed with non-homogenous protein preparations.

1.4 Aims of this study

Cyanogenic glucosides constitute an important class of compounds that is widely distributed throught the biological kingdom. Whilst glycosylation constitutes an integral part of their biosynthesis, no glycosyltransferase with specificity for cyanogenic glucoside precursors had been characterized in molecular terms at the start of this study. The primary aim of this study was therefore to isolate and characterize sbHMNGT (Chapter 2 and 3), and utilize information gained from amino acid sequencing in order to isolated the corresponding cDNA. Isolation of sbHMNGT, either native or recombinant, will then allow sbHMNGT-specific antibodies to be generated. Together, these probes will constitute valuable "tools" that will greatly facilitate further studies into cyanogenic glucoside biology, including topics such as cyanogenic glucoside biosynthetic pathways in other cyanogenic plants, the cellular and intracellular localization of the cyanogenic glucoside biosynthetic pathway, and the ecological significance of cyanogenic glucosides.

A second aim of this study was to increase our current knowledge about plant secondary metabolism glycosyltransferases in general. Specifically, the substrate specificity of isolated recombinant sbHMNGT would be characterized (Chapter 3). At the start of this study, this had only been done comprehensively for one other isolated recombinant glucosyltransferase, vvUFGT (Ford *et al.*, 1998).

A third aim was to test the effect of glucosylation of a secondary metabolite *in planta*. This aim was facilitated by the prior generation of transgenic *Arabidopsis thaliana* functionally expressing the two cytochrome P450s of the biosynthetic pathway. With the availability of *sbHMNGT* it was therefore of interest to investigate if the entire pathway could be transferred to an acyanogenic plant, ie. wild-type *A. thaliana* (Chapter 4). Such plants will provide a platform for studies into the consequences of glucosylation on dhurrin accumulation and stability, as well as future studies on the ecological significance of cyanogenic glucosides.

Lastly, there are still no reports in the literature that describe cyanogenic di-glycosyltransferases. It has been suggested that cyanogenic di-glycosides may constitute a mobilizable transport form (Selmar *et al.*, 1988). An initial attempt to

investigate if such glycosyltransferases are present in *S. bicolor* was therefore initiated at the end of this study (Chapter 5).

Chapter 2

The Isolation of a UDP-glucose: *p*-hydroxymandelonitrile-*O*-β-glucosyltransferase from *Sorghum bicolor*

2.1 Introduction

The biosynthetic pathway of the cyanogenic glucoside dhurrin has been established in etiolated seedlings of *S. bicolor* (Figure 1.1), and is catalyzed by two membrane-bound multi-functional cytochrome P450s and a soluble glucosyltransferase. The two first enzymes of the pathway have been isolated (Sibbesen *et al.*, 1994; Kahn *et al.*, 1997), their corresponding cDNAs isolated (Koch *et al.*, 1995; Bak *et al.*, 1998) and their function verified by heterologous expression in *E. coli* and isolation of the recombinant enzyme (Halkier *et al.*, 1995; Bak *et al.*, 1998). Glucosyltransferases acting towards cyanohydrin acceptor substrates have been partially purified and characterized from several cyanogenic plants (Hahlbrock and Conn, 1970; Reay and Conn, 1974; Hösel and Schiel, 1984; Mederacke *et al*, 1995). Hughes and Hughes (1994) have isolated five putative glycosyltransferase-encoding cDNA clones from cassava using a putative anthocyanidin-glucosyltransferase-encoding cDNA from *Antirrhinum majus* as a probe. However, none of the proteins encoded by these clones where ever characterized. Thus, to date there are no reports of the isolation of a cyanohydrin glucosyltransferase from a cyanogenic plant.

Given the lability of *p*-hydroxymandelonitrile, the massive accumulation of dhurrin (Halkier and Møller, 1989) and the absence of multiple *p*-hydroxymandelonitrile-glucosyltransferase activities in *S. bicolor* (Reay and Conn, 1974), it is most likely that a specific glucosyltransferase is responsible for the synthesis of the cyanogenic glucoside dhurrin, similar to that proposed for anthocyanin accumulation (Boss *et al.*, 1996; Ford *et al.*, 1998). The presence of this specific glucosyltransferase may therefore constitute an obligate requirement for the biosynthesis of cyanogenic glucosides in *S. bicolor*, since other broad substrate specificity glycosyltransferases may not accept *p*-hydroxymandelonitrile to a significant degree. Although other plant species may contain *p*-hydroxymandelonitrile glycosylating enzymes, a successful transfer of the complete biosynthetic pathway to another plant will most likely require that all three enzymes can be expressed simultaneously.

This chapter describes a procedure for the isolation of a *p*-hydroxymandelonitrile-glucosyltransferase from *S. bicolor*. The isolation of this enzyme is essential for the production of tools (cDNAs and antibodies) that will enable in depth studies of the

biology of cyanogenic glucoside biosynthesis to take place, including a detailed characterization of the substrate specificity of the isolated enzyme.

2.2 Materials and methods

2.2.1 Biochemicals, reagents and plant material

All biochemicals were of analytical or higher grade. Substrates and authentic glucosides were obtained from Sigma (St. Louis, MO, USA) and Extrasynthèse (France). Cibacron blue 3G was obtained from Amersham Pharmacia Biotech (Sweden) and all other dye reagents and UDP-glucuronic acid cross linked with beaded agarose were obtained from Sigma (USA). *S. bicolor* seeds were obtained from Pacific Seeds, Australia (cultivar MR31) and from Agripro, Shawnee Mission, USA (cultivar SS1000).

2.2.2 Enzyme assays

General reaction mixtures (total volume 20 µl) included 100 mM Tris·HCl (pH 7.9), 1-5 µM ^{14}C-UDP-glucose (11.0 GBq/mmol, Amersham Pharmacia Biotech, Sweden), 0-200 µM UDP-glucose, 20 mM aglycone dissolved in water, 25 mM γ-gluconolactone (β-glucosidase inhibitor) and 0.5 - 10 µl of protein preparation. At the end of the incubation period (10 min, 30 °C), 2 µl of 10% (v/v) acetic acid were added to terminate the reaction. BSA (1 mg/ml) was included in assays for the assessment of sbHMNGT yield throughout the purification procedure. For TLC, the reaction mixture was applied to Silica Gel 60 F254 plates (Merck, Darmstadt, Germany), dried and developed in a solvent containing ethyl acetate: acetone: dichloromethane: methanol: H_2O (40:30:12:10:8, v/v) for 1 hr. Plates were dried (1 hr, room temperature) and exposed to storage phosphor imaging screens (Molecular Dynamics, Sunnyvale, CA, USA) prior to scanning on a Storm 860 PhosphorImager (Molecular Dynamics, USA). Radioactive products were quantified using the volume function of the ImageQuaNT software (Molecular Dynamics, USA).

For LSC, reaction mixtures were extracted with 400 µl of ethyl acetate to separate glucosides from unincorporated ^{14}C-UDP-glucose. Two ml of Ecoscint A (National Diagnostics, New Jersey, USA) were added to 250 µl of each ethyl acetate extract and analyzed using a liquid scintillation counter. Mandelonitrile was used as substrate to assay fractions generated by liquid chromatography, whilst *p*-hydroxymandelonitrile was employed for assays analyzed by TLC.

2.2.3 Protein purification

Isolation of sbHMNGT

All procedures were carried out at 4 °C except where indicated. Sorghum seedlings were prepared according to Halkier and Møller (1989). Briefly, *S. bicolor* seeds (1 kg) (cultivar MR31) were imbibed in tap water overnight at room temperature and planted between two sheets of cotton gauze strapped over a metal frame. The frame was placed in a plastic container and tap water filled up to ~1 cm below the seeds. Seeds were then allowed to germinate at 30 °C in darkness for 2 days. Seedling shoots (~100 g) were harvested and extracted in 2 volumes of ice-cold extraction buffer (250 mM sucrose; 100 mM Tris-HCl (pH 7.5); 50 mM NaCl; 2 mM EDTA; 5% (w/v) of polyvinylpolypyrrolidone; 200 μM PMSF; 6 mM DTT) using mortar and pestle. The extract was filtered through a nylon mesh prior to centrifugation (20,000 x g, 20 min). The supernatant fraction was subjected to differential ammonium sulfate fractionation (35-70% (w/v)). Ammonium sulfate was slowly added until it had fully dissolved, and each saturation level (35% (w/v) and 70% (w/v)) was maintained for 1 hour with gentle stirring prior to centrifugation (20,000 x g, 15 min). After the first cut (35% (w/v) saturation) the pellet was discarded, whilst after the second cut (70% (w/v) saturation) the pellet was retained. The pellet was resuspended in ~15 ml of buffer A (20 mM Tris-HCl (pH 7.5); 5 mM DTT) and desalted using a Sephadex G-25 (Amersham Pharmacia Biotech, Sweden) or Biogel P-6 (Bio-Rad, Hercules, CA, USA) column (2.5 x 20 cm , flow-rate: 20 ml/min) equilibrated in buffer A. The first UV-absorbing peak (280 nm) was collected and applied to a Q-Sepharose (Amersham Pharmacia Biotech, Sweden) column (2.6 x 23 cm, flow-rate: 60-80 ml/hr) equilibrated in buffer B (buffer A + 50 mM NaCl). The column was washed with buffer B until the baseline had stabilized and proteins were eluted with a linear gradient from 50 to 400 mM NaCl in buffer A (800 ml total). Fractions (10 ml) were collected and 3-5 μl assayed for mandelonitrile glucosyltransferase activity by LSC. To reduce the salt concentration, combined active fractions (50 mg protein, 20 ml) were diluted five-fold in buffer B and concentrated 20-fold using an Amicon YM30 or YM10 membrane prior to storage at -80 °C.

The remainder of the purification was carried out at room temperature. One quarter of the concentrated material from the Q-Sepharose step (~10-15 mg protein, 5 ml) was applied to a column (1 cm x 10 cm, flow-rate: 10-15 ml/hr) containing Reactive Yellow

3 cross-linked onto beaded agarose (Lot 63H9502) (Sigma) equilibrated in buffer B. The column was washed with buffer B until the baseline had stabilized. Proteins were eluted with 10 ml of 2 mM UDP-glucose in buffer B. Active fractions containing essentially pure sbHMNGT were combined and stored at -80 °C with or without addition of 1 mg/ml BSA. SDS-PAGE was performed as below using fractions without BSA.

Separation of sbHMNGT isoforms

Protein purification was performed as described above, except for the use of cultivar SS1000 as plant material and the elution method employed for Yellow 3 chromatography. The two isoforms were eluted from the Reactive Yellow 3 media using a linear gradient (0-4 mM UDP-glucose in buffer B, 30 ml total volume). Fractions (~2.5 ml) were assayed for sbHMNGT activity as described above and subjected to SDS-PAGE using 12% (w/v) acrylamide gels as described below.

2.2.4 Peptide generation and sequencing

sbHMNGT (~ 5 µg, estimated by SDS-PAGE) was subjected to N-terminal sequencing as described below. For peptide digestion, sbHMNGT (~ 100 µg) was precipitated by addition of trichloroacetic acid (10% (w/v) final concentration), resuspended in 50 µl of 50 mM Tris-HCl (pH 8.0), 5 mM DTT and 6.4 M urea, incubated (60 °C, 50 min), cooled to room temperature, and diluted with 3 volumes of 30 mM Tris (pH 7.7) and 1.25 mM EDTA. Endo Lys-C (Promega, Madison, WI, USA) was added (proteinase:substrate ratio 1:25 (w/w)) and the reaction allowed to proceed for 24 hrs at 37 °C. Peptides were purified with Beckman System Gold HPLC equipment fitted with a Vydac 208TP52 C8 column (2.1 mm x 250 mm, flow-rate: 0.2 ml/min). Peptides were applied in buffer C (0.1% (v/v) trifluoroacetic acid) and eluted with a linear gradient from 0 to 80% (v/v) acetonitrile in buffer C. Peptides were collected manually and sequenced using an automated Edman G1000A protein sequencer (Hewlett-Packard, USA).

2.2.5 SDS-PAGE

Protein preparations were concentrated using a speed vac concentrator (Savant) prior to electrophoresis. SDS-PAGE was performed using 12% (w/v) (50:1.35;

acrylamide:bisacrylamide) Tris-glycine or high-Tris linear 8 to 25% (w/v) SDS-Polyacrylamide gradient gels (Mini-Protean II, Bio-Rad) (Fling and Gregerson, 1986). Polypeptides were visualized by staining with Coomassie Brilliant Blue (R-250)(Bradford, 1976).

2.2.6 Generation of antisera and immunoblotting

Anti-sbHMNGT antibodies were produced by Statens Seruminstitut, Copenhagen, Denmark principally as described by Harlow and Lane (1988). Approximately 50 μg of isolated recombinant sbHMNGT (described below, 3.2.3) was injected 4 times into a rabbit (classified as "low immunogenic response" by Statens Seruminstitut, Copenhagen, Denmark) at 10-day intervals. An equal volume of Freund's complete adjuvant was included in the first injection, followed by Freund's incomplete adjuvant in the latter boosts. The rabbit was bled one week after the final boost. 0.1% (v/v) azide was added to the serum of the bleed and aliquots were stored at 4 °C and -80 °C.

Polypeptides were electrophoresed as described above (2.2.5) and blotted onto nitrocellulose membranes using a semi-dry transfer unit (LKB, Bromma, Sweden) as described by (Harlow and Lane, 1988). Proteins were visualised by staining the nitrocellulose membrane with 0.2% (w/v) Ponceau S (Sigma, USA) in 0.2% (w/v) TCA and 3% (w/v) sulfosalicylic acid, followed by destaining with MQ H_2O. Immunoblots were blocked overnight at 4 °C in 5% (w/v) skim milk (Sigma, USA) in TBS-T (137 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl, pH 8.0, 0.1% (v/v) Tween-20 (Sigma, USA)), washed (4x10 min, TBS-T) and probed with anti-sbHMNGT. The immunoblots were subsequently washed (4x10 min, TBS-T) and the primary antibody visualized using secondary anti-rabbit antibodies conjugated with alkaline phosphatase (DAKO, Copenhagen, Denmark) or a chemiluminescent detection system (SuperSignal, Pierce, Rockford) according to the manufacturer's instructions. Immunoblots were incubated with a 1:10,000 (v/v) dilution of primary or secondary antisera in 3% (w/v) BSA in TBS-T for 1 hour.

2.3 Results

2.3.1 Yield and stability of sbHMNGT

Although the two cytochrome P450 enzymes responsible for the conversion of tyrosine into *p*-hydroxymandelonitrile have been isolated and their cDNAs cloned, the enzyme crucial to the stable accumulation of dhurrin, namely sbHMNGT, has like many other glucosyltransferases proven very difficult to identify at the molecular level (Hahlbrock and Conn, 1970; Reay and Conn, 1974; Hösel and Schiel, 1984; Hughes and Hughes, 1994; Mederacke *et al.*, 1995). The difficulty associated with the isolation of plant secondary metabolite glucosyltransferases in general, has been attributed to the apparent lability (Reed *et al.*, 1993) and low concentration of these proteins (Bar-Peled *et al.*, 1991). It is therefore essential to optimize conditions for protein stability and purification speed.

In preparation for sbHMNGT purification, sorghum seeds were germinated in darkness for 1½-5 days and extracts made from seedlings tested for sbHMNGT activity. Under the conditions of growth, a 2-day germination period proved optimal with regards to total sbHMNGT activity, protein concentration and extract volume (data not shown). Although the conversion of tyrosine to cyanide in relation to fresh weight is at a peak as soon as the shoot emerges (~20 hrs), the total yield of biosynthetic activity does not peak until 48 hours under similar growing conditions (Halkier and Møller, 1989). The use of a Waring blender resulted in less than 50% of the activity as compared to extraction with mortar and pestle. sbHMNGT activity was 5 times higher in crude extracts after freezing and thawing at -80 °C than after storage at 4 °C for 2 days. The addition of glycerol to crude extracts did not affect sbHMNGT activity to any great extent, although subsequent work by Kristensen and Hansen (2000) showed that isolated sbHMNGT retained a higher degree of activity upon freezing and thawing with the inclusion of 10% (v/v) glycerol (final concentration). The addition of relatively high concentrations of DTT were required to retain activity. Lowering the concentration of DTT from 5 mM to 2 mM resulted in a ten-fold decrease in activity after storage at 4 °C for 2 days. This pronounced effect of DTT was primarily found in crude preparations, whereas partially purified ion-exchange preparations were less responsive to the concentration of reducing agents in contrast to previous results (Reay and Conn, 1974).

The importance of optimized buffer conditions was highlighted by the strong effect of DTT concentration on sbHMNGT stability in crude extracts. The differential effect of DTT on the maintenance of a reduced environment at different stages throughout purification, may be explained by a higher concentration of low molecular weight radicals and oxidative enzymes in the crude plant extracts. The mechanism by which sbHMNGT activity is affected remains unknown, however, it is interesting to note that the addition of DTT to purified *Zea mays* indole-acetic acid-glucosyltransferase inhibited the formation of inactive multimers (Kowalczyk *et al.*, 1991).

2.3.2 Isolation of sbHMNGT

Mandelonitrile was employed as a substrate for the assay of sbHMNGT activity throughout purification, although the endogenous substrate of sbHMNGT is *p*-hydroxymandelonitrile. Mandelonitrile had previously been shown to be an equally good substrate (Reay and Conn, 1974). The absence of a hydroxyl group at the para-position of the benzene ring also ruled out the possibility of *p*-glucosyloxymandelonitrile synthesis, which would be indistinguishable from dhurrin when the rapid assay based on LSC was employed. Furthermore, the absence of the aromatic hydroxyl-group was also thought to facilitate extraction of the glucoside into the organic phase (ethyl acetate) prior to LSC. The sbHMNGT activity in aliquots of all purification steps were also verified by assays using *p*-hydroxymandelonitrile and TLC. Etiolated seedlings of *S. bicolor* were extracted with mortar and pestle, the protein preparation subjected to ammonium sulfate fractionation and desalted by gel chromatography. Low molecular weight solutes (including cyanide and cyanide-precursors) were effectively removed, although there was no measurable increase in the specific activity of sbHMNGT. All sbHMNGT activity bound to Q-Sepharose and was eluted between 250-300 mM NaCl with a ~4-fold purification (Figure 2.1). Aliquots of combined fractions were stored at -80 °C after desalting and concentrating.

Several pseudoaffinity reagents were subsequently tried in mini-column format, including Cibacron blue 3G, Reactive Green 19, Reactive Yellow 3 and UDP-glucuronic acid cross linked with beaded agarose. Trials with elution using NaCl and UDP-glucose at varying salt concentrations identified Reactive Yellow 3 as the superior column material. sbHMNGT activity bound to Reactive Yellow 3 at 50 mM NaCl and

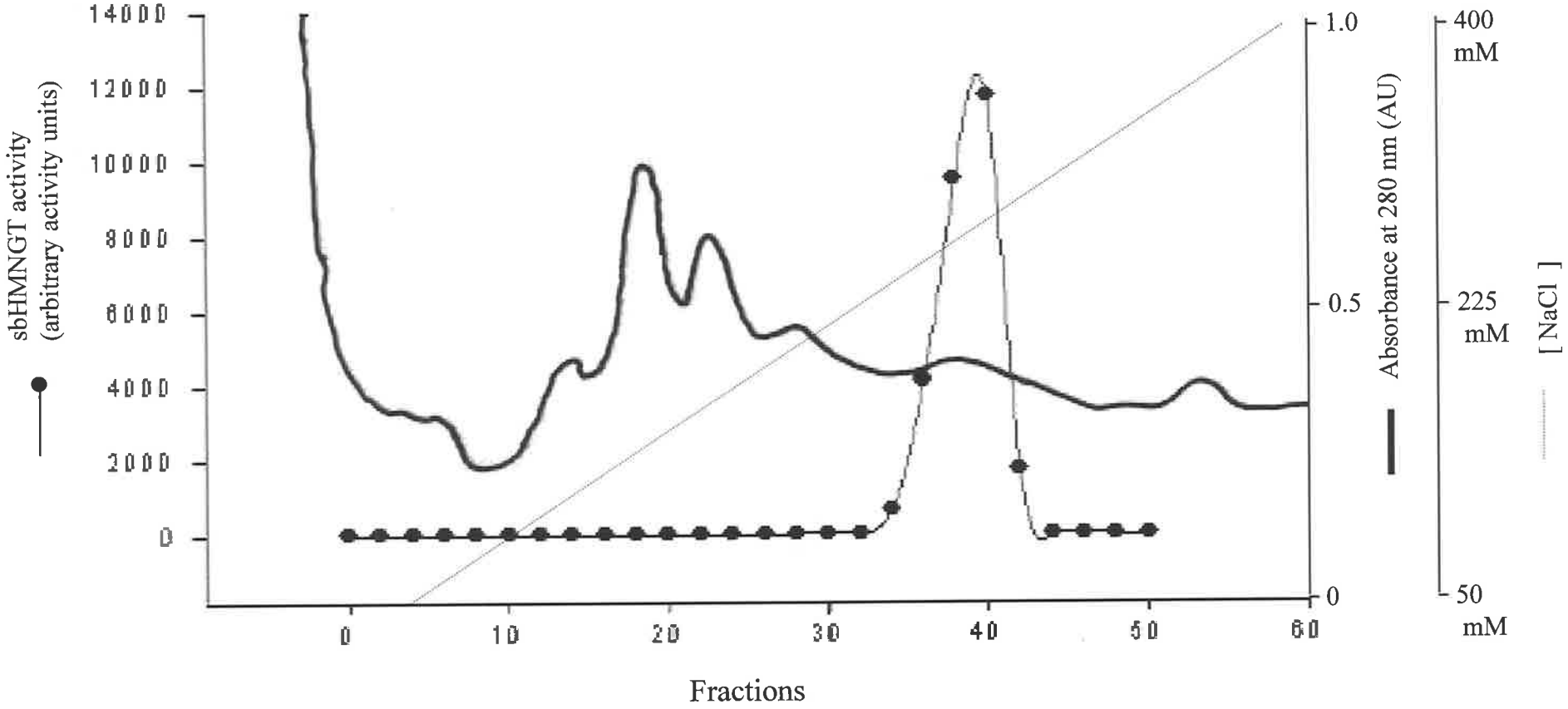


Figure 2.1 Purification of sbHMNGT by Q-sepharose chromatography. Proteins bound to a Q-sepharose (anion-exchange) column were eluted using a linear gradient from 50 to 400 mM NaCl and monitored by absorption at 280 nm. Aliquots (10 µl) of fractions (10 ml) were assayed for mandelonitrile-glucosyltransferase activity by LSC. Only a single activity peak could be identified and active fractions were pooled, desalted with Amicon YM10 ultrafiltration and frozen at -80 °C in multiple aliquots.

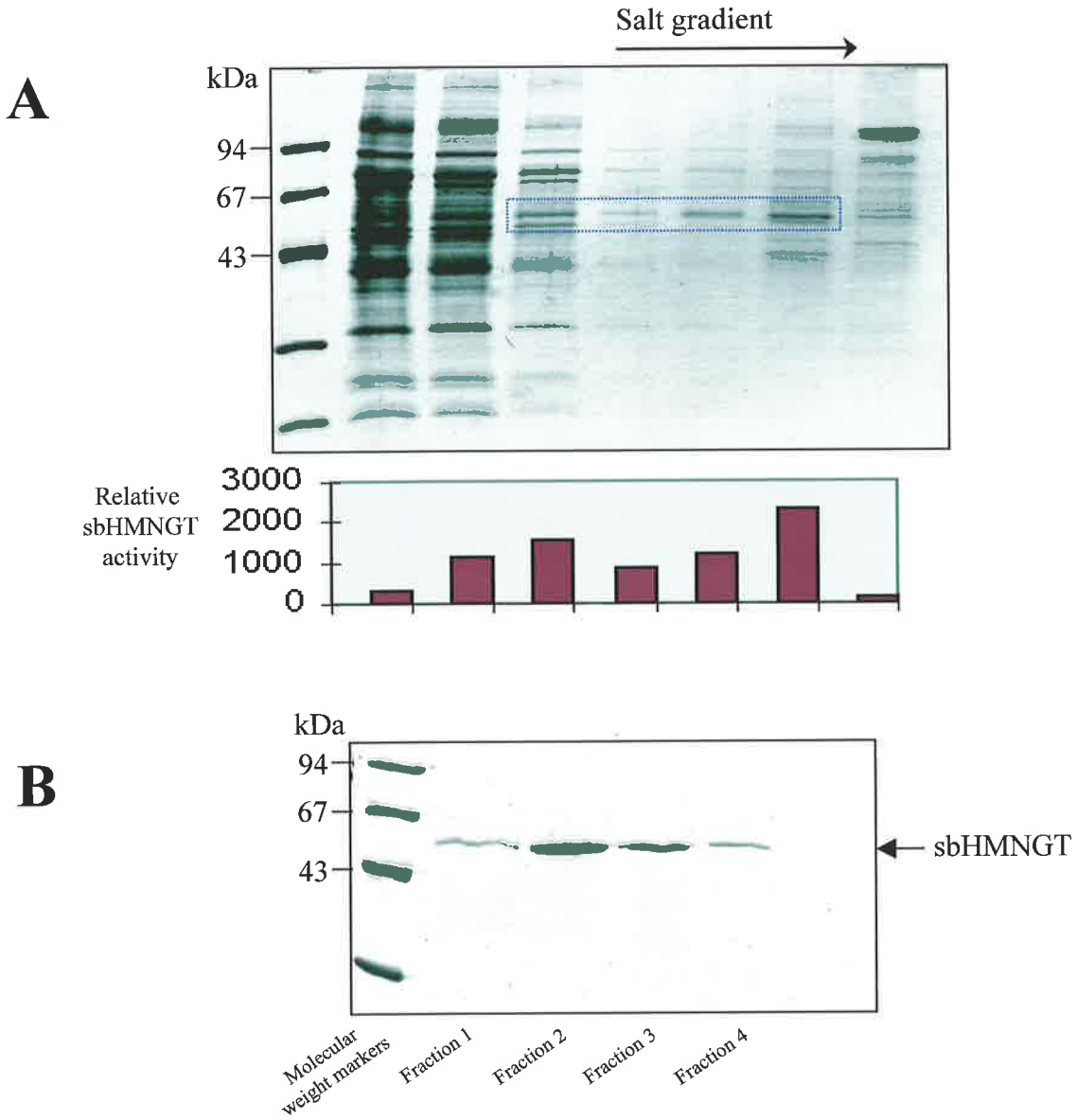


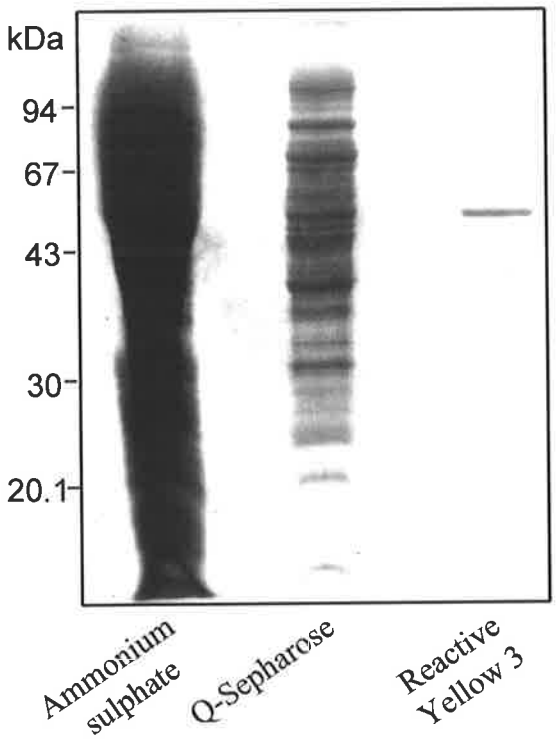
Figure 2.2 Isolation of sbHMNGT by Reactive Yellow 3 dye chromatography. (A) The elution of sbHMNGT activity with NaCl correlates with the abundance of a 50-55 kDa protein, as highlighted by the blue dashed box. Proteins bound to Reactive Yellow 3 were eluted with a linear salt gradient from 0.1 to 2 M NaCl (20 ml total) and monitored by absorption at 280 nm. Aliquots (8 µl) of fractions (~2 ml) were assayed for mandelonitrile activity by LSC. Aliquots of the same fractions (500 µl) were also concentrated and analyzed by SDS-PAGE, followed by Coomassie Brilliant Blue staining. All activity-values are relative only. Lanes containing salt are indicated by the arrow above the SDS-PAGE gel. (B) Elution of sbHMNGT with UDP-glucose. A 50-55 kDa protein could be eluted in apparent homogeneity as visualised by SDS-PAGE and Coomassie Brilliant Blue staining when Reactive Yellow 3 was challenged with 3 ml of 2 mM UDP-glucose instead of gradient of NaCl.

could be eluted after washing with a slight increase in NaCl concentration, without any measurable UV-absorbance in the eluate (Figure 2.2a). sbHMNGT activity correlated with the abundance of a polypeptide migrating around 50-55 kDa by SDS-PAGE, but additional polypeptides were also present. Elution with 2 mM UDP-glucose instead of NaCl resulted in the elution of a similarly migrating polypeptide in apparent homogeneity (Figure 2.2b). Assuming that all of the polypeptide which was visualized by SDS-PAGE was active (and therefore that all inactive protein had been lost), sbHMNGT represented approximately 0.25% (w/w) of the total protein in the ammonium sulfate extract and was purified 420-fold with a maximum yield of 22% (Figure 2.3). The apparent high recovery of sbHMNGT activity after Yellow 3 chromatography may be explained by a lower amount of dhurrin-specific β-glucosidases in the Yellow 3 eluate, compared with the pooled Q-Sepharose fractions. The critical step in sbHMNGT isolation was Reactive Yellow 3 dye-chromatography in association with UDP-glucose elution, which resulted in more than a 100-fold purification in a single step. Reactive Yellow 3 was recently also employed with UDP-glucose elution in the purification of a UDP-glucose:betanidin-6-*O*-glucosyltransferase with similar results as in the present study (Vogt *et al.*, 1997). The use of pseudo-affinity dye chromatography together with substrate specific elution is now emerging as a highly selective key step in plant glucosyltransferase purification (Bar-Peled *et al.*, 1991; Kowalczyk *et al.*, 1991; Li *et al.*, 1997; Kuai *et al.*, 1997; Vogt *et al.*, 1997). This significantly shortens the purification procedure and minimizes the time-dependent loss of activity seen in other glucosyltransferase purification protocols (Sun and Hrazdina, 1991; Reed *et al.*, 1993; Cheng *et al.*, 1994, Guo and Poulton, 1994; Do *et al.*, 1995; Rasmussen and Rudolph, 1997).

2.3.3 Isolation of sbHMNGT isoforms

The initial isolation of sbHMNGT was performed using proteins derived from cultivar MR31. When the developed isolation procedure was repeated using cultivar SS1000, SDS-PAGE resolved two closely migrating polypeptides which could not be separated by additional Cibacron blue 3G chromatography using a linear salt gradient (data not shown). However, the two polypeptides could be differentially eluted when a linear UDP-glucose gradient (0-4 mM) was applied to the Reactive Yellow 3 medium (Figure 2.5a) after the pooled active ion-exchange preparation had been bound and washed. The sbHMNGT activity pattern suggested that both isoforms were active. A comparison

A



B

Step	Specific activity	Purification	Yield
	pmoles/min/mg protein	*fold*	*%*
Crude desalt	36	1	100
Q-Sepharose	141	4	37
Yellow 3	14779	419	36

Figure 2.3 Summary of a simple three-step protocol for the isolation of sbHMNGT. (A) Polypeptide profile of the fractions obtained during the isolation of native sbHMNGT from *S. bicolor* as visualized by Coomassie Brilliant Blue staining. All of the loaded fractions visualised by SDS-PAGE and Coomassie Brilliant Blue staining contained approximately equal sbHMNGT activity. (B) A summary of activities obtained from the three purification steps. The figures are representitve of a typical purification procedure.

A **Peptide sequences**

1	SNAPP (N-terminal sequence)
2	GEAAV RPPAI SSARF RIEVI DDGLS LSVPQ NDVGG LVD
3	NxLHP FRALL RRLGQ EVEEQ DAPPV TTVVG DVVMT FAAAx
4	HAAVG LFVSH CGWNS LLEAT AAGQP VLATP CH
5	GGAS WRNVE RVVND LLLVG GK

B

Figure 2.4 Peptides from isolated and digested sbHMNGT identify one peptide with high sequence similarity to other known secondary plant metabolism glycosyltransferases. (A) Five peptide sequences were obtained from isolated sbHMNGT, one N-terminal (sequence 1), three internal (sequence 2-4) and one C-terminal (sequence 5). (x indicates undeterminable amino acid, underlined amino acids are only putatively identified). (B) A comparison between peptide sequence 4 from sbHMNGT (boxed in milk blue) and deduced peptide sequences from other known (highlighted in yellow) and putative glycosyltransferases. Both the region of comparison and sequence 4 are contained within the PSPG-motif (Figure 1.6). The alignment was created by eye. Highly conserved amino acids between the twelve sequences are shaded in grey. GenBank accession numbers are: meGT60 (*Manihot esculenta* putative glucosyltransferase: X77460); ntGT2 (*Nicotiana tabacum* salicylic acid-inducible glucosyltransferase (not entered into any databanks, (Lee and Raskin, 1999); bnTHGT (*Brassica napus* thiohydroximate-S-glucosyltransferase: Seq. ed. #28 European Patent EP 0 771 878 A1); ntJIGT (*N. tabacum* jasmonic acid inducible putative glucosyltransferase: BAA19155); psGT (*Pisum sativum* putative glucuronosyltransferase: AF034743); meGT64 (*Manihot esculenta* putative glucosyltransferase: X77464); gtUFGT (*Gentiana triflora* flavonoid glucosyltransferase: Q96493). Sequences zmUFGT, zmIAAGT, vvUFGT and stSGT are described in Figure 1.6.

with recombinant sbHMNGT (described below, 3.2.3) and sbHMNGT isolated from cultivar MR31 using 12% (w/v) SDS-PAGE gels implied that cultivar SS1000 contains an additional isoform, $sbHMNGT_2$, which migrates slower than both the recombinant and MR31 isoform (Figure 2.5b). It is interesting to question why there is a second isoform and how it differs from that which is present in cultivar MR31. $sbHMNGT_2$ could either be a novel polypeptide which is encoded by a unique gene, or be the result of post-translational modification. Attempts to obtain molecular masses through mass spectrometry or N-terminal sequence of either of the two isoforms were not successful (data not shown). However, Southern blot analyses indicated that only a single gene copy of *sbHMNGT* (cDNA encoding sbHMNGT) is present in the genome of *S. bicolor* cultivar SS1000 (Kristensen and Hansen, 2000), which leaves open the possibility that $sbHMNGT_2$ and sbHMNGT differ due to a post-transcriptional modification.

2.3.4 Amino acid sequencing of sbHMNGT

N-terminal sequencing of approximately 100 pmole of isolated sbHMNGT from cultivar MR31 yielded PTH-amino acids at a level ten times lower than expected (sequence 1, Figure 2.4a). The low response suggested either partial blockage of the N-terminus or the presence of a co-migrating and fully blocked contaminant. The latter possibility was ruled out because protein digestion by Endo Lys-C, followed by HPLC purification, yielded four major peptides (sequences 2-5, Figure 2.4a) with sequences all contained in a single cDNA, *sbHMNGT* (described below, 3.3.1). Only sequence 4 exhibited high similarity to other known and putative glucosyltransferases, as it contains sequence represented by the PSPG-motif (Figure 2.4b). The absence of peptides that were not encoded by *sbHMNGT* suggested that the preparation containing sbHMNGT was practically homogeneous, although all of the minor peptides resulting from proteolysis were not sequenced.

2.3.5 Immunoblotting

Anti-sbHMNGT antibodies specifically recognized polypeptide(s) of the expected mass in crude extracts of *E. coli* overexpressing recombinant sbHMNGT (described below, 3.2.3) and in crude supernatants of cultivar MR31 and SS1000 homogenates (Figure 2.6a). SDS-PAGE using 12% (w/v) gels, as opposed to gradient gels, confirmed the presence of both isoforms of sbHMNGT in crude protein supernatant extracts of *S. bicolor* (Figure 2.6b). The antisera was unfortunately highly antigenic towards multiple

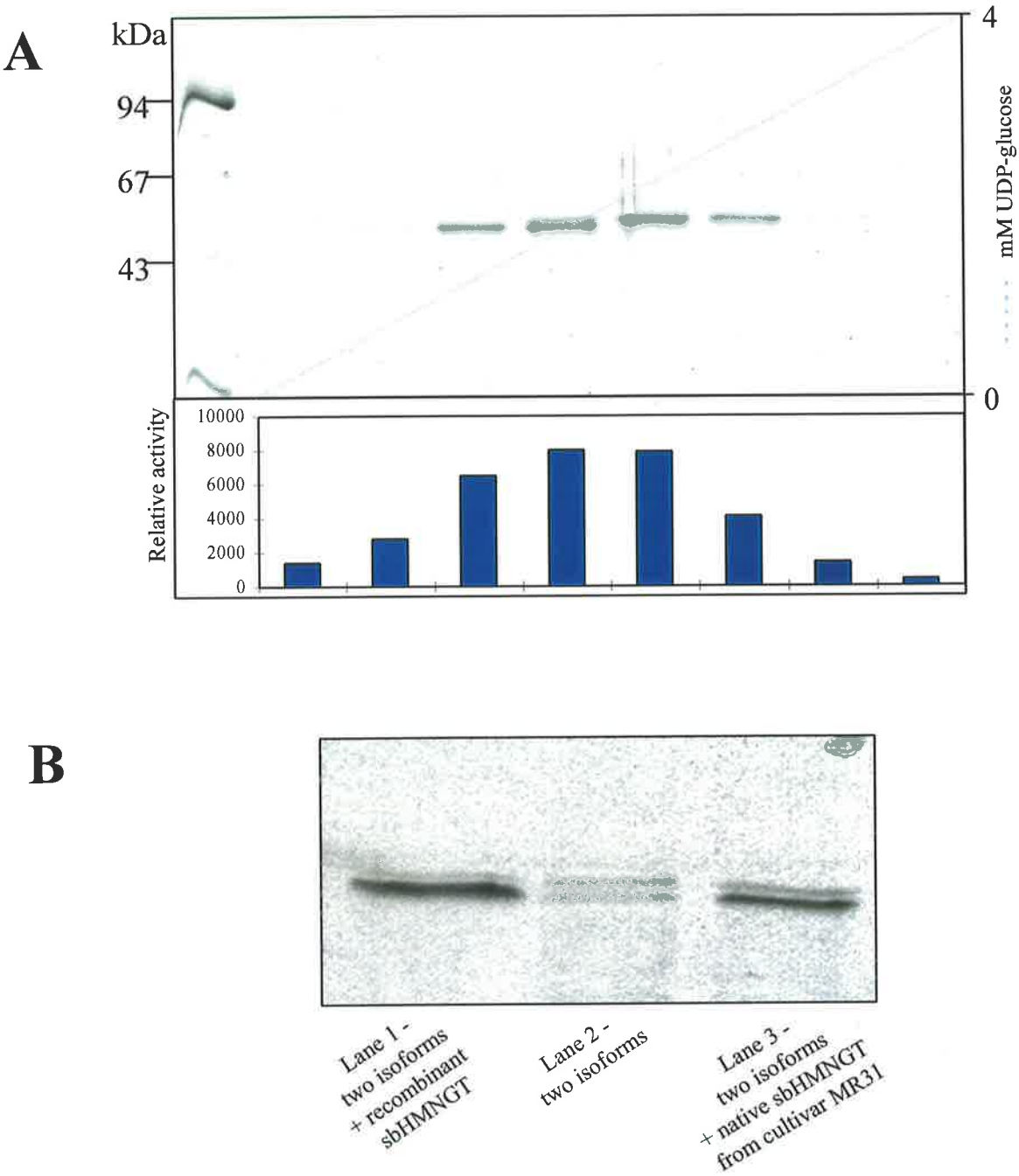


Figure 2.5 The separation of two isoforms of sbHMNGT from cultivar SS1000. (A)Proteins bound to Reactive Yellow 3 were eluted with a linear gradient from 0 - 4 mM UDP-glucose and monitored by absorption at 280 nm. Aliquots of fractions (~5 ml) were assayed for mandelonitrile activity by LSC (5 µl). Aliquots of the same fractions (80 µl) were also concentrated and analyzed by SDS-PAGE using gradient gels as described above (2.2.5), followed by Coomassie Brilliant Blue staining. All activity values are relative only. (B) Isolated isoforms of sbHMNGT from cultivar SS1000 were compared with sbHMNGT isolated from MR31 and recombinant sbHMNGT (described below, 3.2.3). All three lanes contain an equal amount of both native isoforms from cultivar SS1000 (~0.2 µg of each). In addition to this, lane 1 contains isolated recombinant sbHMNGT (~0.8 µg) and lane 3 contains isolated native sbHMNGT from MR31 (~0.2 µg). Polypeptides were subjected to electrophoresis using a 12% (w/v) SDS-PAGE gel as described above (2.2.5).

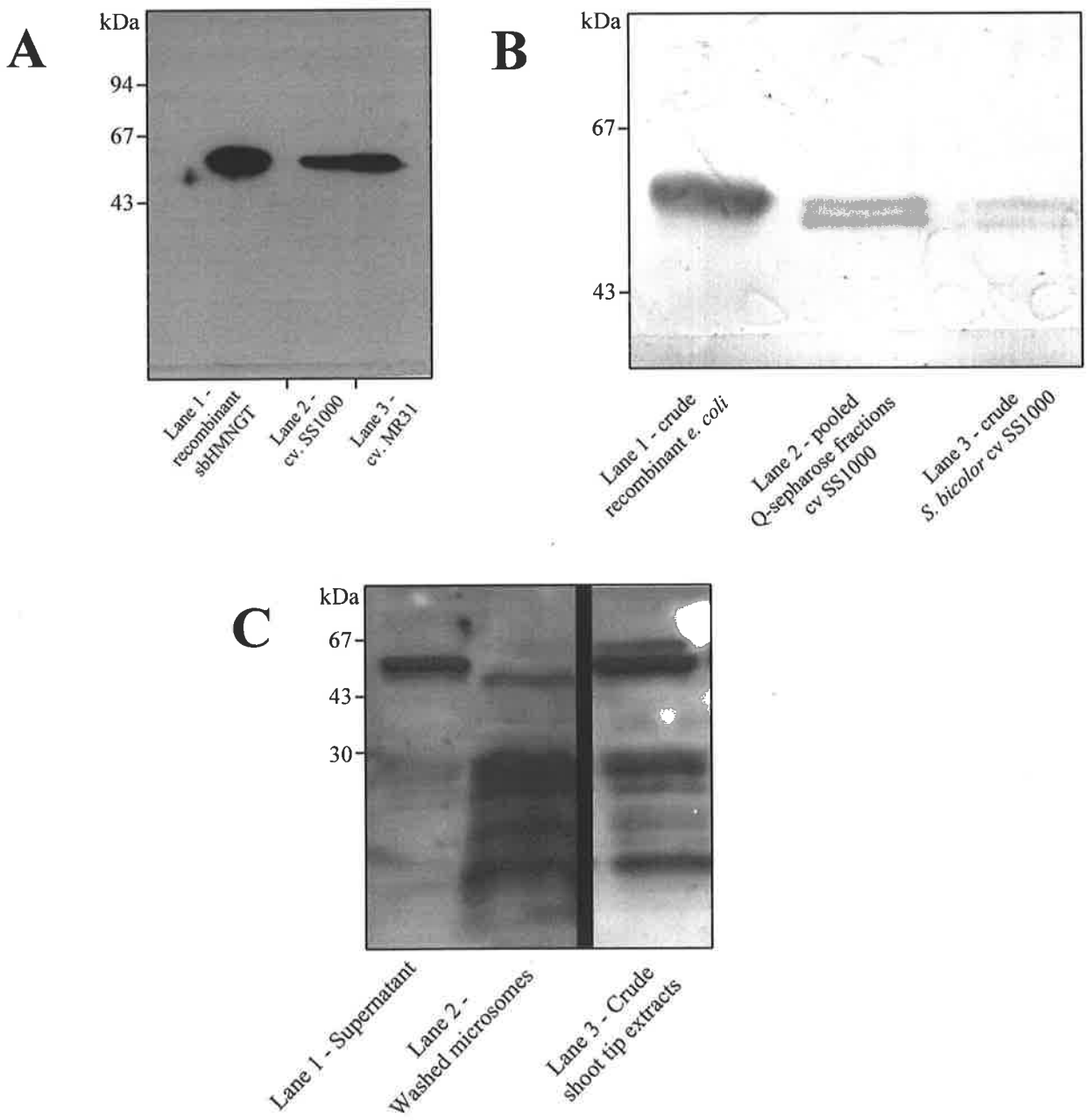


Figure 2.6 Western blotting analyses with anti-sbHMNGT. (A) Immunodetection of sbHMNGT in crude extracts prepared from recombinant *E. coli* expressing sbHMNGT (Lane 1) and *S. bicolor* cultivar SS1000 and MR31, respectively (Lane 2 and 3). (B) SDS-PAGE gels with maximum separation (12% (w/v) gels, described in 2.2.5) separated the two isoforms of sbHMNGT in cultivar SS1000 (Lane 2 and 3). (C) Western blotting analyses of supernatant (Lane 1), washed microsomal extracts (Lane 2) and crude extracts (Lane 3) of *S. bicolor* cultivar SS1000. Panel A and C were visualized using a chemiluminescent detection system, whilst panel B was visualized using alkaline phosphatase (AP) (described in 2.2.6).

polypeptides in microsomal extracts of *S. bicolor* (Figure 2.6c), although anti-sbHMNGT displayed high specificity towards sbHMNGT in the presence of other soluble proteins (Figure 2.6, panels A and B). Initial studies suggest that anti-sbHMNGT recognizes one major polypeptide in both cassava (*M. esculenta*) and *T. maritima* of the expected molecular mass (Kristensen and Hansen, 2000). The antisera could therefore be employed for immunoscreening in these two cyanogenic cultivars, although it would need to be further purified for immunolocalization and cross-linking studies with CYP79A1 and CYP71E1.

2.4 Discussion

The isolation of sbHMNGT was the most critical achievement for this entire study. With the information gathered from amino acid sequencing it was subsequently possible to obtain the corresponding cDNA, characterize the substrate specificity of recombinant sbHMNGT (chapter 3) and to reconstitute the entire cyanogenic glucoside biosynthetic pathway in an Eppendorf tube and in another plant (chapter 4). It is obvious that the choice of model plant was very important at the beginning of the study into the biosynthesis of cyanogenic glucosides (Akazawa *et al.*, 1990; McFarlane *et al.*, 1975). Secondary plant metabolites are generally present at low concentrations in plants and only in rare instances, such as with dhurrin in *S. bicolor*, do these metabolites accumulate to a significant degree in a short space of time (Halkier and Møller, 1989). There are now numerous examples of glycosyltransferases that have been isolated from plants where their products are present at wide ranging levels (Dixon *et al.*, 1989; Bar-Peled *et al.*, 1991; Kowalczyk *et al.*, 1991; Reed *et al.*, 1993; Hasegawa *et al.*, 1997; Li *et al.*, 1997; Vogt *et al.*, 1997; Lee and Raskin, 1999; Miller *et al*, 1999; Arend *et al*, 2000).

One of the most time-consuming tasks in plant science today is the search for tools, commonly in the form of cDNAs and antibodies, which can be utilised to gain information about particular systems within the plants. It is tempting to here take the opportunity to compare the two main options for the isolation of these molecular tools, the direct approach (protein -> cDNA) and the reverse approach (cDNA -> protein). This is particularly relevant now that genomic sequencing has almost been completed for *Arabidopsis thaliana*, and expressed sequence tags have been isolated from numerous important crop plants (Bouchez and Höfte, 1998; Somerville and Somerville, 1999). It has been estimated that approximately 120 putative glycosyltransferases may be present in the genome of *A. thaliana* (Li *et al.*, 2000). Over 50 individual expressed sequence tags coding for putative glycosyltransferases have been identified, suggesting that a large proportion of these glycosyltransferase-encoding genes are expressed, and therefore utilised by the plant (Søren Bak, personal communication). Despite this wealth of readily available information, the direct approach will most often be far more fruitful in the search for a specific glycosyltransferase until functional genomics has provided more information (Bouchez and Höfte, 1998), since to date there has been no way in

which the sequence of glycosyltransferase-encoding genes can be related to the substrate specificity of the encoded enzyme (Vogt and Jones, 2000). In favour of the direct approach, it is reasonably easy to develop an assay for most glycosyltransferases and most are soluble with few exceptions (Leah *et al.*, 1982; Warnecke and Heinz, 1994; Vogt and Taylor, 1995). Several glycosyltransferases have been isolated to date (detailed above), and despite being present at low concentrations they are often sufficiently stable in optimized buffers to allow purification to a state in which amino acid sequence can be obtained. An alternative direct method is that of positive selection in yeasts, which can be employed due to the detoxifying nature of certain glycosyltransferases. This was sucessfully illustrated by the isolation of a cDNA encoding a solanidine-glucosyltransferase in potato (Moehs *et al.*, 1997). Nevertheless, the reverse approach does have merits when protein purification is not possible, particularly in woody species such as cassava and grapevine, where long-term protein stability is difficult to achieve in the absence of complex buffers which may hinder certain chromatographic procedures (Andersen *et al.*, 1999; Chris Ford, pers. comm.). Two notably successful examples of the reverse approach are the anthocyanidin-glucosyltransferases from *V. vinifera* (Sparvoli *et al.*, 1994; Ford *et al.*, 1998) and *Perilla frutescens* (Yamazaki *et al.*, 1999).

A tool is of limited use unless we know the function of that tool, or the proteins associated with it, within the plant. The function of a glycosyltransferase would need to be related to the substrate specificity of that enzyme, and in a broader sense, to the impact on overall plant biology of the glycosylation of those substrates which it will conjugate *in planta*, as discussed by Bouchez and Höfte (1998). Since the direct approach utilises the substrate specificity of the protein as an indicator, a direct approach is more likely to produce a tool of the desired function, if function is specified by substrate specificity. Unfortunately, even though a particular enzyme has the ability to glycosylate a certain substrate, it may not necessarily have that function *in planta*. For example, Fraissinet-Tachet *et al.* (1998) analyzed the isolated recombinant polypeptides encoded by two salicylic acid inducible genes containing the PSPG-motif. They were unable to suggest a function of these two proteins despite in-depth analyses of mRNA expression and *in vitro* substrate specificity. One of the enzymes conjugated all 15 of 15 tested substrates! The fields of plant metabolism in which these 15 phenylpropanoid substrates are associated include cell wall biosynthesis, intracellular

communication (plant hormones) and pathogen and pest defence! Thus, what function do these glycosyltransferases have *in planta*? Are knock-out mutants or transgenic native plants with anti-sense expression ultimately the only means for verifying the link between enzyme and function?

In this chapter it has been assumed that sbHMNGT is the only enzyme which glucosylates *p*-hydroxymandelonitrile to form dhurrin, and this was based on the fact that only one enzyme with acceptance towards *p*-hydroxymandelonitrile was isolated from cultivar MR31. The function of sbHMNGT is most likely primarily that of dhurrin biosynthesis as described above (2.1) and as shown by further characterization of the substrate specificity of recombinant sbHMNGT in chapter 3. However, sbHMNGT may perform other roles in *S. bicolor*, and there may be other enzymes in *S. bicolor* that can glucosylate *p*-hydroxymandelonitrile, but these questions may perhaps best be answered by analyzing transgenic cultivars of *S. bicolor* with reduced sbHMNGT activity.

Chapter 3

Cloning, heterologous expression and characterization of the substrate specificity of recombinant UDP-glucose:*p*-hydroxy-(*S*)-mandelonitrile-*O*-β-glucosyltransferase from *Sorghum bicolor*

3.1 Introduction

Although a large range of substrates can be glycosylated by plants, as outlined in Chapter 1, the exact role of any single glycosyltransferase relates to the substrate specificity and, to the temporal and tissue-specific expression and localization of that enzyme. An *in vitro* substrate specificity profile can be generated by testing isolated glycosyltransferases against a wide range of substrates. It is difficult to test the more than 1000 potential substrates that plants are capable of producing, and in practice only a small subset of potential aglycones can feasibly be tested. Nevertheless, such a profile will provide information about the potential role of that glycosyltransferase within the plant although the range of substrates towards which the enzyme exhibits activity *in vitro* may be more limited *in vivo* due to differential regulation of expression, differential localization of the enzyme in relation to potential acceptor substrates, competing enzymes and substrates and differences between *in vivo* and *in vitro* conditions.

With the information gained from peptide sequencing of native sbHMNGT it was possible to clone the corresponding cDNA, *sbHMNGT*. *sbHMNGT* was then expressed in *E. coli*, isolated and characterized with respect to substrate specificity. The qualitative and quantitative substrate specificity confirmed that *sbHMNGT* encodes a UDP-glucose:*p*-hydroxymandelonitrile-*O*-glucosyltransferase.

3.2 Materials and methods

3.2.1 Materials and general methods

All biochemicals and reagents were obtained from Sigma (USA) except where indicated.

DNA sequencing reactions were carried out using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (7-deaza dGTP) (Amersham Pharmacia Biotech, Sweden) and analyzed using an ALFexpress DNA Sequencer (Amersham Pharmacia Biotech, Sweden). Sequence computer analysis was performed using programs in the Genetic Computer Group (Madison, WI) Sequence Analysis Package, NCBI BLAST (Altschul *et al.*, 1997) and ExPaSy (Swiss Institute of Bioinformatics; Appel *et al.*, 1994). All gel purifications of DNA were carried out using the Qiaex II gel extraction kit (Qiagen, USA) after 1.5% (w/v) agarose gel electrophoresis.

3.2.2 PCR amplification, cloning and library screening

First round PCR amplification reactions (total volume: 40 µl) were carried out using 2 units of Taq DNA polymerase (Amersham Pharmacia Biotech, Sweden), 4 µl of 10xTaq DNA polymerase buffer (Amersham Pharmacia Biotech, Sweden), 5% (v/v) dimethyl sulfoxide, 1 µl dNTPs (10 mM), 80 pmoles each of primers C2EF (5′-TTYGTIWSICAYTGYGGITGGAA-3′) and T7 (5′-AATACGACTCACTATAG-3′) and ~10 ng of plasmid DNA template. The plasmid DNA template was prepared from a unidirectional pcDNAII (Invitrogen, Carlsbad, CA, USA) plasmid library made from mRNA isolated from 1-2 cm high etiolated *S. bicolor* seedlings (Bak *et al.*, 1998). Thermal cycling parameters were 95 °C, 5 min, 3 x (95 °C for 5 s, 42 °C for 30 s, 72 °C for 30 s), 32 x (95 °C for 5 s, 50 °C for 30 s, 72 °C for 30 s) and a final 72 °C for 5 min. Second round PCR amplifications were carried out as above, except using primers C2DF (5′-GARGCIACIGCIGCIGGICARCC-3′) and T7, and 1 µl of the reaction solution from the first round reaction was employed as DNA template. Thermal cycling parameters were 95 °C, 5 min, 32 x (95 °C for 5 s, 55 °C for 30 s, 72 °C for 30 s) and a final 72 °C for 5 min. The PCR reaction mixtures were subjected to gel electrophoresis using a 1.5% (w/v) agarose gel and a ~600 bp band was excised and purified as described above (3.2.1). The cleaned PCR product was then ligated into the pGEM-T

vector and used to transform the *E. coli* JM109 strain according to the manufacturers instructions (Promega, USA). The Nucleic acid sequence of PCR clone 15#44 encoded peptide sequences obtained from purified native sbHMNGT. The PCR clone 15#44 was employed as template to generate a 306 bp digoxigenin-11-dUTP-labelled probe by PCR using primers 441F (5′-GAGGCGACGGCGGCGGGGCAG-3′) and 442R (5′-CATGTCACTGCTTGCCCCCGACCA-3′) according to the manufacturer′s instructions (Boehringer Mannheim, Germany). The labelled probe was gel purified and employed to screen approximately 50,000 colonies of the above mentioned plasmid library. Hybridizations were carried out O/N at 65 °C in 5xSSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS and 1% (v/v) blocking reagent (Boehringer Mannheim, Germany). Membranes were washed (3 x 15 min) in 0.5xSSC at 60 °C and positive clones identified visually after detection according to manufacturer's instructions (Boehringer Mannheim, Germany). Seven hybridizing clones were isolated, after repeated screening of 14 primary positives, and one full-length clone, *sbHMNGT*, was chosen for further characterization.

3.2.3 Heterologous expression and isolation of recombinant sbHMNGT

Primers EXF1 (5'-AATAAAAGCATATGGGAAGCAACGCGCCGCCTCCG-3') and EXR1 (5'-TTGGATCCTCACTGCTTGCCCCCGACCA-3') were employed to amplify a 1500 bp full-length *sbHMNGT* insert by PCR, using the *sbHMNGT* plasmid as template. The primers contained 5' recognition sites for restriction endonucleases NdeI (EXF1) and BamHI (EXR1). PCR reaction conditions were essentially as above, except for thermal cycling parameters: 95 °C, 3 min, 30 x (95 °C for 5 s, 53 °C for 30 s, 72 °C for 90 s) and a final 72 °C for 5 min. The PCR product was gel purified, digested with NdeI and BamHI, gel purified once again and ligated into the plasmid expression vector pSP19g10L (kindly supplied by Dr. Henry Barnes) (Halkier *et al.*, 1995) which also had been digested with the same restriction enzymes and gel purified. The ligation reaction mixture was then used to transform *E. coli* JM109 cells according to the manufacturer's instructions (Promega, USA). After selection of successfully cloned cells, expression was initiated as per Ford *et al.* (1998). Briefly, 600 µl of an overnight 37 °C culture was added to 300 ml LB broth containing ampicillin (100 µg/ml). The culture was allowed to grow at 28 °C (150 rpm) for 5 hours and isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 0.4 mM. After induction, the culture

was allowed to continue growing O/N and cells were harvested by centrifugation (2500 x g, 10 min). The pellet was resuspended in 9 ml of 200 mM Tris-HCl pH 7.9, 1 mM EDTA, 5 mM DTT and 0.1 mg/ml lysozyme. An equal volume of ice-cold water was added and the mixture allowed to incubate (10 min at room temperature, 20 min on ice). After the addition of 18 μmoles of PMSF and 100 units of DNaseI/ml (Sigma, USA), the suspension was subjected to three freeze and thaw cycles at -20 °C. PMSF was adjusted to 1.5 mM final concentration and the preparation centrifuged at 15,000 x g for 15 min. Negative controls, containing no insert in the plasmid vector, were prepared as above. Using as starting material two lysed 300 ml cultures, the recombinant protein was isolated essentially as described for the native protein (described above, 2.2.3). Isolated recombinant sbHMNGT was stored at -80 °C in multiple aliquots.

3.2.4 Enzyme assays

Qualitative analysis of recombinant sbHMNGT activities were performed as outlined above (2.2.2) except for the incubation period (20 min) and concentrations of reagents: 1.25 mM aglycone (dissolved in ethanol except for flavonoids which were dissolved in ethylene glycol monoether), 1.25 μM ^{14}C-UDP-glucose, 12.5 μM UDP-glucose, 100 ng recombinant sbHMNGT, 4 μg BSA. Quantitative analyses were performed as outlined for qualitative analysis except for incubation period (4 min), and concentrations of reagents: 1, 5 or 10 mM aglycone, 5 μM ^{14}C-UDP-glucose, 0.2 mM UDP-glucose, 200 ng recombinant sbHMNGT, 24 μg BSA. Reaction mixtures for analysis by NMR spectroscopy (total volume 0.5-1 ml) included 2 mM *p*-hydroxymandelonitrile or 6.5 mM geraniol, 3 mM UDP-glucose, 2.5 μg recombinant sbHMNGT, 0.5 mg BSA. After incubation (2 hrs), glucosides were extracted with ethyl acetate and lyophilized using a speed vac concentrator (Savant) prior to NMR analysis as described below (5.2.5).

3.3 Results

3.3.1 Cloning of a full-length cDNA encoding sbHMNGT

The amino acid sequences obtained from peptides generated by Endo Lys-C digestion of native sbHMNGT was utilised to clone the cDNA encoding sbHMNGT. Degenerate oligonucleotides derived from peptide sequence 4 (Figure 2.5a) and a plasmid T7 primer, were employed in nested PCR reactions using a unidirectional *S. bicolor* seedling plasmid library as template. A ~600 bp PCR fragment representing the C-terminal portion of sbHMNGT was isolated and shown to encode peptide sequences 4 and 5 (Figure 2.5a). The partial PCR fragment was then employed to screen the same library that had been used as a template for the PCR reactions. Approximately 50,000 clones were screened, resulting in seven positive isolates, of which 4 were full-length. Sequencing indicated that they all represented identical mRNAs and therefore almost certainly an identical gene. One clone, *sbHMNGT*, was chosen for further study and the nucleic acid sequence of this sbHMNGT-encoding cDNA was deposited in GenBank™ with the accession number AF199453. The deduced translation product (Figure 3.1) comprises 492 amino acid residues and has a predicted molecular mass of 52.9 kDa (52915.81 dalton (gram/mole)) and a theoretical isoelectric point of 5.5. All of the five amino acid sequences generated from peptide sequencing of native sbHMNGT (Figure 2.4a) were contained within the *sbHMNGT*-translation product, and their positions in the sequence are highlighted in Figure 3.1. Minor discrepancies between the peptide sequence of native sbHMNGT and the deduced amino acid sequence of *sbHMNGT* can be explained by errors in peptide sequencing, since Southern blot analyses indicated that *S. bicolor* only contains a single copy of *sbHMNGT* in the genome (Kristensen and Hansen, 2000). A search of the PROSITE sequence motif database using the ExPaSy proteomics server (Swiss Institute of Bioinformatics) revealed no extended stretches of identical sequence, except for a UDP-glucosyltransferase signature sequence between residues 368-411. The *sbHMNGT* translation product was compared with the deduced protein sequences of four known (zmIAAGT, zmUFGT, vvUFGT and psGT) and one putative (meGT) glycosyltransferase-encoding cDNAs (Figure 3.1). The highest degree of similarity between the different glycosyltransferase-encoding sequences was found in the C-terminal region, whereas the N-terminal half only contained a few short segments of high similarity, in support of the theory that the C-terminal end encodes the UDP-

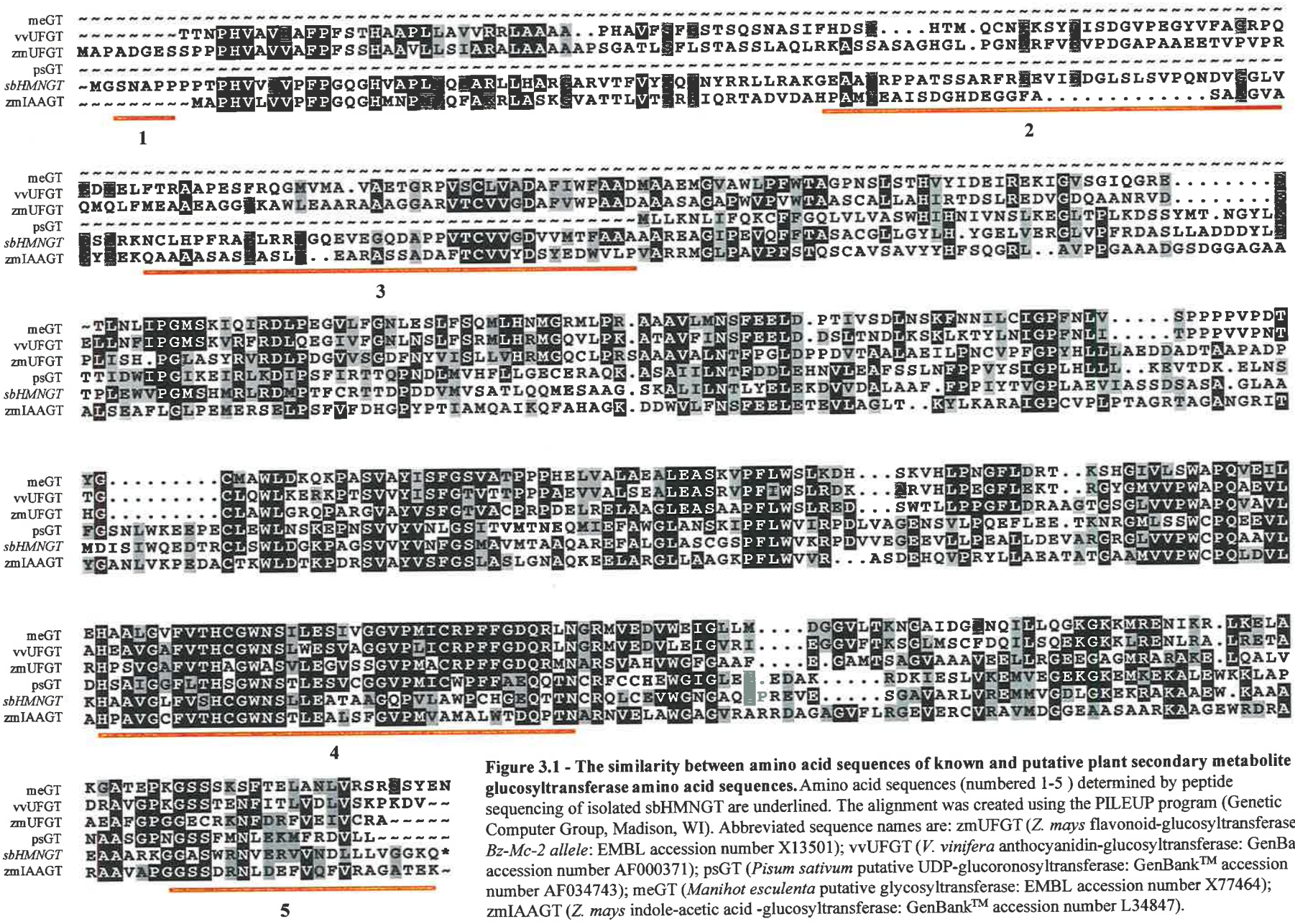


Figure 3.1 - The similarity between amino acid sequences of known and putative plant secondary metabolite glucosyltransferase amino acid sequences. Amino acid sequences (numbered 1-5) determined by peptide sequencing of isolated sbHMNGT are underlined. The alignment was created using the PILEUP program (Genetic Computer Group, Madison, WI). Abbreviated sequence names are: zmUFGT (*Z. mays* flavonoid-glucosyltransferase, *Bz-Mc-2 allele*: EMBL accession number X13501); vvUFGT (*V. vinifera* anthocyanidin-glucosyltransferase: GenBank™ accession number AF000371); psGT (*Pisum sativum* putative UDP-glucoronosyltransferase: GenBank™ accession number AF034743); meGT (*Manihot esculenta* putative glycosyltransferase: EMBL accession number X77464); zmIAAGT (*Z. mays* indole-acetic acid -glucosyltransferase: GenBank™ accession number L34847).

TABLE 3 . 1 Identity and similarity between sbHMNGT and deduced translation products of cDNA sequences encoding several known and putative glucosyltransferases. The N-terminal region is arbitrarily defined as the sequence situated N-terminal of consensus sequence xCLxWL with a split-point at residue 291/292 in sbHMNGT. Calculations of percent similarity and identity were based on pairwise comparisons of cDNA translation products using the Gap program (Genetic Computer Group, Madison, WI). Similar residues are defined as A/G, Y/F, S/T, V/I/L, R/K/H, and D/E/N/Q. Abbreviated sequence names are psGT (*Pisum sativum* partial putative UDP-glucoronosyltransferase: GenBank™ accession number AF034743); zmUFGT (*Z. mays* flavonoid-glucosyltransferase, *Bz-Mc-2 allele*: EMBL accession number X13501); zmIAAGT (*Z. mays* indole-acetic acid -glucosyltransferase: GenBank™ accession number L34847); meGT (*Manihot esculenta* putative glycosyltransferase: EMBL accession number X77464); bnTHGT (*Brassica napus* thiohydroximate-S-glucosyltransferase: Seq. ed. #28 European Patent EP 0 771 878 A1); vvUFGT (*V. vinifera* anthocyanidin-glucosyltransferase: GenBank™ accession number AF000371), and stSGT (*Solanum tuberosum* solanidine-glucosyltransferase: GenBank™ accession number U82367).

	Overall		N-terminal region	
	% Identity	% Similarity	% Identity	% Similarity
psGT	41.6	51.5	32.9	46.3
zmUFGT	36.7	41.5	32.6	37.1
zmIAAGT	34.9	41.3	27.8	35.0
meGT	31.3	41.6	25.3	36.8
bnTHGT	30.7	38.0	24.7	33.3
vvUFGT	30.0	38.7	23.8	33.3
stSGT	28.9	38.0	23.6	31.0

glucose binding domain as discussed in the introductory chapter (1.3.3). A comparison between *sbHMNGT* and a range of known and putative plant glycosyltransferases[1] revealed that *sbHMNGT* shares highest overall identity (41.6%) and similarity (51.5%) with a glucuronosyltransferase-encoding cDNA from *Pisum sativum* (Table 3.1). The acceptor substrate specificity of the glucuronosyltransferase has not been determined, although it has been shown to be required for normal growth and development in pea and alfalfa (Woo *et al.*, 1999). The overall relative similarities and identities are also reflected in the N-terminal comparisons, although at approximately 10% lower levels (Table 3.1). None of the six putative glucosyltransferase-encoding cDNAs isolated from cassava (*M. esculenta*)(Hughes and Hughes, 1994) shared any strong identity with sbHMNGT. There are three possible explanations for this apparent discrepancy. First, none of the clones isolated by Hughes and Hughes (1994) encode a cyanohydrin-glucosyltransferase. Second, one of the cassava clones does encode a cyanohydrin-glucosyltransferase, and the higher degree of similarity between sbHMNGT and monocotyledonous glycosyltransferases (zmUFGT and zmIAAGT) indicates a stronger co-evolution between glucosyltransferases of monocotyledonous plants than between cyanohydrin-glucosyltransferases of different species. Third, cyanohydrin glucosyltransferases specific for aromatic and aliphatic nitriles, respectively, have evolved independently. The latter explanation is supported by the fact that acetone cyanohydrin, the non-aromatic precursor of the cyanogenic glucoside linamarin present in cassava, was not glucosylated by sbHMNGT (Table 3.2). Notably, the two cytochrome P450s, CYP79D1 and CYP79D2, that catalyze the first step in the biosynthesis of cyanogenic glucosides in cassava share 54% positional identity with CYP79A1 from *Sorgum* (Andersen *et al.*, 2000).

3.3.2 Functional expression in *E. coli* and characterization of substrate specificity

Active soluble recombinant sbHMNGT was synthesized in *E. coli* after transformation with an *sbHMNGT*-expression vector and induction with IPTG (Figure 3.2a). There was no measurable difference in the amount of recombinant sbHMNGT-activity, in crude sbHMNGT-expressing *E. coli* extracts made from bacteria grown at 16 °C or 28 °C. This was in contrast to earlier results in which the expression of active soluble

[1] All of the known and putative glycosyltransferase-encoding sequences that were included in the comparison are described in the legends of Figure 2.4, Figure 3.1 and Table 3.1. The following sequences were also compared with sbHMNGT: *Manihot esculenta* putative glycosyltransferases: EMBL accession number X77459, X77461, X77462 and X77463).

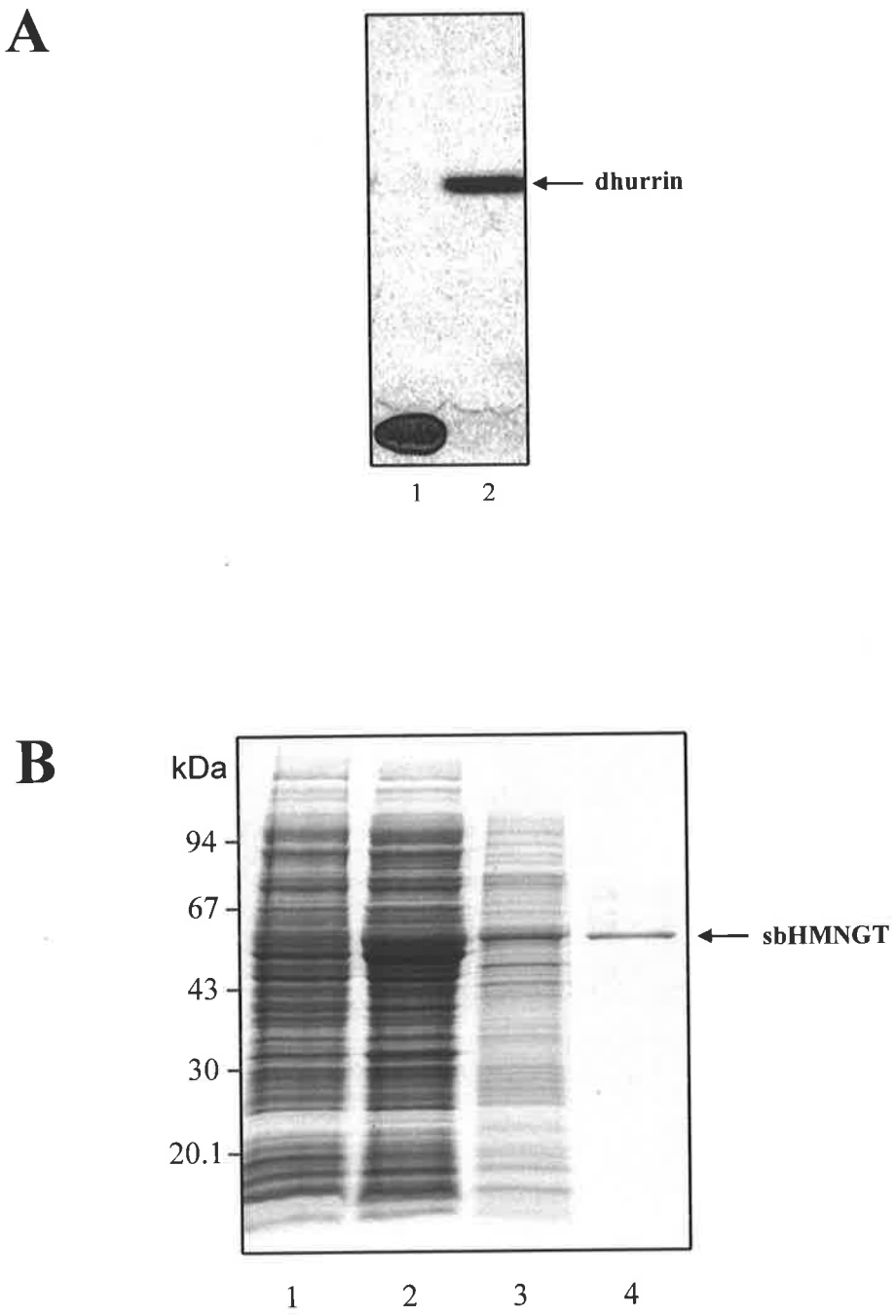


Figure 3.2 - Synthesis and isolation of recombinant sbHMNGT. (A) TLC of *p* hydroxymandelonitrile-glucosyltransferase activity assays conducted using ^{14}C-UDP-glucose as a radioactive marker. The protein preparations used for the assay were composed of crude extracts of *E. coli* transformed with (Lane 1) an empty expression vector pSP19g10L carrying no insert and (Lane 2) the sbHMNGT expression vector construct as detailed in 3.2.3. (B) Polypeptide profile of the fractions obtained during the isolation of recombinant sbHMNGT from *E. coli* as visualised by Coomassie Brilliant Blue staining. The protein preparations consisted of crude extracts of *E. coli* transformed with empty expression vector pSP19g10L carrying no insert (Lane 1) and sbHMNGT-expression vector (Lane 2), pooled Q-Sepharose fractions with sbHMNGT activity (Lane 3), and isolated recombinant sbHMNGT after Yellow 3 chromatography (Lane 4).

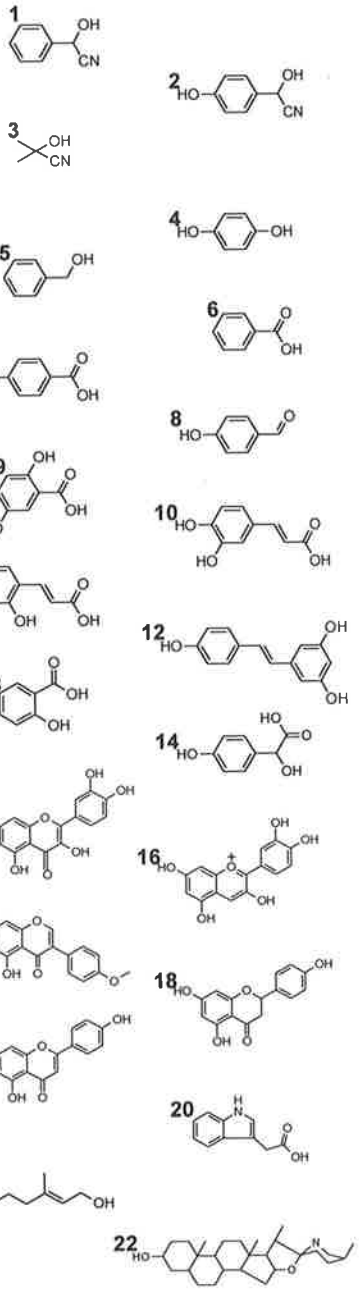
recombinant *V. vinifera* anthocyanidin-3-*O*-glucosyltransferase in *E. coli* could only be achieved at cold growth conditions (16 °C) (Ford *et al.*, 1998). The enzyme was isolated using the same procedure as for the native protein (Figure 3.2b), implying that the recombinant and native protein shared the features which were necessary for this highly selective purification protocol. The yields and specific activities obtained during the isolation of both native and recombinant sbHMNGT were highly variable due to the loss of activity upon freezing and storage at 4 °C, particularly with homogenous preparations. Activity measurements were also affected by side-activities in crude preparations (β-glucosidases and other glucosyltransferases) and the presence of varying amounts of "cold" UDP-glucose in the Yellow 3 eluate. The active recombinant sbHMNGT protein preparations contained the metal ion chelating compound EDTA and the sbHMNGT activity of crude extracts of *S. bicolor* was not stimulated by the addition of 10 mM $MnCl_2$, $MgCl_2$ or $CaCl_2$ (data not shown). Together, these results suggest that sbHMNGT does not require metal ions in order to function in contrast with many other eukaryotic and prokaryotic glycosyltransferases (Busch *et al.*, 1998). Final yield estimates varied between 6-22% with a maximum purification factor of 60 for recombinant sbHMNGT and 420 for native sbHMNGT (Figure 2.3). The apparent V_{max} of isolated recombinant sbHMNGT was approximately 3 nkatals/mg (3 moles dhurrin/mg sbHMNGT/s) in comparison to approximately 0.25 nkatals/mg for native isolated sbHMNGT (Figure 2.3b)[2]. Discrepancies may be explained by the difficulty in accurately estimating the number of active sbHMNGT molecules in a given preparation, since inactive sbHMNGT may co-migrate with active sbHMNGT upon SDS-PAGE.

The substrate specificity of recombinant sbHMNGT was compared to part of the complement of glucosyltransferase activities present in crude desalted extracts of etiolated *S. bicolor* seedlings (Table 3.2). Fifteen of the twenty two substrates tested were glucosylated by the crude extract, whereas only six of these substrates were accepted by the recombinant enzyme. Hence, additional glycosyltransferases with differing substrate affinities to sbHMNGT must also be present in young *Sorghum* seedlings. Hydroquinone (1,4-benzenediol) and *p*-hydroxybenzaldehyde were not glucosylated by sbHMNGT although both compounds reported to serve as substrates when non-homogenous preparations were used (Reay and Conn, 1974). In addition to

[2] The turnover number quoted for sbHMNGT in Jones *et al.* (1999) was incorrectly calculated. The correct figure is given in this text.

Table 3.2 - Comparison of the substrate specficity of recombinant sbHMNGT with that of a desalted crude extract from etiolated Sorghum seedlings. Glucosyltransferase activity was determined by TLC using ^{14}C-UDP-glucose as detailed in 3.2.3. Filled boxes (■) indicate that a radiolabelled product was visualised after incubation with the respective aglycone substrate. Empty boxes (❑) indicate that no radiolabelled substrate could be detected under the experimental conditions employed. Figures in brackets indicated relative V_{max} (percent), in comparison to *p*-hydroxymandelonitrile, for each respective aglycone with calculated standard deviations. The estimated V_{max}-value for *p*-hydroxymandelonitrile was 3 nkatals/mg of sbHMNGT (3 moles dhurrin/mg sbHMNGT/s). n.d., not determined.

SUBSTRATES	ACTIVITY	
	Crude *Sorghum* extract	Recombinant sbHMNGT
cyanohydrins		
1) mandelonitrile	■	(77.8 ± 8.6 %) ■
2) *p*-hydroxymandelonitrile	■	(100 ± 7.2%) ■
3) acetone cyanohydrin	❑	❑
benzyl derivatives		
4) hydroquinone	■	❑
5) benzyl alcohol	■	(13.1 ± 2.1%) ■
6) benzoic acid	■	(4.2 ± 0.8%) ■
7) *p*-hydroxybenzoic acid	■	(n.d.) ■
8) *p*-hydroxybenzaldehyde	■	❑
9) gentisic acid	❑	❑
10) caffeic acid	■	❑
11) 2-hydroxy cinnamic acid	■	❑
12) resveratrol (stilbene)	■	❑
13) salicylic acid	■	❑
14) *p*-hydroxymandelic acid	❑	❑
flavonoids		
15) quercetin (flavonol)	❑	❑
16) cyanidin (anthocyanidin)	❑	❑
17) biochanin A (isoflavone)	■	❑
18) naringenin (flavanone)	■	❑
19) apigenin (flavone)	❑	❑
others		
20) indole acetic acid (plant hormone)	❑	❑
21) geraniol (monoterpenoid)	■	(11.0 ± 0.5%) ■
22) tomatidine (alkaloid)	■	❑



mandelonitrile and *p*-hydroxymandelonitrile, benzyl alcohol, benzoic acid and the monoterpenoid geraniol also acted as substrates for sbHMNGT. NMR spectroscopy confirmed the sbHMNGT mediated *in vitro* synthesis of geraniol glucoside and dhurrin (*p*-hydroxy-(S)-mandelonitrile-β-D-glucopyranoside) (data not shown). The multiple substrate specificity of sbHMNGT prompted analyses of the relative efficiency of utilisation between the differing aglycones. It was not possible to accurately determine comparative Michaelis constants for the different substrates of sbHMNGT since *p*-hydroxymandelonitrile rapidly dissociates in aqueous solutions to *p*-hydroxybenzaldehyde and cyanide, forming an equilibrium which is dependent on the initial concentration of the substrate (Reay and Conn, 1974). Furthermore, *p*-hydroxybenzaldehyde, and possibly *p*-hydroxymandelonitrile and dhurrin, are potent concentration-dependent inhibitors of isolated sbHMNGT, although this effect was negated by the inclusion of BSA (1 mg/ml) in the reaction mixture (data not shown). Accordingly, addition of BSA to the reaction mixtures rendered it possible to estimate V_{max}-values for the substrates that were accepted by sbHMNGT. The enhanced activity of sbHMNGT in the presence of BSA may be explained by the ability of albumin to bind hydrophobic molecules (Zucker *et al.*, 1995). Preliminary experiments in which *p*-hydroxybenzaldehyde or cyanide was added to the sbHMNGT reaction mixture indicated that the aromatic degradation product of *p*-hydroxymandelonitrile, but not cyanide, was capable of inhibiting sbHMNGT activity (data not shown). BSA may therefore enhance sbHMNGT activity by binding *p*-hydroxybenzaldehyde and/or *p*-hydroxymandelonitrile.

Initial tests indicated that sbHMNGT displayed maximal activity towards all tested substrates at a 5 mM concentration of aglycone in the assay mixtures (data not shown). In the absence of comparative Michaelis constant values for the different substrates, and non-saturating concentrations of UDP-glucose, the results of the qualitative assay are only indicative at best. Nevertheless, the quantitative measurements of recombinant sbHMNGT demonstrated a greater preference for *p*-hydroxymandelonitrile, the endogenous substrate, compared to mandelonitrile (Table 3.2). This is in contrast to previous results obtained using nonhomogenous preparations in the absence of BSA (Reay and Conn, 1974). The other three non-cyanogenic substrates were only utilised at less than a fifth of the maximal rate observed with the cyanohydrins. However, the acceptance of benzyl alcohol and benzoic acid as substrates indicates that sbHMNGT is

only partially specific for the nitrile group and that the stereochemistry and/or interactive chemistry of the additional groups present on the hydroxyl-bearing carbon also influence sbHMNGT acceptance. The importance of the correct stereochemistry for sbHMNGT acceptance is highlighted by earlier studies which found that sbHMNGT is stereospecific for the (S)-enantiomer of *p*-hydroxymandelonitrile (Reay and Conn, 1974).

Nevertheless, sbHMNGT displays high selectivity for substrates which are closely similar to mandelonitrile, given that aglycones with only slight differences in chemical structure, such as hydroquinone, gentisic acid and acetone cyanohydrin, do not serve as acceptor substrates. It was, therefore, surprising to find that sbHMNGT also conjugated the monoterpenoid geraniol, with equal efficiency to that of benzyl alcohol (13.1 % of the activity exhibited against *p*-hydroxymandelonitrile compared with 11%). Control reactions with crude extracts of *E. coli* transformed with the expression vector (pSP19g10L) carrying no insert showed no evidence of geraniol conjugation (data not shown). To date there are no reports of the isolation of a monoterpenoid glucosyltransferase, despite the obvious importance of this enzyme class in relation to the aroma of processed fruits and vegetables (Stahl-Biskup *et al.*, 1993).

3.4 Discussion

The presence of a conserved PSPG-motif within all functionally verified plant β-glycosyltransferases suggests that it is possible to predict that any sequence that contains an identifiable PSPG-motif most likely encodes a glycosyltransferase. Whilst it almost certainly is possible to predict the general glycosyltransferase activity from sequence comparisons, this cannot be extended to predicting substrate specificities from sequence comparisons, as no determinant residues or regions of residues have been established. Currently, there are several hundred different putative plant glycosyltransferase-encoding gene and cDNA sequences available in international data banks such as Genbank™. Over one hundred of these sequences have been derived from the *Arabidopsis* genomic sequencing project (Li *et al.*, 2000), and only a handful of these sequences have been functionally verified (Jackson *et al.*, 2000; Lim *et al.*, 2000). With so many putative glycosyltransferases in *Arabidopsis*, it would be highly advantageous to be able to assign a likely substrate specificity to an encoded protein when searching for a glycosyltransferase with a particular function within the plant. The difficulty in assigning glycosyltransferase acceptor substrate specificity is highlighted by the current study. For example, there was almost an equal degree of overall positional identity between sbHMNGT and zmIAAGT (34.9%) as that found between sbHMNGT and zmUFGT (Table 3.1). Yet, grapevine vvUFGT (dicot) and maize zmUFGT (monocot), which both glucosylate anthocyanidins *in vivo* (Ford *et al.*, 1998), have only a marginally greater positional identity (42%). A larger set of functionally verified glycosyltransferase-encoding cDNAs are required to further our understanding on this matter. Substantial progress in this area may be dependent on determination of the three-dimensional structures of plant glycosyltransferases. In recognition of this, preliminary attempts at crystallizing sbHMNGT were undertaken, albeit, with no success so far (data not shown).

Notwithstanding the above observations, two potential candidates for acceptor substrate specificity determining regions at the N-terminus of eukaryotic glycosyltransferases merit further study. Moehs *et al.* (1997) identified a region of 34 amino acids (residues 109 - 143 of potato solanidine-glucosyltransferase; residues 134 - 168 of mammalian steroid glucuronosyltransferases) in which the steroid solanidine-glucosyltransferase from potato exhibited a high degree of positional similarity (56 - 62%) with three

mammalian steroid-glucuronosyltransferases. Ciotti *et al.* (1998) also identified a region of 28 amino acids (residues 152 - 180) that has unique and identifying characteristics in three classes of detoxifying mammalian glucuronosyltransferases with different aglycone substrates. It will be interesting to reexamine the equivalent regions in sbHMNGT once additional cyanohydrin-glucosyltransferase-encoding sequences have been isolated from other plant species.

Investigations into the qualitative and quantitative substrate specificity of recombinant sbHMNGT showed a strong preference for the cyanohydrin present in *S. bicolor*. Similarly, when recombinant *V. vinifera* anthocyanidin-glucosyltransferase was assayed against a wide range of different aglycones, it was found to be strictly specific for flavonols and anthocyanidins only, with a strong preference for the latter (Ford *et al*, 1998). Both sbHMNGT and *V. vinifera* anthocyanidin-glucosyltransferase are involved in the final stages of predominant secondary metabolite biosynthetic pathways. Their presence coincides with the highly tissue- and development-specific accumulation of their respective glucosides (Reay and Conn, 1974; Halkier *et al.*, 1989; Boss *et al.*, 1996). A possible scenario then is that the sole *in vivo* function of these enzymes is related to the glucosylation of unique and single secondary metabolites. It is, nevertheless, likely that enzymes present at the end of biosynthetic pathways have a broader substrate specificity than those preceding upstream. Such broad substrate specificity would facilitate evolution of novel pathways for secondary metabolite biosynthesis and for catabolism of xenobiotics. This is illustrated by the finding that CYP71E1 and sbHMNGT also accept phenylalanine-derived oximes and cyanohydrins (mandelonitrile) respectively, whilst the first enzyme of the pathway, CYP79A1, is specific for tyrosine (Kahn *et al.*, 1999).

Although sbHMNGT can glucosylate mandelonitrile with a high degree of efficiency (Table 3.2) *Sorghum* does not accumulate sambunigrin and prunasin (the glucosides of mandelonitrile (Mao *et al.*, 1965; Rosen *et al.*, 1975)). This is due to the limiting substrate specificity of CYP79A1 (Kahn *et al.*, 1999), the first enzyme of the pathway. Hence, a glycosyltransferase will only ever glycosylate those substrates that it **meets** and **accepts**. It is therefore possible that glycosyltransferases with broad substrate specificities *in vitro* may exhibit exclusive activity against fewer aglycones *in vivo*. For example, a recombinant salicylic-acid inducible glucosyltransferase in *N. tabacum* that

accepts both salicylic acid and indole acetic acid and glucosylates hydroxybenzoic acid derivatives at both the carboxyl- and aromatic hydroxyl-function was isolated by Lee and Raskin (1999). It is difficult to believe that such a promiscuous enzyme serves a particular function in tobacco, if it is not restricted further *in planta* with respect to the range of aglycones which it is presented with. One mechanism for such a restriction is differential compartmentalization between substrate and enzyme. This concept is discussed further in the next chapter (Chapter 4).

Chapter 4

Reconstitution of the cyanogenic glucoside biosynthetic pathway *in vitro* and in transgenic *Arabidopsis thaliana*

4.1 Introduction

The three enzymes required for the conversion of tyrosine to dhurrin have been isolated (see Chapter 2) and their corresponding cDNAs have been cloned and expressed in an active state in *E. coli* (see Chapter 3). Therefore, it is now possible, in principle, to transfer the entire pathway to alternative systems to gain further insight into the biology of cyanogenic glucosides. Such reconstitutions have previously been done in the absence of sbHMNGT, by employing crude extracts or reconstituting only part of the pathway (Møller and Seigler, 1998). McFarlane *et al.* (1975) were first to report both the separation and reconstitution of individual enzyme activities required for the biosynthesis of cyanogenic glucosides. This simple experiment entailed centrifugal separation of microsomes from the supernatant of a crude extract of *S. bicolor*, followed by recombination in a test tube to effect the conversion of ^{14}C-tyrosine to ^{14}C-dhurrin. In a more sophisticated experiment, Kahn *et al.* (1997) reconstituted the pathway using isolated recombinant CYP79A1, native CYP71E1 and NADPH-P450 oxidoreductase, and a crude *Sorghum* extract with glucosyltransferase activity. These studies suggest that the cyanogenic glucoside biosynthetic pathway only requires a limited set of polypeptides that can interact functionally in the absence of a plant cell structure. Recently, part of the pathway was transferred to acyanogenic plants when Bak *et al.* (1999) and Bak *et al.* (2000) transformed *A. thaliana* and tobacco (*Nicotiana tabacum*) with CYP79A1 (Bak *et al.*, 1999; Bak *et al.*, 2000) and CYP79A1 together with CYP71E1 (Bak *et al.*, 2000). A large range of glycosidic metabolites of the cytochrome P450-generated intermediates were found to accumulate *in planta* in the absence of native *p*-hydroxymandelonitrile glucosyltransferase activities (Figure 4.1).

This chapter describes a full reconstitution of the biosynthetic pathway with a defined composition *in vitro* and in transgenic *A. thaliana*, and therefore verifies the earlier suggestion that the pathway only requires a limited set of proteins to function. These experiments illustrate that entire secondary plant product metabolic pathways may be plastic in relation to their environmental requirements. Furthermore, the ability to generate cyanogenic cultivars of acyanogenic plants can form the basis of an experimental system with highly defined controls in the study of the biological role of cyanogenic glucosides.

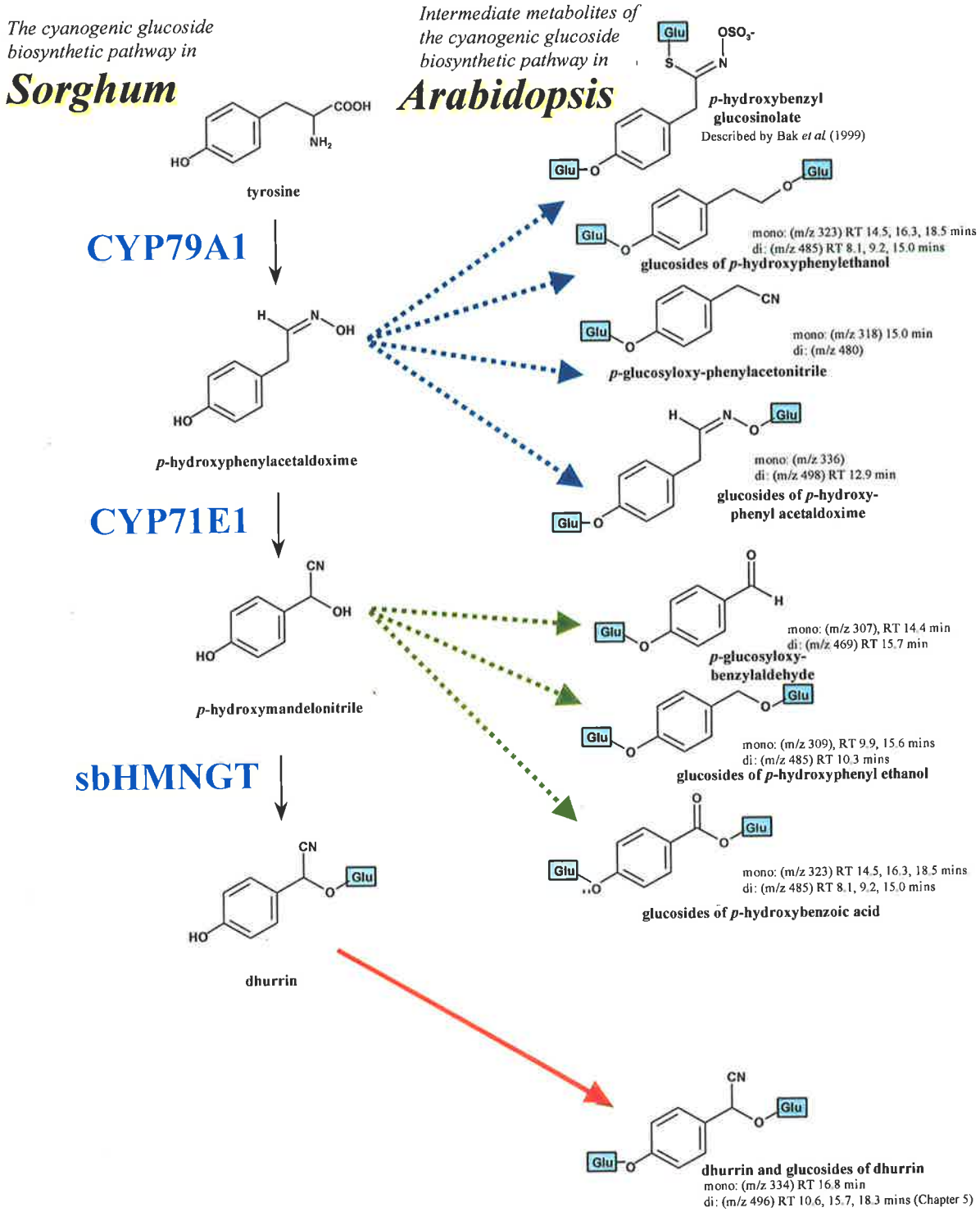


Figure 4.1 A schematic representation of glucosidic metabolites which may be generated from intermediates by the cyanogenic glucoside biosynthetic pathway in transgenic *Arabidopsis*. The aglycone structures of the metabolites in 2x-lines have been identifed by Bak *et al.* (2000). The expected mass $[M + Na]^+$ of monoglucosides and diglucosides of each metabolite are indicated next to the name of each metabolite, together with the retention time of peaks with dominant ions of the corresponding mass observed in extracts of 2x- and 3x-lines by LC-MS. The positions of glucosyl-moeities have been added to all potential conjugation sites and are highlighted in milk blue. The identification and characterization of *p*-hydroxybenzylglucosinolate is detailed in Bak *et al.* (1999).

4.2 Materials and Methods

4.2.1 Materials

Recombinant CYP79A1 isolated according to Halkier *et al.* (1995) was a kind gift from Theo Fahrendorf. Recombinant CYP71E1 isolated according to Bak *et al.* (1998) was a kind gift from Søren Bak. *S. bicolor* NADPH-P450 oxidoreductase isolated according to Kahn *et al.* (1997) was a kind gift from Rachel Kahn. *p*-Glucosyloxy-benzoic acid and *p*-hydroxybenzoic acid-glucoside were kind gifts from Dr. Lutz Heide (Universität Tübingen, Germany). *p*-Glucosyloxyphenylacetonitrile was kindly synthesized by Dr. M. Saddik Motawia (Royal Veterinary and Agricultural University, Copenhagen). *p*-Hydroxyphenylacetaldoxime was a kind gift from Eric Conn (University of California, USA). All biochemicals and reagents were obtained from Sigma (USA) except where indicated.

4.2.2 Reconstitution of the cyanogenic glucoside biosynthetic pathway *in vitro*

General reaction mixtures (total volume 50 µl) included 100 mM Tris-HCl (pH 7.5), 2.2 µM ^{14}C-tyrosine (0.05 µCi/µl, 443 mCi/mmol, Amersham Pharmacia Biotech, Sweden), 100 µM UDP-glucose, 1.5 mM NADPH, 2 mM DTT, 1 mg/ml of sonicated L-α-dilauroyl phosphatidylethanolamine micelles, 1 mg/ml BSA, ~250 ng recombinant sbHMNGT, ~125-250 ng recombinant CYP79A1, ~250-500 ng recombinant CYP71E1 and 0.075 U isolated NADPH-P450 oxidoreductase (Kahn *et al.*, 1997). ^{14}C-tyrosine was added last and the reactions incubated for 10 min at 30 °C. Controls were prepared with various enzyme components replaced by MQ H_2O. Reaction mixtures were cooled on ice, applied directly to TLC plates and TLC performed as described above (2.2.2). A solvent containing ethyl acetate:toluene (1:5 v/v) (solvent system 2) was employed to separate hydrophobic intermediates of the cyanogenic glucoside biosynthetic pathway. The relative mobility of pathway intermediates in the reconstitution preparations were confirmed by co-migration with authentic standards of *p*-hydroxyphenylacetaldoxime, *p*-hydroxybenzaldehyde and *p*-hydroxyacetonitrile. Solvent system 1 refers to the solvent described in section 2.2.2. As a positive control of the microsomal component of the pathway, all of the added polypeptides were replaced in the above reaction mixture with 8 µl (~150 ng of protein) of a microsomal preparation from 2-day old 'green-grown' seedlings of *S. bicolor*. Microsomes were prepared as described above (2.2.3),

except as follows; following the first centrifugation the supernatant was discarded and the yellow precipitate resuspended in buffer A with a small paint brush, followed by storage at -80 °C.

4.2.3 Transformation of *Arabidopsis thaliana* with *sbHMNGT*

Preparation of 35S*-sbHMNGT *vector-construct

Two vector-constructs were prepared: pPZP111-GT and pPZP221-GT contain *sbHMNGT* fused to the cucumber mosaic virus 35S promoter and polyadenylation site (Töpfer *et al.*, 1987) inserted into binary *Agrobacterium/E. coli* vectors pPZP111 and pPZP221, respectively (Hajdukiewicz *et al.*, 1994). Briefly, *sbHMNGT* cDNA was excised with *Eco*RI and *Xba*I from the pCDNAII clone of *sbHMNGT* (described above, 3.2.2) and ligated into pRT101 (Töpfer *et al.*, 1987), linearized with *Eco*RI and *Xba*I, to introduce the 35S promoter and polyadenylation site. *sbHMNGT* together with the 35S promoter and polyadenylation site were then excised from the pRT101-GT construct with *Hind*III and ligated into pPZP111 and pPZP221 linearized with *Hind*III. All linearized vectors were dephosphorylated with shrimp alkaline phosphatase (Boehringer Mannheim, Germany) prior to ligation.

Transformation of* A. thaliana *with pPZP111-GT and pPZP221-GT

A. thaliana containing CYP79A1- and CYP71E1-encoding cDNAs (2x.79A1.71E1, Bak *et al.*, 2000) were selected with kanamycin and transformed with the pPZP221-GT plasmid preparation. Wild-type *A. thaliana* L. (cultivar ecotype Columbia) was transformed with the pPZP111-GT plasmid preparation. All transformations were done using the floral dip method (Clough and Bent, 1998). *Arabidopsis* transformed with *sbHMNGT*, CYP79A1- and CYP71E1-encoding cDNAs, and all three cDNA constructs, were termed 1x-, 2x-, and 3x-*Arabidopsis*, respectively. Briefly, *Agrobacterium tumefaciens* (C58C1/pGV3850) was transformed with pPZP111-GT and pPZP221-GT by electroporation (Wen-Jun and Forde, 1989). Two 200 ml cultures of YEP (1% (w/v) bactopeptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) containing 100 μg/ml rifampicin, 100 μg/ml chloramphenicol (pPZP111-GT) or 75 μg/ml spectinomycin (pPZP221-GT)) were inoculated with freshly plated *Agrobacterium* transformed with pPZP111-GT and pPZP221-GT, respectively, and propagated (24 hrs, 28 °C). Cells were pelleted (1000 x g, 20 min) and resuspended in 400 ml of 10 mM

$MgCl_2$, 5% (w/v) sucrose and 0.005% (v/v) Silwet L-77. Stems of potted *A. thaliana* (2x.79A1.71E1 for pPZP221-GT; wt for PZP111-GT) with newly developed siliques were immersed upside down in the appropriate *Agrobacterium* cultures for 5 minutes. The pots were then enclosed in plastic bags, inflated by mouth, closed with rubber bands and placed in *Arabidopsis* growth cabinets (AR-60L, Percival, Boone, IA) (12 hr light, 20 °C, 70% relative humidity, 100-120 µmol photons/m^2/s) for two days. The plastic bags were removed and the seedlings allowed to grow in the same growth cabinets until the siliques had become dry and pale yellow, when the seeds were harvested and stored at 4 °C.

Seeds were selectively germinated in the above mentioned growth cabinets on Murashige and Skoog medium (Sigma, USA) containing 2% (v/v) sucrose, 200 µg/ml gentamycin (PZP221-GT) or 50 µg/ml kanamycin sulfate (PZP111-GT), and 0.8% (v/v) agar. Putative transgenic plants were selected visually on the basis of growth and health, followed by transplantation to soil and growth in a chamber as described above. Primary transformants with sbHMNGT activity were identified by dhurrin analyses as described below (4.2.4, 4.2.6).

4.2.4 Plant growth, protein and glucoside extraction

Seeds of putative 3x-lines, heterozygotic for *sbHMNGT*, were selected on Murashige and Skoog media (Sigma, USA) containing the appropriate antibiotics as described above (4.2.3), transferred to pots containing soil and grown in growth cabinets for 3-4 weeks as described above (4.2.3), except for an 8 hr light period. Leaf tissue (50-100 mg) was harvested, weighed and placed in 2.0 ml Sarstedt safe seal tubes. Methanol (85% (v/v), 1 ml) was added, incubated (100 °C, 5 min), cooled and centrifuged (5 min, 15,000 x g). The solution was evaporated in a speed vac concentrator (Savant) and resuspended in 62.5 µl of H_2O per 10 mg of plant material (f.w.), followed by centrifugation (5 min, 15,000 x g) and storage at -80 °C. Proteins were extracted from 50 mg lots of plant material as described for *S. bicolor* (see 2.2.3). Glucoside extracts of 3 day-old 'green-grown' *Sorghum* seedlings (28 °C, >95% relative humidity) were prepared as described below (5.2.2). The glucoside extracts of *Sorghum* were resuspended in 20-40 µl of H_2O per 10 mg of plant material (f.w.) after evaporation with a speed vac concentrator (Savant).

4.2.5 Synthesis of ^{14}C-dhurrin in transgenic leaves

Leaves from fully developed transgenic *Arabidopsis* plants (3-4 weeks old) were excised at the base of the petiole and placed in 2.0 ml Sarstedt safe seal tubes containing 10 µl of ^{14}C-tyrosine (0.05 µCi/µl, 443 mCi/mmol, Amersham Pharmacia Biotech, Sweden). Twenty µl of H_2O was added to each tube after most of the radioactive tracer had been absorbed (1-2 hr). Leaves were then left O/N under light in a humid chamber, extracted as described above (4.2.4) and subjected to TLC and phosphorimager analyses as described above (2.2.2).

4.2.6 Analytical methods

sbHMNGT assays and TLC were conducted as described above (2.2.2). For TLC, authentic (unlabelled) dhurrin (Extrasynthèse, France) was added to samples of negative controls (wt *Arabidopsis* transformed with pPZP111 (empty, no cDNA insert) and pPZP221 (empty, no cDNA insert)), and the relative mobility recorded after visual inspection under UV-light.

Liquid chromatography mass spectrometry (LC-MS)

LC-MS was done on a HP1100 LC coupled to a Bruker Esquire-LC ion trap mass spectrometer. The reversed-phase LC conditions were as follows: A C_{18} column (Chrompack Inertsil 3 ODS-3 S15x3 COL CP 29126) was used with the mobile phases A: 50 µM sodium acetate, B: methanol. The flow rate was 0.25 ml/min and the gradient program was: 0-30 min: linear gradient 10-60% B; 30-35 min: isocratic 60 % B; 35-40 min: linear gradient 60-90% B; 40-45 min: isocratic 100% B.The mass spectrometer was run in the positive ion mode. Twenty µl of each preparation was injected. The TIC (total ion current), RICs (reconstructed ion chromatograms) and UV traces (230, 260 and 280 nm) were used to locate peaks, and the $[M+Na]^+$ adduct ions were used for identifications and quantified using the Bruker DataAnalysis Esquire-LC (1.6m) analytical program (Bruker Daltonik). Samples of dhurrin (Extrasynthèse, France), *p*-glucosyloxy-benzoic acid, *p*-hydroxybenzoic acid-glucoside and *p*-glucosyloxy-phenylacetonitrile were used as standards. Each reconstructed ion chromatogram (RIC) corresponding to a particular mass was 23 units larger than expected, due to the addition of sodium acetate to every sample and the resultant formation of a sodium-glucoside complex. Hence, an RIC of

mass 334 corresponded to a glucoside ion with a molecular weight of 311, the expected molecular mass of dhurrin (Figure 4.1). The increase in apparent molecular weight was confirmed by LC-MS analysis of authentic standards of dhurrin (Extrasynthèse, France) and amygdalin (Sigma, USA).

Cyanide assays

Cyanide assays of potentially cyanogenic *A. thaliana* were conducted as follows: Approximately 20 mg of leaf material taken from 1x, 2x and 3x-*Arabidopsis* plants, respectively, were cut into small pieces and quickly added to a 2.0 ml Sarstedt safe seal tube containing 200 µl of 50 mM MES buffer (pH 6.5). A 0.5 ml thin walled PCR tube (with lid and rim removed) containing 200 µl of 1 M NaOH was then quickly placed and enclosed in each 2.0 ml Sarsted safe seal tube. The tubes were then frozen in liquid nitrogen before incubation O/N at 37 °C. Cyanide liberated from the leaf tissue was subsequently trapped in the separate tube of 1 M NaOH. One hundred µl of the 1 M NaOH solution was removed and 50 µl of acetic acid added. The cyanide was detected as follows: Two hundred µl of reagent A (2.5 g/l succinimide, 1 g/l N-chlorosuccinimide) and 200 µl of reagent B (300 ml/l pyridine, 60 g/l barbituric acid) were added sequentially to the cyanide preparation followed by one volume of MQ H_2O. The colour reaction was then allowed to develop for at least 5 minutes before the absorbance at 585 nm was measured using a Shimadzu spectrophotometer. Samples of dhurrin (Extrasynthèse, France) and potassium cyanide (Sigma, USA) were used as standards.

Cyanide assays for selection of 3x-lines were conducted as follows: Ten µl of emulsin (almond β-glucosidase type II, 10 mg/ml, Sigma) and 10 µl of phosphate-buffer (1 M, pH 6.5) were added to 100 µl of crude plant extracts, prepared as described above (2.2.3), and allowed to hydrolyze in 1.5 ml Eppendorf tubes for 2 hours at 30 °C. Tubes containing hydrolyzed glucosides were frozen in liquid nitrogen, opened and 40 µl of 6 M NaOH added. The samples were thawed and stored at room temperature for 20 minutes before addition of 50 µl of acetic acid. Detection of cyanide was performed as described above.

Quantification of dhurrin

Dhurrin was quantified in extracts from six independent 3x-lines prepared as described above (4.2.4). One leaf was taken from four different plants of each line, extracted as described above (4.2.4) and quantified by HPLC as described below (5.2.5), using amygdalin (Sigma, USA) as an internal standard for extraction and analysis. Dhurrin (Extrasynthèse, France) was employed as a quantitative standard.

4.3 Results

4.3.1 Reconstitution of the cyanogenic glucoside biosynthetic pathway *in vitro*

The biosynthetic pathway for the cyanogenic glucoside dhurrin was reconstituted by combining isolated recombinant CYP71E1, CYP79A1 and sbHMNGT with isolated NADPH-P450 oxidoreductase in the presence of lipid micelles. ^{14}C-dhurrin was synthesized from administered ^{14}C-tyrosine (Figure 4.2a), accompanied by the two intermediates *p*-hydroxyphenylacetaldoxime, *p*-hydroxyphenylacetonitrile and the breakdown-product of *p*-hydroxymandelonitrile, *p*-hydroxybenzylaldehyde (Figure 4.2b). Whilst *p*-hydroxyphenylacetaldoxime and *p*-hydroxymandelonitrile are released following completion of the catalytic function of CYP71E1 and CYP79A1, respectively, *p*-hydroxyphenylacetonitrile is released from CYP71E1 prior to completion of the enzyme's synthesis of *p*-hydroxymandelonitrile (Figure 1.1). Despite several attempts at optimizing the relative proportion of the three enzymes, it was not possible to synthesize dhurrin without generating the two intermediates released by the cytochrome P450s (data not shown). Furthermore *p*-hydroxybenzaldehyde accumulated in all experiments although a highly active fraction of isolated sbHMNGT was employed. Together, these data suggest that the pathway was only partially operational. In particular, the generation of *p*-hydroxyphenylacetaldoxime and *p*-hydroxyphenylacetonitrile can be explained by a poorly functioning CYP71E1 component as earlier studies had shown that isolated recombinant CYP71E1 was highly labile in comparison with CYP79A1 (Kahn *et al.*, 1999), and that native CYP71E1 also was more labile than CYP79A1 in dialysed membrane preparations (Møller and Conn, 1980). An alternative reason for the release of intermediates during reconstitution could be that the pathway requires an additional component, an alternative organization of compartments and enzymes or a different membrane structure to secure channeling and/or avoid release of intermediates. In comparison, microsomal extracts of *Sorghum* did not generate any intermediates except for the end-product *p*-hydroxybenzaldehyde (Figure 4.2b, lane 3) as had been shown earlier (McFarlane *et al.*, 1975; Møller and Conn, 1980). If the absence of additional components or the absence of a scaffolding structure is the main reason for the imperfect reconstitution, then one would expect that reconstitution within a plant cell would be more optimal. This issue was addressed next.

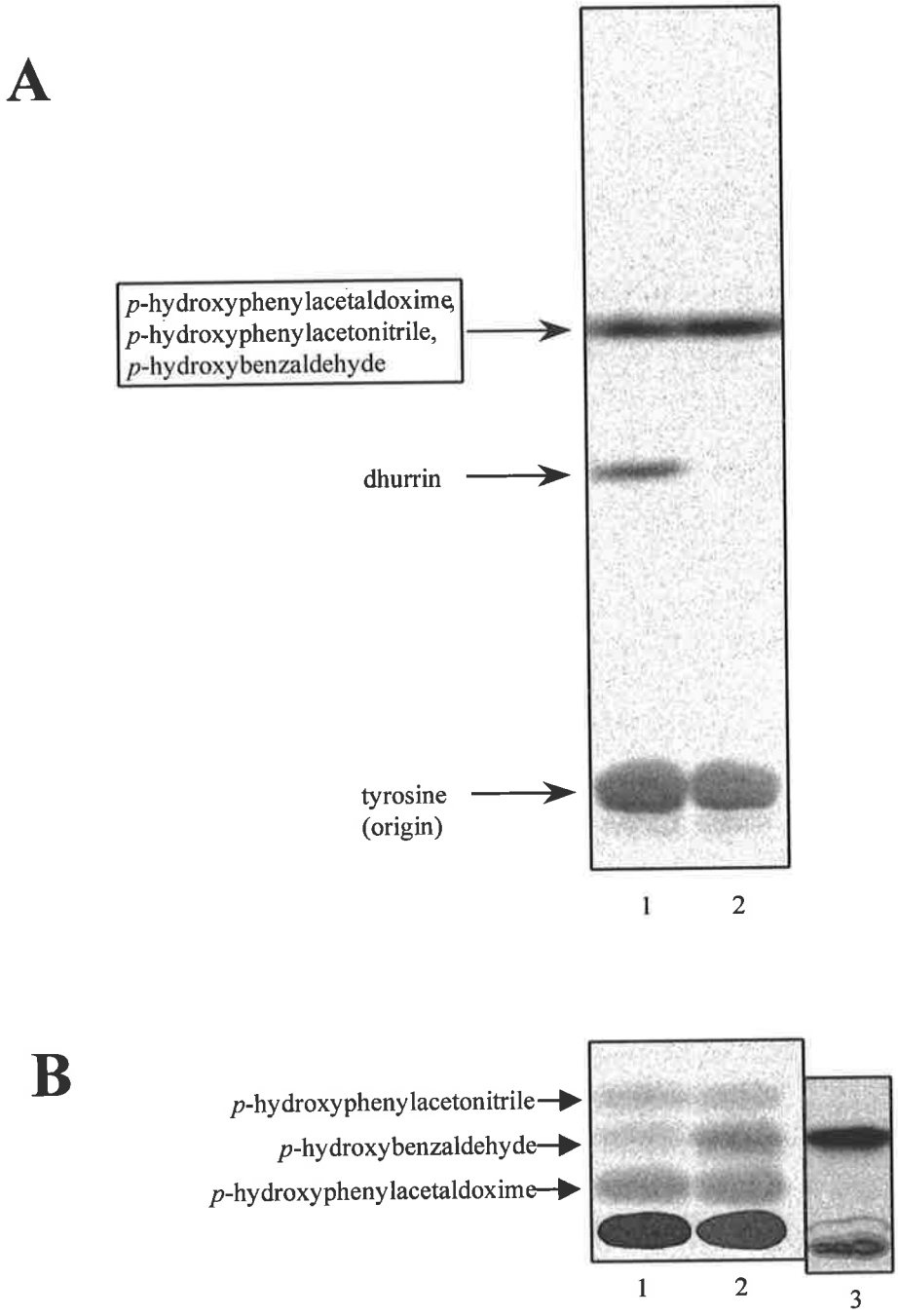


Figure 4.2 Reconstitution of the cyanogenic glucoside biosynthetic pathway *in vitro* using isolated recombinant CYP79A1, CYP71E1 and sbHMNGT. (A) TLC analysis of products from reconstitution assays with (Lane 1) or without (Lane 2) sbHMNGT (solvent system 1 (4.2.2)). (B) TLC analysis of products from reconstitution assays with separation of hydrophobic intermediates (solvent system 2 (4.2.2)). Lane 1: CYP79A1, CYP71E1, sbHMNGT, Lane 2: CYP79A1, CYP71E1, Lane 3: A microsomal extract of young *Sorghum* seedlings as a source of protein. Reaction mixtures contained an approximate molar ratio between CYP79A1, CYP71E1 and sbHMNGT of 1 (Lane 1 and 2; panel A and B). ^{14}C-tyrosine was employed as a radioactive marker in all reaction mixtures. Dhurrin and the three intermediates were identified by co-migration with authentic standards.

4.3.2 Reconstitution of the cyanogenic glucoside biosynthetic pathway in transgenic *A. thaliana*

***Transfer of the cyanogenic glucoside biosynthetic pathway to* A. thaliana**

Two homozygotic lines of transgenic *A. thaliana* (2x.79A1.71E1; termed 2x-lines; Bak *et al.*, 2000), functionally expressing CYP79A1 and CYP71E1 under the regulation of constitutive 35S promoters, were transformed with *sbHMNGT* fused to the cucumber mosaic virus 35S promoter and polyadenylation site (Figure 4.3). The resulting transformants that contained all three genes of the pathway were termed 3x-plants. Wild-type *Arabidopsis* was also transformed with the *sbHMNGT*-35S construct, and these were termed 1x-plants. Potentially transgenic seed lines of 3x and 1x, respectively, were identified by selective growth in Murashige and Skoog medium containing the appropriate plant antibiotics. Lines expressing sbHMNGT only (1x) were identified by sbHMNGT-activity assays (data not shown), whilst 3x-lines were selected on the basis of cyanide tests using emulsin as described above (4.2.6) (data not shown). Protein preparations of leaves from the potentially cyanogenic 3x-lines were then analyzed for sbHMNGT activity, which showed that the majority of lines contained sbHMNGT activity at varying levels (Figure 4.4a). Selected lines of the same plants were subsequently fed ^{14}C-tyrosine and TLC analyses of glucoside extracts showed that a significant amount of the applied ^{14}C-tyrosine had been converted into ^{14}C-labelled dhurrin (Figure 4.4b). The apparent discrepancy between the sbHMNGT-activity assay and the leaf feeding study of 3x.8.7.1.2 can possibly be explained by a high threshold for minimum activity in the sbHMNGT assay (Figure 4.4a: Lane 5; Figure 4.4b: Lane 4).

Seedlings of sucessfully transformed 1x-, 2x- and 3x- lines were allowed to grow for 3-4 weeks in a growth cabinet prior to harvest. Glucoside extracts were prepared from leaves and subjected to LC-MS analysis. The entire LC-MS profiles of the analyzed 2x- and 3x-lines were scanned for novel peaks with single dominant ions that were not present in the profile of the 1x-lines, and only these major novel peaks are discussed below. Figure 4.5 illustrates the typical UV and ion $[M + Na]^+$ profiles obtained with the 1x-, 2x- and 3x-lines. There was no detectable dhurrin in 1x- or 2x-plants. In contrast, 3x-plants contained both dhurrin (RT 17.0 min; m/z 334 $[M + Na]^+$) and three peaks with dominating ions of the expected mass of dhurrin-glucosides (RT 10.6, 15.7

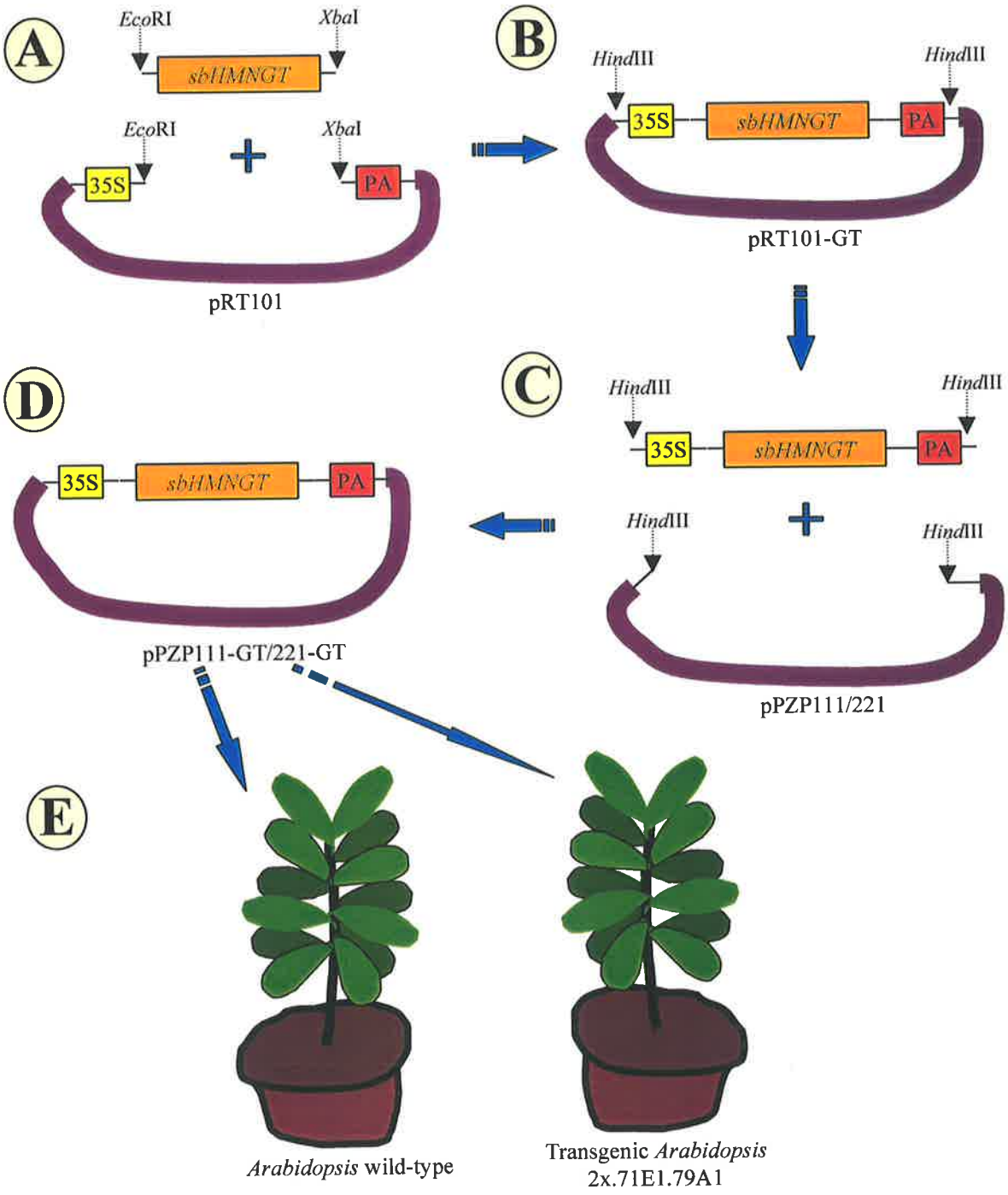


Figure 4.3 Construction of pPZP/pRT101/*sbHMNGT*-vector constructs. (A) *sbHMNGT* was ligated into the internal polylinker of pRT101 excised with *Eco*RI and *Xba*I. (B) *sbHMNGT* including the 35S promoter and polyadenylation site (PA) were excised with *Hind*III from pRT101-GT and (C) ligated into the *Hind*III site of pPZP111 and pPZP221 to generate (D) pPZP111-GT and pPZP221-GT, respectively. (E) The two vector constructs were used to transform *Agrobacterium tumefaciens* and selected with antibiotics towards *Agrobacterium tumefaciens* for successful transformants. Wild-type and 2x.71E1.79A1 *A. thaliana* were then inoculated with cultures of *Agrobacterium tumefaciens* transformed with pPZP111-GT and pPZP221-GT, respectively.

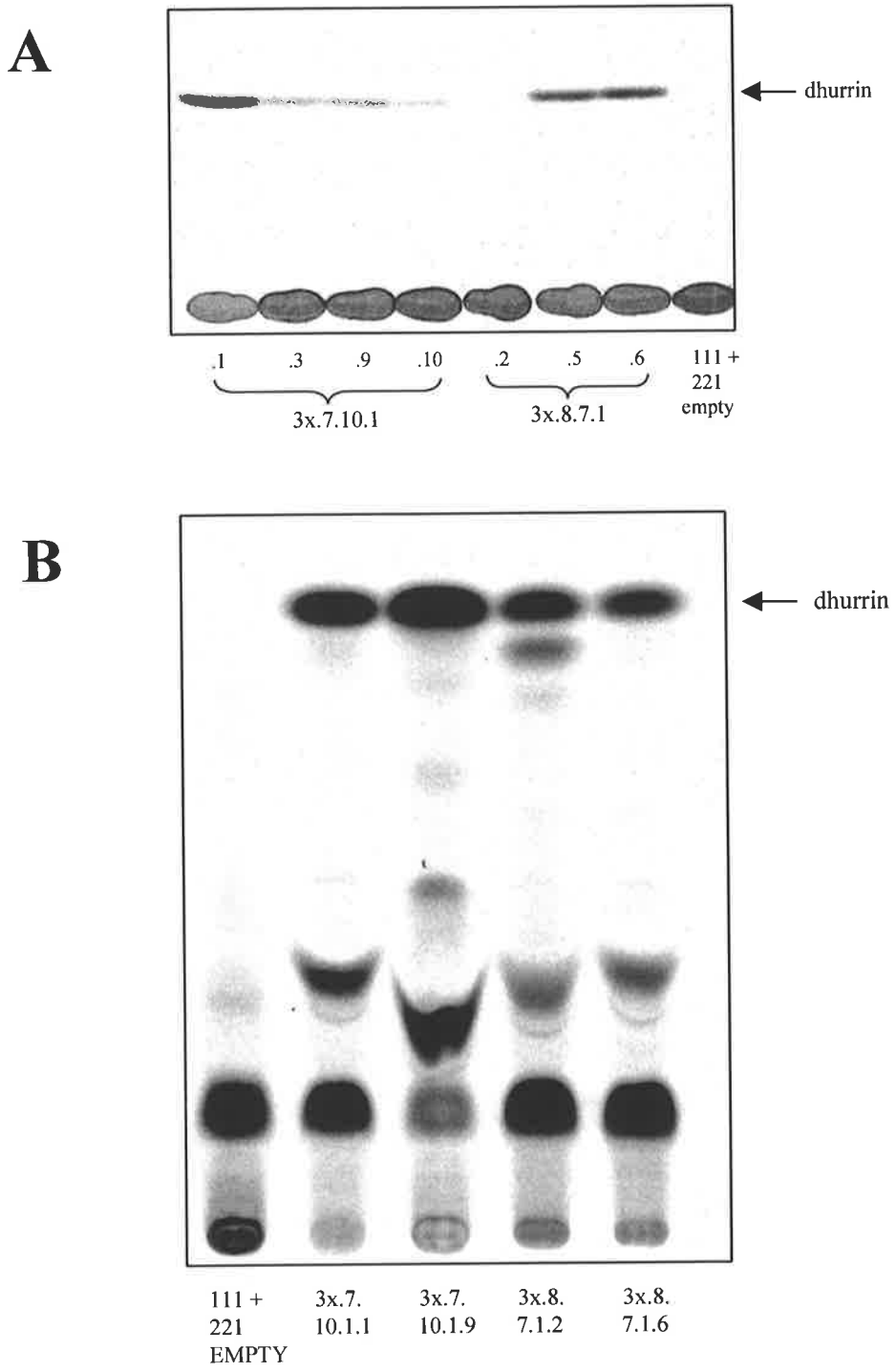


Figure 4.4 sbHMNGT activity and dhurrin synthesis in transgenic *A. thaliana*. (A) TLC analysis of sbHMNGT activity assays performed using crude protein extracts of transformed plants, selected as described above (4.2.3). ^{14}C-UDP-glucose and *p*-hydroxymandelonitrile were employed as substrates. (B) TLC analysis of glucoside extracts made from leaves of transgenic 3x-lines that were excised and fed with ^{14}C-tyrosine O/N. Unlabelled authentic samples of dhurrin were added to lane one and the relative migration of this standard is indicated with an arrow. Transgenic *A. thaliana* transformed with empty pPZP111 and pPZP221 carrying no insert were used as controls.

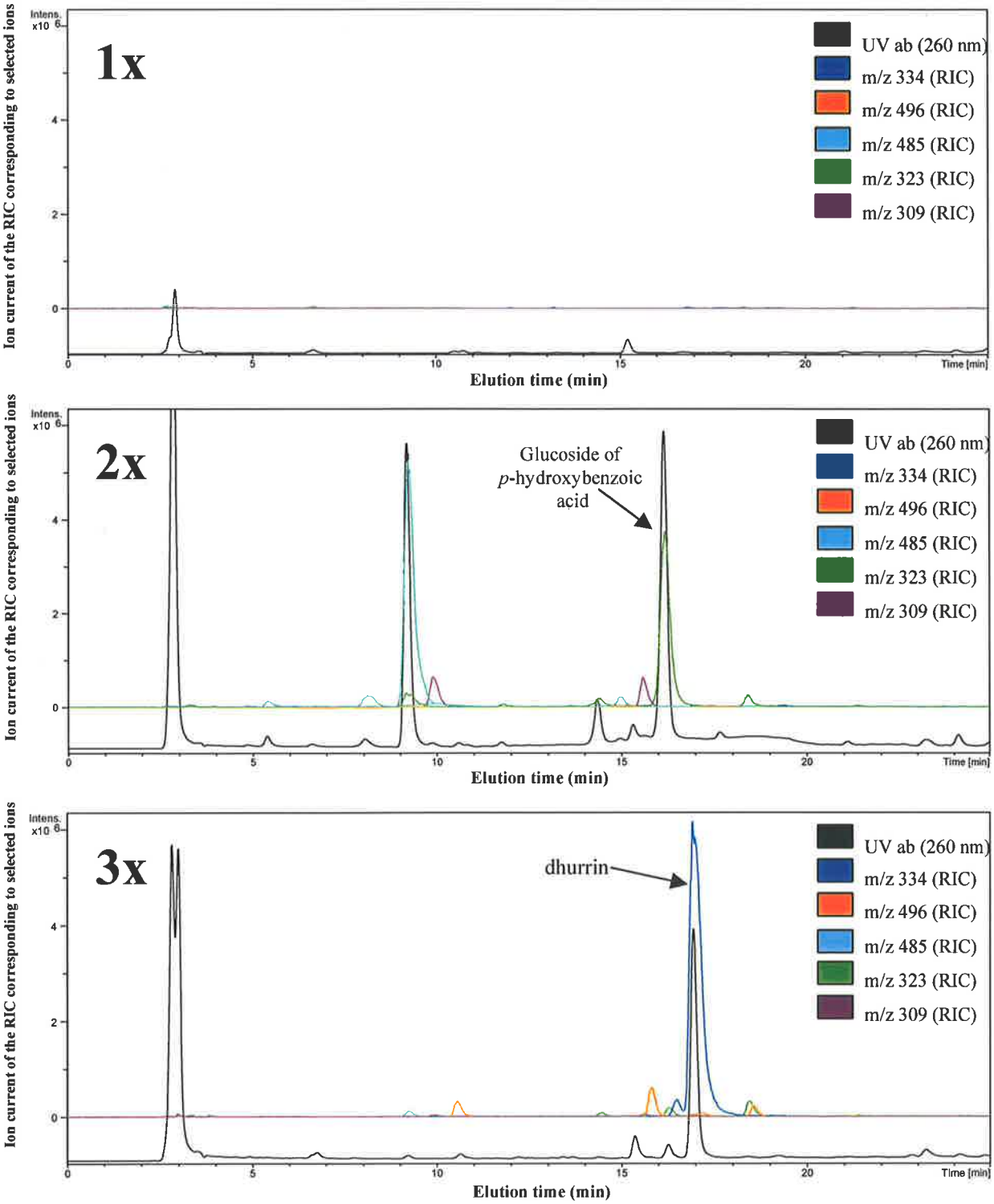


Figure 4.5 LC-MS of extracts prepared from 1x-, 2x- and 3x-plants. Metabolites recovered from approximately equivalent amounts of 1x (top panel), 2x (middle panel), and 3x (lower panel) plant material were subjected to LC-MS analyses as described in section 4.2.6. The mass spectrometer was operated by scanning from mass 100 to mass 500 and the traces for selected masses extracted. The different ion-masses of the RIC-traces are indicated by the coloured boxes to the right of each panel. Hydroxybenzoic acid glucosides absorb poorly at 230 nm and dhurrin absorbs poorly at 280 nm. The use of 260 nm for the UV-trace was therefore chosen as a compromise. The total ion-trace was noisy and is not shown. The 3x-line that was used in this comparison was derived from an independent transformation, with pPZP221-GT, of the same 2x-line that was used to represent the 2x-plants.

and 18.4 mins; m/z 496 $[M + Na]^+$; Figure 5.2). Several peaks with the expected mass of mono- and di-glucosides of intermediate-metabolites previously shown to accumulate in 2x-lines (Bak *et al.*, 2000; Figure 4.1), could also be detected by LC-MS analysis in 2x- and 3x-lines, but not in the 1x-lines (Figure 4.5). The major novel products in the 2x-lines, as indicated by the integrated area of the RIC peaks, were putative mono- and di-glucosides of *p*-hydroxybenzoic acid metabolites, derived from the breakdown of *p*-hydroxymandelonitrile (Figure 4.5; panel B and C)(Bak *et al.*, 2000). The identity of the monoglucoside of *p*-hydroxybenzoic acid (RT 16.3 min; m/z 323 $[M + Na]^+$) was confirmed by co-elution with an authentic standard, whilst the peak with an ion of m/z 485 $[M + Na]^+$ as the dominant ion (RT 9.3 min; Figure 4.5b) possibly represents a diglucoside of *p*-hydroxybenzoic acid. Both of these metabolites were markedly reduced in 3x-lines, most likely as a result of stabilization of *p*-hydroxymandelonitrile through glucosylation by sbHMNGT. There were no peaks of the expected mass of *p*-hydroxybenzaldehyde glucosides, indicating that the metabolism of *p*-hydroxybenzaldehyde to *p*-hydroxybenzoic acid may be more rapid than glucosylation at the aromatic hydroxyl-group. The studies by Bak *et al.* (1999) and Bak *et al.* (2000) revealed that 2x-lines of *Arabidopsis* did not contain any non-glycosylated intermediates. The relatively low level of accumulation of the major glucosylated intermediates in comparison with dhurrin in 3x-lines (Figure 4.5-6), therefore suggests that dhurrin synthesis was more efficient in *Arabidopsis* than *in vitro*. However, such a conclusion can only be tentative until quantitative data is obtained from both reconstitution systems.

The amount of dhurrin present in the different 3x-lines was estimated by HPLC and found to vary between 1 and 8 mg/g (f.w.) (data not shown), a similar level to that found in 'green-grown' *Sorghum* seedlings (~5 mg/g (f.w.); Halkier and Møller, 1989). Thus, the entire pathway had been transferred functionally from *Sorghum* to *A. thaliana*. Hence, as previously suggested by Bak *et al.* (2000), *A. thaliana* contains NADPH P450 oxidoreductases (Urban *et al.*, 1997) that function properly with the two cytochrome P450s, as well as other compartments and proteins with a sufficiently broad specificity to allow the novel secondary plant metabolite, dhurrin, to accumulate. A higher concentration of dhurrin could possibly be obtained if transgenic 3x-*Arabidopsis* were generated by a single transformation event with all three genes in the same vector, since 2x-lines with a high expression of CYP79A1 and CYP71E1 may not have survived the

antibiotic screening process due to the potential toxicity of pathway intermediates. This is highlighted by the fact that the 2x-plants were smaller, and grew more slowly, than wild-type and vector control plants. The subsequent production of 3x-plants restored the phenotype to near wild-type appearance.

A comparison between 3-day old 'green-grown' *Sorghum* seedlings grown in a tropical environment (28 °C, >95% relative humidity, described in 5.2.2) and 3x-*Arabidopsis* showed that all of the three peaks with the dominant ion of a mass of 496 $[M + Na]^+$ ((RT 10.6, 15.7, 18.4 mins) in 3x-*Arabidopsis* have a similar (if not identical) elution time to three peaks in *Sorghum* with the dominant ion of a mass of 496 $[M + Na]^+$ (Figure 4.6; panel A compared with panel B). These three peaks are absent in wild-type plants transformed with *sbHMNGT* (1x) (Figure 4.5a) and in *Arabidopsis* expressing only the two cytochrome P450s (2x) (Figure 4.5b). Hence, it is therefore most likely that these peaks are metabolites of dhurrin, since they are dependent upon the expression of all three enzymes of the dhurrin biosynthetic pathway. Alternatively, these peaks may be related to intermediates that initially were generated by the two cytochrome P450s and subsequently glycosylated by sbHMNGT. Interestingly, a peak with an ion of m/z 485 $[M + Na]^+$ as the dominant ion (RT 9.3 min; Figure 4.6b) was also found in *Sorghum*. Even if this peak does represent a di-glucoside of *p*-hydroxybenzoic acid, the question still remains if it was formed from *p*-hydroxymandelonitrile, or from the phenylpropanoid pathway.

Are 3x*-Arabidopsis *cyanogenic?

When 3x-*Arabidopsis* was tested for it's ability to liberate cyanide upon maceration, a weak, but clear, reaction in the colorimetric cyanide assay resulted (data not shown). In contrast, no cyanide was detected in assays involving 1x- or 2x- plant material (data not shown), indicating that these plants contain no, or undetectable levels, of cyanide-liberating compounds that are able to be hydrolyzed by *Arabidopsis*. Indeed, no dhurrin has been detected in 1x- or 2x- plant extracts. Extracts of *S. bicolor* were also tested as a control and these showed a significantly stronger evolution (10-fold or more) of cyanide than 3x-*Arabidopsis* extracts (data not shown). Given the long incubation time employed in the experiment (O/N incubation), and the ability of 3x-*Arabidopsis* to accumulate similar levels of dhurrin as *S. bicolor*, these preliminary experiments indicate that only a proportion of the accumulated dhurrin in 3x- *Arabidopsis* was

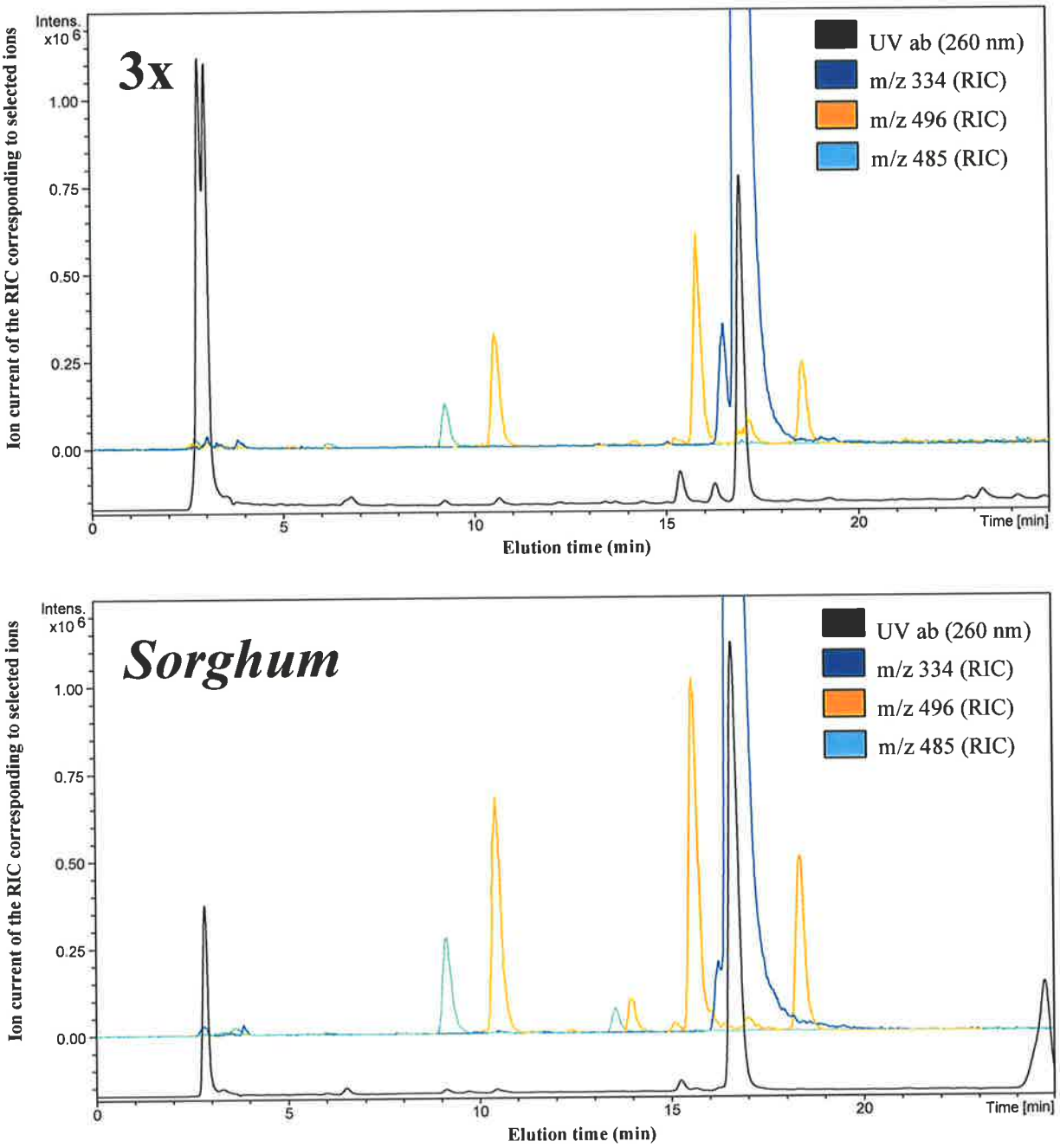


Figure 4.6 A comparison between extracts from 'green-grown' *Sorghum* and 3x-*Arabidopsis*. Glucoside extracts were prepared from 3-day old seedlings of 'green-grown' *Sorghum* (upper panel) grown at 28 °C as described in Chapter 5 (5.2.1) and compared with an extract of 3x *Arabidopsis* (lower panel). Glucoside extracts of approximately double the amount of plant material (f.w.) was injected for the *Sorghum* sample compared to 3x-*Arabidopsis*. Only peaks with dominant ions of the expected mass of sodium adducts of dhurrin (m/z 334 $[M + Na]^+$), dhurrin-glucoside (m/z 496 $[M + Na]^+$) and di-glucosides of *p*-hydroxybenzoic acid (m/z 485 $[M + Na]^+$) are included in the figure.

hydrolyzed. These initial results therefore suggest that *A. thaliana* does not contain native β-glucosidases and/or α-hydroxynitrilases that are capable of hydrolyzing dhurrin and breaking down *p*-hydroxymandelonitrile, respectively, to any significant extent. Hence, even though 3x- *Arabidopsis* accumulate large amounts of dhurrin, they are not highly cyanogenic. Further investigations into the dhurrin-hydrolyzing and *p*-hydroxymandelonitrile dissociating capability of *A. thaliana* will need to be performed in order to confirm this tentative conclusion.

4.4 Discussion

Metabolic engineering is currently a 'hot' topic (Dixon and Steele, 1999; Mann *et al.*, 2000). Principally, research is aimed at modulating the accumulation of particular primary or secondary metabolites in transgenic food crops in an attempt to improve crop yield and quality or to use the crop as a factory for production of a single plant metabolite in large quantities (Mann *et al.*, 2000). There are several notable reports of metabolic engineering where single genes have been inserted into various crop plants, resulting in the redirection of a particular metabolite into desired and novel end-products (Hain *et al.*, 1993; Siebert *et al.*, 1996; Schwinn *et al.* (1997); Bak *et al.*, 1999; Mann *et al.*, 2000). The successful generation of potentially cyanogenic cultivars of acyanogenic *A. thaliana* is the first reported instance of the transfer of an entire biosynthetic pathway from one plant species to another. The plasticity of the pathway in terms of environmental requirements is highlighted by the accumulation of dhurrin to a similar level to that found in *Sorghum*.

There are several interesting issues relating to the insertion of complete pathways into non-native species. For example, the number of foreign promoters and genes that can be inserted may be limited due to sense suppression (Al-Kaff *et al.*, 2000). Another issue relates to the potential interaction between proteins of different pathways with intermediates and end-products of other pathways. If several genes of a pathway are inserted into an organism by transgenic technology, will the intermediates released by the various enzymes be utilised by other native pathways within the transgenic plant or by the next transgene-encoded enzyme in the pathway sequence? One answer to this question was provided by Bak *et al.* (1999) and Bak *et al.* (2000). The enzymes of the cyanogenic glucoside biosynthetic pathway are able to share intermediates with the native glucosinolate biosynthetic pathway in *Arabidopsis* (Bak *et al.*, 1999; Bak *et al.*, 2000). Transgenic *Arabidopsis* expressing CYP79A1, that synthesizes *p*-hydroxyphenylacetaldoxime from tyrosine, could utilise the novel oxime and generate the novel glucosinolate, *p*-hydroxybenzylglucosinolate. When the next enzyme of the cyanogenic glucoside biosynthetic pathway, CYP71E1, was inserted together with CYP79A1, a competition for the oxime resulted, and the accumulation of the novel glucosinolate was greatly reduced (Bak *et al.*, 2000).

As was reviewed in the introduction (see 1.3.3.3), there is evidence to suggest that certain pathway intermediates are channelled as a result of a co-localization of pathway enzymes. In the absence of a physical association between CYP71E1 and sbHMNGT, or an exclusive compartmentalization of the cyanogenic glucoside biosynthetic pathway, it is expected that the biosynthesis of dhurrin would also generate metabolites of *p*-hydroxybenzaldehyde in both *S. bicolor* and *A. thaliana* (Figure 1.7). Interestingly, there is now evidence to suggest that such metabolites may accumulate in both 3x-*Arabidopsis* and *Sorghum* (Figure 4.6). However, this does not rule out the possibility that the cyanogenic glucoside biosynthetic pathway is channelled *in vivo*, since only a proportion of the three enzymes may be co-localized. Similarly, the interaction observed between the glucosinolate biosynthetic pathway and CYP79A1 and CYP71E1, does not exclude that sbHMNGT may be co-localized with CYP71E1. A proportion of the CYP79A1 molecules may even be co-localized together with the CYP71E1 equivalent of the *A. thaliana* glucosinolate biosynthetic pathway (Bak *et al.*, 1999; Bak *et al.*, 2000). In future studies, different forms of cDNAs encoding variants of green fluorescent proteins will be fused in various combinations to the three cDNAs encoding for the cyanogenic glucoside biosynthetic pathway, in an attempt to prove or disprove the formation of a metabolon in 3x-*Arabidopsis*. These studies can now be carried out since the present study has shown that the pathway can be transferred functionally to another plant species, and that there are striking similarities in the metabolic profile between 3x-*Arabidopsis* and *Sorghum*, suggesting that if the entire pathway forms a metabolon in *Sorghum*, the same may very well apply to 3x-*Arabidopsis*.

Notwithstanding the significance of the transfer of a whole metabolic pathway from *S. bicolor* to *A. thaliana*, perhaps the most exciting insight gained from this investigation is direct experimental evidence in support of the hypothesis that glycosylation of secondary metabolites is a key prerequisite for their stable accumulation in plants. A comparison between the metabolic profiles of 2x- and 3x-*Arabidopsis* illustrate this point very clearly (Figure 4.5). At least two distinct consequences of glycosylation are envisaged to contribute to the enhanced accumulation of secondary metabolites. First, glycosylation stabilizes the aglycone against further enzymatic or chemical modification as seen when comparing the fate of *p*-hydroxymandelonitrile in 2x- and 3x-lines. Such a stabilizing effect can also be inferred from *in vitro* and *in vivo* studies of other secondary metabolites, such as anthocyanidins and thiohydroximates, as discussed in

the introduction (see 1.3.2). Second, glycosylation of secondary metabolites may be linked to their intact transfer into storage compartments such as vacuoles, and the establishment of a driving force for the pathway by continual removal of the end-product. Evidence for the existence of glucoside-specific vacuolar transporters in plants have already been presented (Klein *et al.*, 1996; Klein *et al.*, 2000). Whether or not glycosylation affects the turnover rate of the whole pathway can now be tested by investigating if there are changes in the consumption of tyrosine by CYP79A1 as a result of the insertion of sbHMNGT.

Chapter 5

A dhurrin-glucosyltransferase in *S. bicolor*

5.1 Introduction

It was first suggested by Clegg *et al.* (1979) that cyanogenic glucosides are mobilized within cyanogenic plants. They found that the cyanogenic glucoside linamarin disappeared from Costa Rican wild lima beans seeds upon germination, only to accumulate in roots, cotyledons and shoots, whilst the total content of linamarin per plant remained constant throughout this process. It was concluded that linamarin had been transported rather than degraded and resynthesized, given that the formation of linamarin from the parent amino acid valine is not directly reversible. Dirk Selmar and co-workers subsequently proposed a theory upon how cyanogenic glucosides can be mobilized and showed that the essential components of this system, the 'linustatin pathway', are present in several cyanogenic plants (Selmar *et al.*, 1988; Selmar, 1993; Gruhnert *et al.*, 1994). Briefly, the theory involves di-glucosylation of the cyanogenic glucoside in the source cell, followed, by mobilization and subsequent storage or complete or partial breakdown in the sink tissue (Figure 5.1). The monoglucosides are restricted from entering the apoplastic space in an intact state, due to the presence of apoplastic mono-β-glucosidases in the source tissue. However, the corresponding diglucosides can enter the apoplastic space and be transported to other tissues since there are no measurable di-β-glucosidase activities in the apoplast of biosynthetic tissue (Gruhnert *et al.*, 1994). Most studied cyanogenic plants contain both mono- and di-glucosides of cyanohydrins (Frehner *et al.*, 1990; Lechtenberg and Nahrstedt, 1999). Recently, it was found that *S. bicolor* also accumulates a cyanogenic di-glucoside in the form of dhurrin-6'-glucoside (Figure 5.2) (Selmar *et al.*, 1996). The di-glucoside was found to be highly abundant in apoplasmic exudates, *ca* 10-20 nmol dhurrin-6'-glucoside per ml of exudate, indicating a possible role in dhurrin mobilization.

The present study was undertaken in order to characterize the enzyme(s) responsible for the synthesis of dhurrin-glucosides. An assay to detect the di-glucosyltransferase was developed. Subsequently, inconsistencies between the outcomes of this study and the work of Dirk Selmar prompted further analysis into the identity and accumulation of dhurrin-glucoside(s) in the SS1000 cultivar of *S. bicolor* used within our laboratory. Finally, an attempt was made to purify the responsible enzyme(s). Although not completed, the progress made, indicates that it may be possible to purify the responsible protein(s) to a homogenous state.

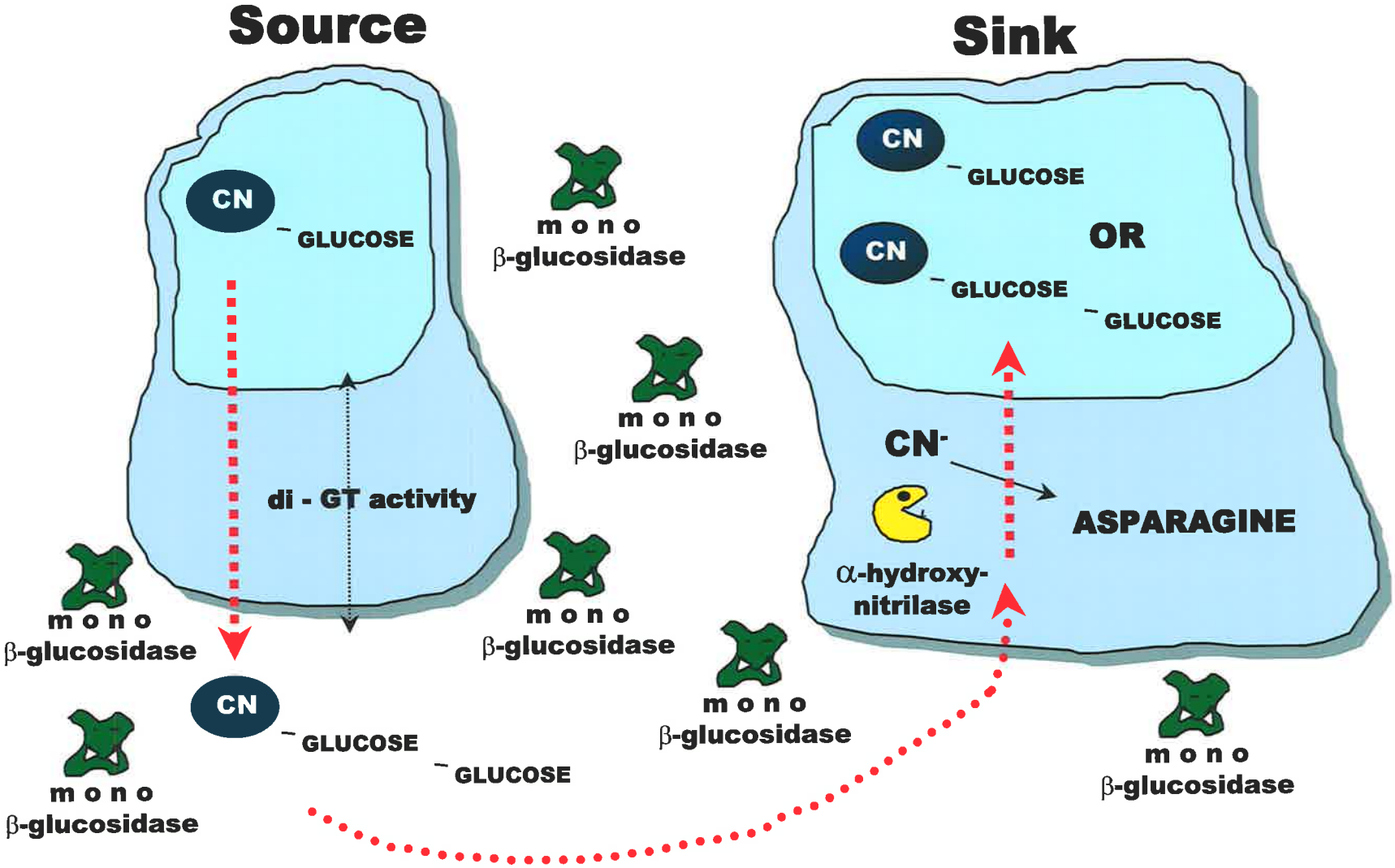


Figure 5.1 The mobilization of cyanogenic glucosides through the 'Linustatin pathway'. The 'Linustatin pathway' has been proposed by Selmar *et al.* (1988). Briefly, the cyanogenic monoglucoside (marine blue) is di-glucosylated prior to exiting the source cell (vacuole: turquoise; cell: milk blue). Outside of the source cell there are β-glucosidases (green) that specificially cleave the cyanogenic monoglucoside, but not the corresponding diglucoside. The diglucoside is apoplastically transported within the plant and enters the sink cell. There it is either stored as is in the vacuole, or cleaved to the corresponding monoglucoside, or broken down completely, aided by α-hydroxynitrilases (yellow).

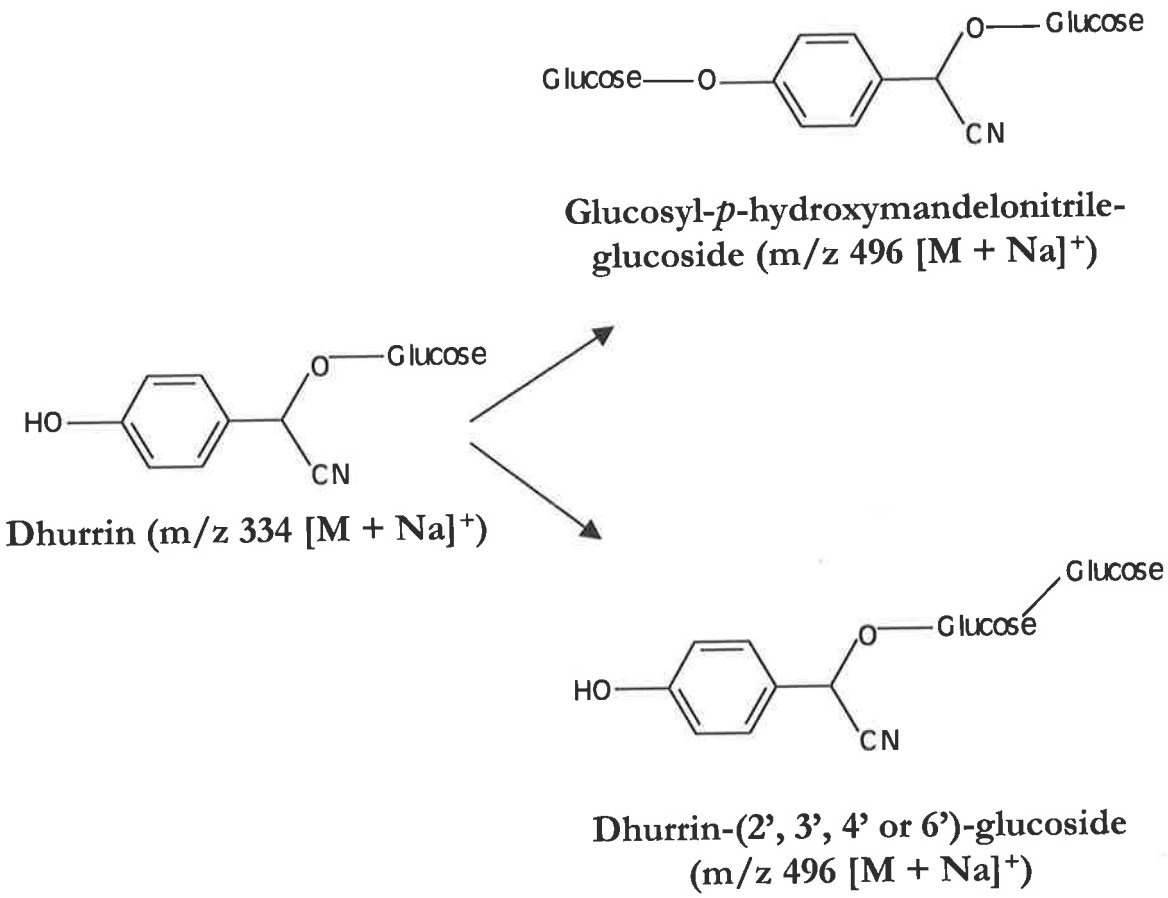


Figure 5.2 Five potentially different forms of dhurrin-*O*-β-glucosides. Dhurrin has five hydroxyl-groups that may be conjugated: (1) the aromatic ring, yielding glucosyl-*p*-hydroxy-mandelonitrile-glucoside (proteacin) and (2-5) the glucose-moiety, yielding dhurrin-(2', 3', 4' or 6')-glucoside, depending on which of the four hydroxyl-groups of the first glucose-moeity that the second glucose-moeity is bound to. Only proteacin and dhurrin-6'-glucoside are described to date (Lechtenberg and Nahrstedt, 1999). The detailed chemistry of dhurrin-6'-glucoside is shown in Figure 5.4

5.2 Materials and Methods

5.2.1 Materials and plant growth

Sambunigrin was a kind gift from Dr. Eric Conn (University of California, USA). All other chemicals, except where indicated, were obtained from Sigma (USA).

S. bicolor seeds (variety SS1000) were obtained from Agripro (Shawnee Mission, USA). Seeds were allowed to germinate and grow in darkness or in light at room temperature or in a greenhouse under tropical conditions (28 °C day, 25 °C night, 100% humidity) as described above (2.2.3).

5.2.2 Extraction of glucosides and proteins from plants

Approximately 20 to 100 mg of each sample of seedlings was harvested using a scalpel and weighed. Plant material was either used directly or frozen in liquid nitrogen and stored at –20 °C in 1.5 ml Eppendorf tubes. For glucoside extraction: One ml of methanol was added to the plant material, the tubes sealed with Capp-Lock (USA SCIENTIFIC) and placed in a water bath at 70-80 °C for 15 minutes. The tubes were subsequently cooled on ice, centrifuged (10,000 x g, 4 °C , 10 min) in a conventional benchtop centrifuge and the supernatant transferred to clean Eppendorf tubes. The methanol extract was then evaporated to dryness using a speed vac concentrator (Savant), dissolved in 200 µl of 20 mM Tris-HCl (pH 7.5) and stored at –20 °C prior to LC-MS analysis. All solubilisations were done with multiple pipetting and all evaporations were stopped promptly at or near dryness. Wherever possible, glucosides were kept on ice throughout the procedure. For protein extraction: plant material was homogenized with mortar and pestle in 2 volumes of ice-cold buffer (250 mM sucrose, 50 mM NaCl, 2 mM EDTA, 100 mM Tris-HCl (pH 7.5), 5 mM DTT, 200 µM PMSF, 5 g PVPP/100 ml), centrifuged (10,000 x g, 4 °C , 10 min) and stored at –80 °C.

5.2.3 Dhurrin-glucosyltransferase activity assay

General reaction mixtures (total volume 50 µl) included 100 mM Tris-HCl (pH 7.5), 250 µM UDP-glucose, 5 mM dhurrin (Extrasynthèse, France), sambunigrin or prunasin, 50 mM γ-gluconolactone (β-glucosidase inhibitor) and 5-25 µl of protein preparation. One to 5 µM ^{14}C-UDP-glucose (11.0 GBq/mmol, Amersham Pharmacia Biotech,

Sweden) were added to reaction mixtures that were analyzed by TLC. Reaction mixtures were allowed to incubate for 1 hour at 30 °C, cooled on ice and evaporated using a speed vac concentrator. The diglucosides were subsequently extracted with 100 µl of methanol, evaporated using a speed vac concentrator, dissolved in 50 µl of 20 mM Tris-HCl (pH 7.5) and stored at -20 °C prior to LC-MS analysis (4.2.6) or TLC (2.2.2). All solubilisations were done with multiple pipetting and all evaporations were stopped promptly at or near dryness. Where possible, glucoside preparations were kept on ice throughout the procedure.

5.2.4 Isolation of dhurrin-glucoside by TLC

Glucoside extracts from 40-50 mg of plant tissue, prepared as described above (5.2.2), or from five pooled dhurrin-glucosyltransferase assays, prepared as described above (5.2.3), were subjected to TLC as described above (2.2.2). A UV-absorbing band with an approximate R_f of 0.4 was scraped from each silica-sheet, pulverized using mortar and pestle and dissolved in 3 ml of 20 mM Tris-HCl (pH 7.5). The silica was deposited by centrifugation (10,000 x g, 10 min) and 2.5 ml of the supernatant recovered. One ml of the partially purified dhurrin-glucoside from plant extracts was then repurified as described above, except for the volume of solution employed to resolve the silica-extract (1 ml). Purified dhurrin-glucosides were stored at -20 °C prior to analysis.

5.2.5 HPLC, LC-MS, MS-MS and NMR analyses of diglucosides

HPLC analysis

HPLC was performed on a Shimadzu HPLC system with a SPD-M10AVP (Shimadzu) diode array detector. The reversed-phase LC conditions were as follows: A C_{18} column (Supelco LC-ABZ-3 (25x4.6 mm) 59142) was used with the mobile phases A: water, B: methanol. The flow rate was 1 ml/min and the gradient program was: 0-2 min: isocratic 0% B; 2-20 min: linear gradient 0-20% B; 20-30 min: linear gradient 20-100% B; 30-40 min: linear gradient 100-0% B; 40-45 min: isocratic 0% B. The UV-absorbance was recorded at 230, 250 and 280 nm and analysed using the Shimadzu HPLC analysis package.

LC-MS and MS-MS analysis

LC-MS was performed as described above (4.2.6). Peaks with ions at m/z 480 or 496 $[M + Na]^+$ as the dominant ions, synthesized by enzymatic assays employing sambunigrin and dhurrin as substrates (see section 5.2.3), respectively, were isolated in the ion trap and then subjected to fragmentation in the trap prior to reapplication to MS.

The amount of dhurrin-glucoside in a given sample was quantified by LC-MS accordingly: The integrated area of products, corresponding to a peak with an ion at m/z 496 (RT ~18.4 min) as the dominant ion, in each sample, and in an aliquot of the TLC purified dhurrin-glucoside, were compared using the Bruker Data Analysis program. The concentration of the TLC purified dhurrin-glucoside preparation that was applied to LC-MS was quantified by cyanide and glucose analysis (see below, 5.2.6). The amount of dhurrin-glucoside in each sample could thus be estimated in relation to the TLC-purified di-glucoside.

NMR analysis

NMR was performed on a Bruker Avance 400 NM spectrometer. Samples were evaporated to dryness using a speed vac concentrator and dissolved in methanol-d4 prior to injection. The chemical shift values indicated in Figure 5.4 are relative to internal tetramethylsilane.

5.2.6 Cyanide and glucose assays of isolated dhurrin-glucoside

Ten µl of emulsin (almond β-glucosidase type II, 10 mg/ml, Sigma) and 10 µl of phosphate-buffer (1 M, pH 6.5) were added to 100 µl of TLC-purified dhurrin-glucoside (unknown concentration) and hydrolysis progressed in 1.5 ml Eppendorf tubes for 2 hours at 30 °C. Standards, composed of 0, 2, 4 and 6 µg of dhurrin (Extrasynthèse, France) in 100 µl of Tris-HCl (pH 7.5), were hydrolyzed as described above. Tubes containing hydrolyzed glucosides were frozen in liquid nitrogen, opened and 40 µl of 6 M NaOH added. The samples were thawed and stored at room temperature for 20 minutes before addition of 50 µl of acetic acid. Cyanide was detected as described above (4.2.6).

Glucose assays

Four hundred µl of assay buffer (50 mM MOPS·KOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1.25 mM ATP, 1.25 mM NAD^+, 2 U hexokinase) was added to each sample of glucosides, hydrolyzed as described above, mixed and allowed to incubate for 15 minutes. The absorbance at 340 nm was recorded as above (A1) and 0.5 U of glucose-6-phosphate dehydrogenase added to the reaction. The reaction mixture was mixed and allowed to incubate for 15 minutes, prior to a new recording of the absorbance at 340 nm (A2). The difference between A2 and A1 was used to calculate the amount of free glucose in the sample.

5.2.7 Partial purification of a dhurrin-glucosyltransferase from *S. bicolor*

The purification protocol for the di-glucosyltransferase essentially followed that developed for sbHMNGT (2.2.3). Briefly, a crude extract was prepared from ~100 g of 2-3 day old green seedlings (28 °C day, 25 °C night, 100% humidity, 5-10 cm high) and subjected to ammonium sulfate precipitation (90%) followed by desalting using a Sephadex G-25 (Amersham Pharmacia Biotech, Sweden) gel filtration column in buffer A (10 mM NaCl, 50 mM Tris-HCl pH 7.5, 5mM DTT) as described above (2.2.3). Proteins were applied to a Q-Sepharose column (1 cm x 10 cm, flow-rate 40-50 ml/hr) in buffer A and eluted using a linear gradient from 50 mM to 400 mM NaCl in buffer A (400 ml total volume). Fractions (2 x 5 ml) and aliquots (15 µl) thereof were collected and stored at -80 °C. Aliquots were subsequently thawed and assayed for dhurrin-glucosyltransferase activity as described above (5.2.3). Active fractions (5 ml each) were thawed and desalted using an Amicon YM10 ultrafiltration membrane in buffer A to give a final salt concentration 15 fold lower. The preparation was split into two equal parts and applied to a Reactive Brown 10 (Sigma, USA) column (batch 98F9635) (1 cm x 7 cm) and a Reactive Yellow 3 (Sigma, USA) column (1/3 batch 63H9502, 2/3 batch 87F9687) (1 cm x 10 cm) equilibrated in buffer B (20 mM NaCl, 20 mM Tris-HCl pH 7.5, 5mM DTT). The columns were washed with 30 ml of buffer B, followed by elution with 1 ml of UDP-glucose (5 mM) in buffer B and further washing with 10 ml of buffer B. A step-wise salt gradient was then applied in 5 ml portions, 70 mM, 120 mM and 420 mM NaCl in buffer B, followed by 10 ml of washing in 420 mM NaCl in buffer B. Aliquots (2 x 100 µl) of fractions (5 ml) were collected manually, adjusted to 10% (v/v) glycerol immediately and assayed as described above (5.2.3) after storage at -80 °C.

5.3 Results

5.3.1 The identification of a dhurrin-glucosyltransferase activity *S. bicolor*

The primary task was to investigate if extracts of sorghum contained a dhurrin-glucosyltransferase activity and how an assay for this activity could be optimized. A product with the expected characteristics of a dhurrin-glucoside is formed by sorghum homogenates, however, in the absence of a commercially available standard, it was necessary to certify that the product in fact was a glucoside of dhurrin. Crude and desalted protein extracts from young etiolated seedlings were assayed using dhurrin and ^{14}C-UDP-glucose as substrates, separated by TLC, and detected by exposure to phosphorimager plates (Molecular Dynamics). Despite the presence of dhurrin β-glucosidases, which hydrolyze the substrate, and sbHMNGT, which consumes the radioactive donor, it was possible to identify a radioactive band with slower mobility than dhurrin (Figure 5.3a). The putative diglucoside was not formed in the absence of dhurrin (lane 1, Figure 5.3b), the activity peaked at pH 7.5 (lane 3, Figure 5.3a), and was positively influenced by the presence of β-glucosidase inhibitors in the assay mixture (lane 5 compared with lane 3, Figure 5.3a). When the assay was conducted with sambunigrin (mandelonitrile glucoside) - which is identical to dhurrin except for the absence of an hydroxyl-group at the *para*-position of the benzene-ring (Lechtenberg and Nahrstedt, 1999) - there was a shift in the mobility of the putative diglucoside of sambunigrin compared with dhurrin-glucoside (lane 6, Figure 5.3a). This mobility shift was of a similar magnitude to that observed between radioactive dhurrin and sambunigrin, that were formed by hydrolysis and re-glucosylation with ^{14}C-UDP-glucose. The majority of the dhurrin-glucosyltransferase activity was found to be soluble after centrifugal separation of microsomes (data not shown). There was no activity in pooled ion-exchange fractions containing sbHMNGT (lane 7, Figure 5.3a), indicating that sbHMNGT was not responsible for di-glycosylation and that the two glucosyltransferases did not co-purify. An increased incubation time (Lane 2 (1 hr) compared with Lane 3 (10 min), Figure 5.3b) resulted in increased amount of product, as would be expected in the absence of a β-glucosidase with specificity for dhurrin-glucosides (Selmar *et al.*, 1996).

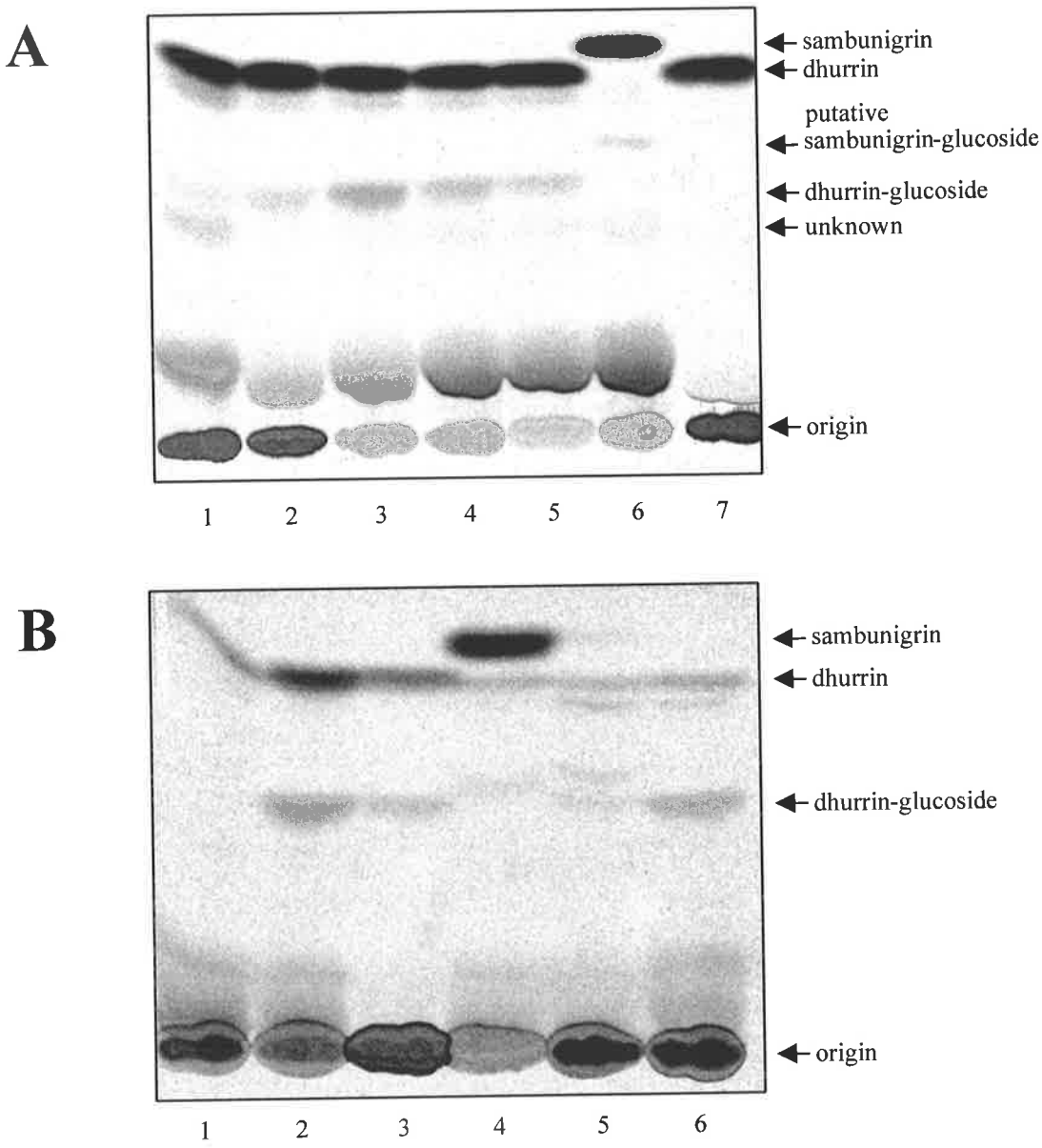


Figure 5.3 The synthesis of dhurrin-glucoside shown by assays using ^{14}C-UDP-glucose and analysis by TLC. (A) Dhurrin-glucosyltransferase activity assays using desalted (30-70%) ammonium sulfate precipitate of crude extracts of etiolated *S. bicolor*. Lanes: (1) pH 6.5; (2) pH 7.0; (3) pH 7.5; (4) pH 7.9; (5) identical to lane 3, except no β-glucosidase inhibitor; (6) identical to lane 3, except sambunigrin was used as substrate instead of dhurrin; (7) identical to lane 3, except pooled ion-exchange fractions with sbHMNGT activity was used as protein preparation instead of desalted crude extracts. (B) Dhurrin-glucosyltransferase activity assays using crude extract of 'green-grown' *S. bicolor* seedlings. Lanes: (1) identical to lane 2, except dhurrin was omitted from the assay; (2) 1 hour incubation, pH 7.5; (3) identical to lane 2, except 10 min incubation; (4) identical to lane 2, except mandelonitrile was used as substrate instead of dhurrin; (5) identical to lane 2, except prunasin (stereoisomer of sambunigrin) was used as substrate instead of dhurrin; (6) identical to lane 2, except pH 6.0. Arrows indicate the relative migration of standards and putative sambunigrin.

TLC was then performed with a large-scale dhurrin-glucosyltransferase activity assay and a UV-absorbing band that co-migrated with the radioactively labelled putative di-glucoside was extracted and subjected to NMR analysis (Figure 5.4). A sharp singlet at ~5.90 ppm, which is characteristic for the *p*-hydroxyphenyl group of dhurrin, suggested that the putative di-glucoside was a metabolite of dhurrin. Sharp singlets are rarely found in this range of the ^{1}H-NMR spectrum, in which signals normally are due to protons associated with carbon atoms. The same singlet was also found in ^{1}H-NMR spectra recorded with an authentic standard of dhurrin (green box, Figure 5.4). Two equally intense doublets in the NMR spectra of the putative diglucoside (4.58 ppm and 4.65 ppm; J = ~7.8 Hz; Figure 5.4) indicated that two β-hexoses most likely were present in the sample. However, the accurate position of one of them (4.58 ppm) was 0.13 ppm higher than that reported for dhurrin-6'-glucoside by Selmar *et al.*, (1996). There were also other inconsistencies between the ^{1}H-NMR spectrum reported for the dhurrin-glucoside isolated by Selmar *et al.* (1996) and that of the current study, although both contained the singlet at 5.90 ppm. The differences suggested that the two NMR-spectra were recorded from preparations that contained two different forms of dhurrin-glucosides, possibly with the second sugar moeity attached to different positions of the innermost glucose unit, or that one of the preparations was contaminated. However, the amount of sample was not sufficient for further structural determination by 2-dimensional NMR spectroscopy. Additional attempts at large-scale purification of the putative di-glucoside by repetitive HPLC purification and rotary evaporator concentration were unsuccessful. This was most likely due to oxidation, as the glucoside preparation rapidly turned red-brown. Initial attempts at synthesizing large quantities of a glucoside of sambunigrin suggested that it may be better suited as a substrate for the preparation of a high concentration of pure cyanogenic diglucoside, since this glucoside-preparation remained white throughout the purification procedure. Hence, the aromatic hydroxyl group may be responsible for the observed oxidative vulnerability of the dhurrin-glucoside preparation.

The di-glucosyltransferase assay preparation was then subjected to LC-MS analysis. One major (~18.4 min) and four minor (10.6, 11.4, 15.7, 17.1 mins) peaks with an ion at m/z 496 as the dominant ion were eluted (Figure 5.5a). The lower ratio of UV-absorbance:RIC (mass) of the putative dhurrin-glucoside (~18.4 min) compared with dhurrin could be explained by saturation of the ion-current detection system. Peaks with

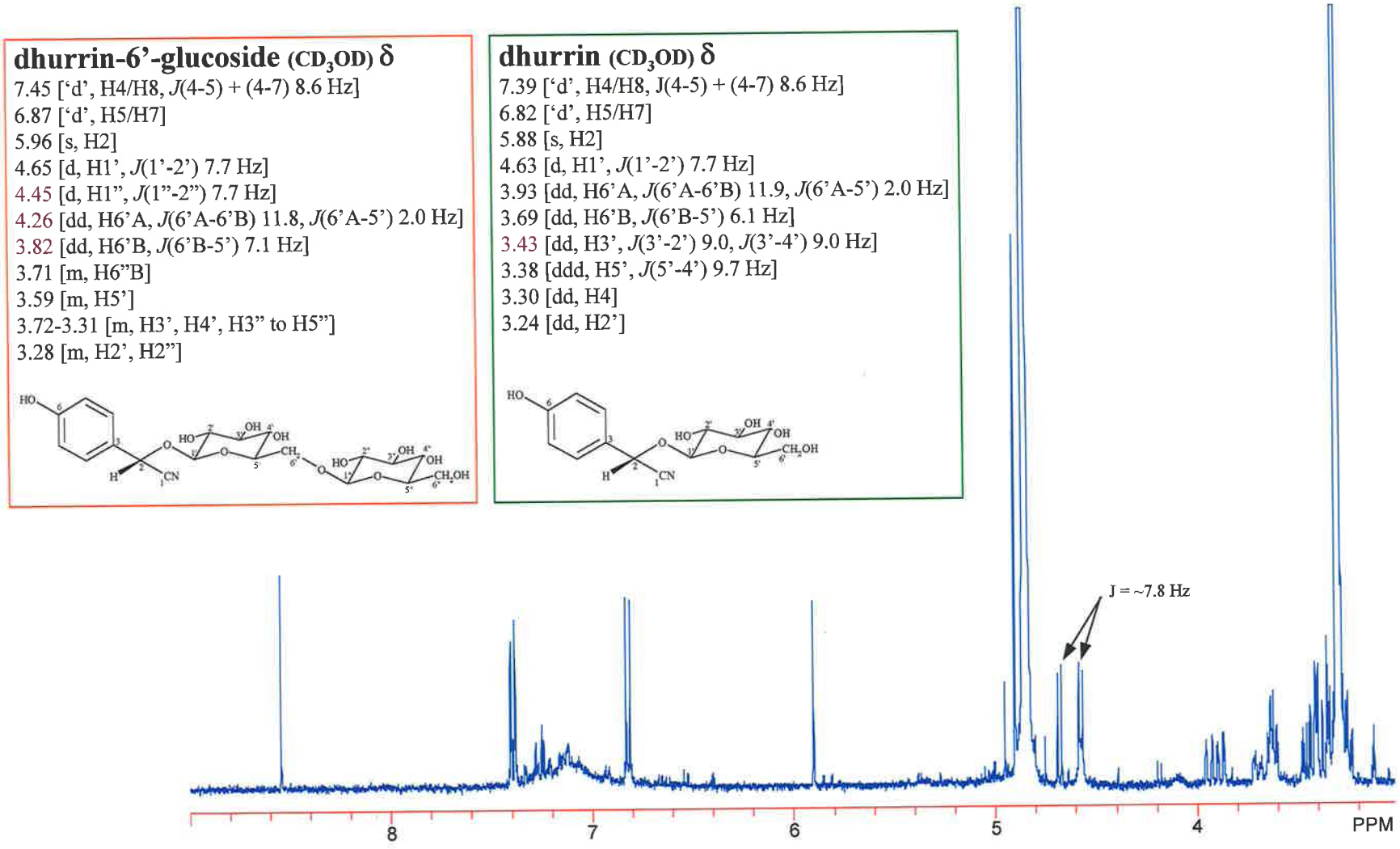


Figure 5.4 ^{1}H NMR spectra recorded with a TLC-purified dhurrin-glucoside synthesized from crude extracts of *S. bicolor*. The red and green boxes illustrate the chemical structure of dhurrin-6'-glucoside and dhurrin, respectively. A list of the characteristic peaks for each of the compounds obtained by ^{1}H-NMR by Selmar *et al.* (1996) are included. A list of characteristic peaks for dhurrin (Extrasynthèse, France) was also generated by NMR in the present study (data not shown). Those peaks which are clearly different from the results obtained in the present study are highlighted in purple. Both Selmar *et al.* (1996) and the results of the present study were obtained with methanol as a solvent.

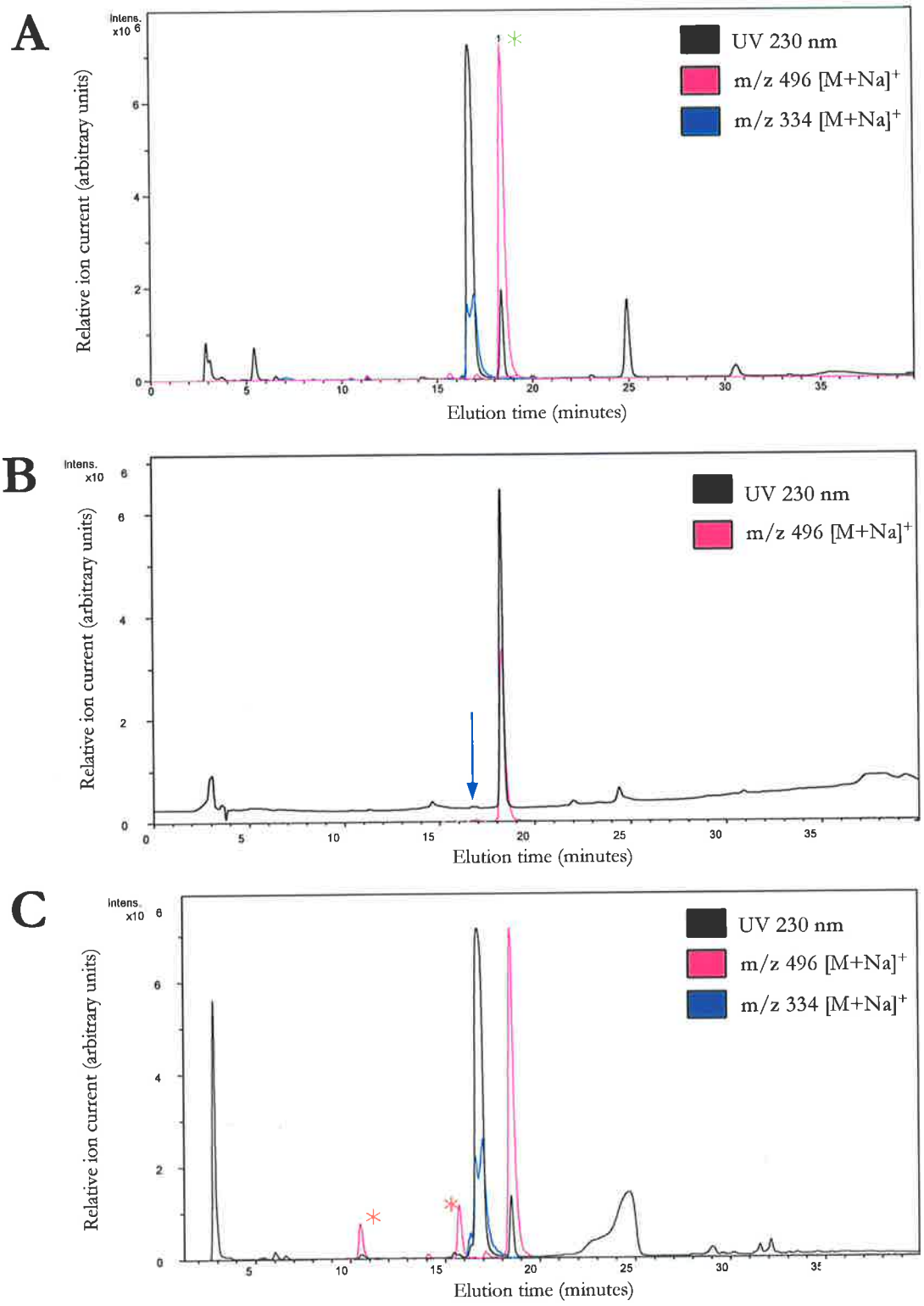


Figure 5.5 Identification of dhurrin-glucoside by LC-MS analysis of enzymatic assays, TLC purified glucosides and plant extracts. (A) A typical example of a dhurrin-glucosyltransferase activity assay using crude extract of 6-day old 'green-grown'*S. bicolor* seedlings as enzyme source. The peak representing dhurrin-glucoside is highlighted with a lime-green star. (B) Approximately 0.24 µg of TLC-purified dhurrin-glucoside. The blue arrow indicates the expected elution time of dhurrin. (C) Glucoside extract from 3-day old etiolated seedlings. Two minor peaks with the expected retention time of dhurrin-glucoside (m/z 496 $[M + Na]^+$) are indicated by red stars. The different mass traces are presented at an equal but arbitrary scale that was identical between all LC-MS samples of this study. The UV-profile (230 nm) has been scaled for visibility.

ions at m/z 496 $[M + Na]^+$ and m/z 480 $[M + Na]^+$ as the dominant ions that were isolated by LC-MS from samples of putative dhurrin-glucoside and putative sambunigrin-glucoside respectively, were isolated in the ion trap and subjected to fragmentation. Peaks with an ion of 347 $[M + Na]^+$ as the dominant ion was obtained after fragmentation in both cases, which is consistent with the expected molecular mass of a sodium adduct of two linked glucose molecules.

In summary, a product could be synthesized *in vitro* using crude sorghum protein, dhurrin and UDP-glucose as reactants, and it was not formed in the absence of dhurrin. The product was most likely composed of dhurrin (as shown by the 5.90 ppm singlet in the NMR spectra), had the expected mass of a dhurrin-glucoside and, through MS-MS analysis, was believed to contain a diglycosyl unit. It was therefore concluded that an extract prepared from sorghum was able to synthesize a glucoside of dhurrin, and that the second glucose-molecule had been conjugated to the first glucose unit. Unfortunately, it was not possible to determine whether the identified dhurrin-glucoside had the same structure as the di-glucoside that was isolated by Selmar *et al.* (1996).

5.3.2 Dhurrin-glucosides in *S. bicolor* - isolation and identification

Was the diglucoside, formed by the crude extracts, also present as a natural constituent in the plant? Since the reason for this study was to investigate the role of di-glucosylation in cyanogenic glucoside mobilization, there was no point in attempting to isolate an enzyme that does not produce the product *in vivo*. Glucoside extracts were prepared from shoots of young, etiolated and 'green-grown' seedlings and analysed by TLC. A product with similar mobility to the di-glucoside identified above (5.3.1), with an ~R_f of 0.4, was extracted and subjected to LC-MS analysis (data not shown). A peak with the same elution time as the *in vitro* synthesized dhurrin-glucoside (18.4 min) and the expected mass of a sodium adduct of dhurrin-glucoside (496 $[M + Na]^+$) could be identified. The remaining portion of the glucoside extract was re-purified by TLC, and LC-MS analysis indicated that the preparation was practically homogenous (Figure 5.5b). Aliquots of the re-purified glucoside were then hydrolyzed and quantified by cyanide and glucose analysis. The molar ratio of glucose:cyanide was estimated to be 2.00 : 0.97, and approximately 1.2 μg of dhurrin-glucoside had been isolated from 10 mg of plant tissue. The availability of a dhurrin-glucoside preparation of known

concentration made it possible to quantify the amount of dhurrin-glucoside in a given sample.

When glucoside extracts prepared from etiolated or 'green-grown' *Sorghum* seedlings were subjected to LC-MS analyses, a peak (RT ~18.4 min) with an ion of m/z 496 $[M + Na]^+$ as the dominant ion eluted (Figure 5.5c). This suggests that the enzymatically synthesized dhurrin-glucoside, obtained in the *in vitro* reaction, also is naturally present in the plant (Figure 5.5, panel A compared with panel C). However, it remains unknown which of the four possible β-di-glucosides (Figure 5.2) this major dhurrin-glucoside in *S. bicolor* (cv SS1000) represents. Interestingly, there were two minor additional peaks, with ions of m/z 496 $[M + Na]^+$ as the dominant ion, that could also represent glucosides of dhurrin. The link between dhurrin and these two minor peaks is further strengthened by their exclusive accumulation in transgenic *A. thaliana* that express all three enzymes of the cyanogenic glucoside biosynthetic pathway as shown in Figure 4.6. The importance of any of these potential di-glucosides for cyanogenic glucoside mobilization is unknown, and for practical reasons it was decided that only the major dhurrin-glucoside eluting at ~18.4 min should be employed as a marker in assays of dhurrin-glucosyltransferase activity.

5.3.3 The *in vivo* accumulation of dhurrin-glucoside in response to differing growth conditions

The accumulation of the major dhurrin-glucoside in *Sorghum* (RT ~18.4), grown under different conditions, was investigated in order to choose the most optimal tissue for dhurrin-glucosyltransferase purification. *Sorghum* seedlings were allowed to germinate in soil and grown for three weeks in a cool laboratory (~20-22 °C) in the presence of light (see section 5.2.1 for detailed growth conditions). Plant material was taken at three time points and analyzed for dhurrin-glucoside accumulation and dhurrin-glucosyltransferase activity (Figure 5.6a). The concentration of dhurrin-glucoside peaked at the earliest sample time-point (3 days) and declined thereafter. This accumulative pattern is broadly similar to that of dhurrin in etiolated seedlings (Halkier *et al.*, 1989), and this was also the conclusion of Selmar *et al.* (1996) although the data on which that conclusion was made, were not disclosed. However, the concentration of dhurrin-glucoside may peak somewhere in between 3 and 6 days after germination, since a comparison between plants grown at 23-25 °C showed a higher

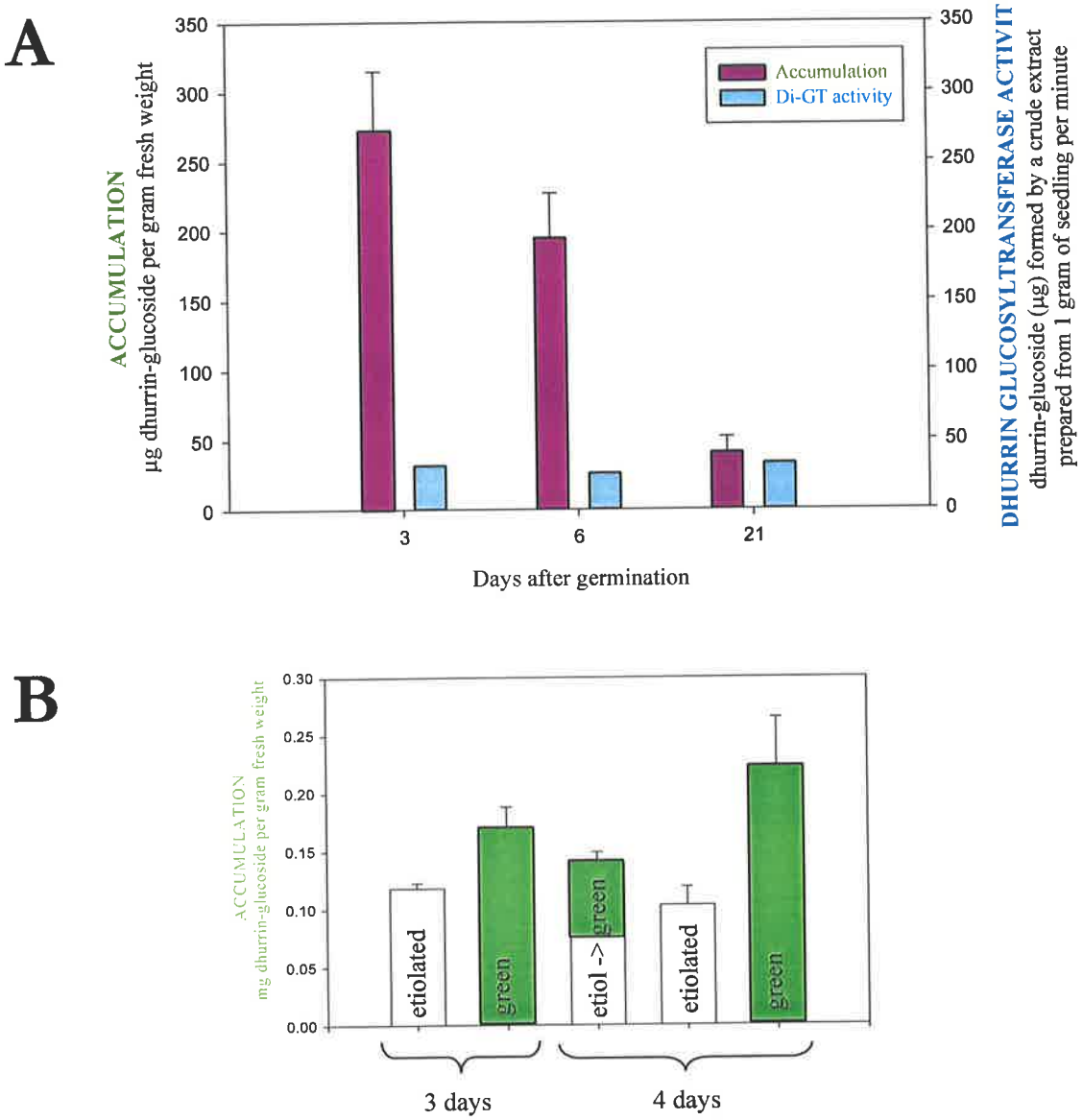


Figure 5.6 The dhurrin-glucosyltransferase activity and accumulation of dhurrin-glucoside in *S. bicolor* seedlings in response to varying growth conditions. (A) The dhurrin-glucosyltransferase activity and accumulation of dhurrin-glucoside at 3, 6 and 21 days after germination, grown in a cool (20-22 °C) laboratory. The amount of dhurrin-glucoside in a given sample was quantified as outlined above (5.2.5). The concentration of dhurrin-glucoside in each sample is indicated by the purple bars, whilst the amount of dhurrin-glucosyltransferase present in each tissue sample is indicated by the milk blue bars. (B) The accumulation of dhurrin-glucoside in response to light and darkness. Four trays of seeds were grown as described above (2.2.3), two in darkness and two in light, except that seedlings were allowed to germinate at 23-25 °C as described below (5.3.3). One of the dark-grown trays was transferred to light (Bar 3) at day three. Samples were prepared and analyzed in triplicate as described above. The error bars indicate standard deviation between samples.

concentration of dhurrin-glucoside in 4 day old plants compared with 3 day old plants (Figure 5.6b). The maximum concentration of dhurrin-glucoside (0.3 mg/g (f.w.)) was approximately 20-fold lower than the maximum concentration of dhurrin, as found in an earlier study made with etiolated seedlings of the same cultivar (Halkier *et al.*, 1989). The concentration of dhurrin-glucoside in *Sorghum* seedlings, of approximately the same height, varied in response to different growth conditions. For example, initial experiments in which etiolated seedlings were grown in a dark growth chamber at 30-32 °C showed no sign of dhurrin-glucoside accumulation (data not shown). In contrast, seedlings grown in light at 20-22 °C accumulated the highest observed levels of dhurrin-glucoside (Figure 5.6a). However, when seedlings were allowed to simultaneously grow in darkness, or in light, at the same temperature (~23 - 25 °C), there were only minor differences in the amount of accumulated dhurrin-glucoside between 'green-grown' and etiolated seedlings (Figure 5.6b). The concentration of dhurrin-glucoside in 'green-grown' seedlings at ~23-25 °C was approximately half of that in plants grown under cooler conditions (20 - 22 °C) as shown in Figure 5.6. Similarly, seedlings grown under tropical conditions (28 °C day, 25 °C night, 100% humidity) accumulated only 5-10% of the dhurrin-glucoside accumulated under cooler conditions (20-22 °C) (data not shown). As shown by comparisons between Figure 4.6 and 5.5, the relative proportion of the dhurrin-glucoside and the two putative dhurrin-glucosides was also different at different temperatures of growth. It was not possible within the time-frame of this study to perform a controlled experiment in which the effect of temperature on dhurrin-glucoside accumulation could be tested. However, the results obtained from these initial studies suggest that the accumulation of dhurrin-glucoside is diminished at relatively high growth temperatures.

Notably, the dhurrin-glucosyltransferase activity did not vary with growth temperature. For example, there were equal amounts of dhurrin-glucosyltransferase activity (per gram f.w.) in etiolated seedlings compared with those found in 'green-grown' seedlings grown under cool conditions (20-22 °C), despite no evidence of dhurrin-glucoside accumulation in the former plants (data not shown). The variation in dhurrin-glucoside accumulation between 'green-grown' and etiolated seedlings grown under cooler conditions was practically paralleled by the difference in dhurrin-glucosyltransferase activity (data not shown). The extractable *in vitro* activity was 25-40 μg dhurrin-glucoside/g (f.w)/hr (Figure 5.6a), which theoretically would be sufficient to synthesize

all of the observed dhurrin-glucoside, at 3 days after germination, in approximately 10 hours. Since the accumulation of dhurrin-glucoside decreases after germination, except for a possible peak between 3 and 6 days, and the amount of dhurrin far exceeds that of dhurrin-glucoside at all developmental stages (data not shown), and the amount of extractable di-glucosyltransferase activity remains essentially stable (Figure 5.6a), it is plausible that the enzyme(s) is(are) restricted in access to the substrate, or that there is a significant degree of removal or turnover of the dhurrin-glucoside. Selmar *et al.* (1996) suggested that dhurrin-glucoside was present at high concentrations in guttation droplets. One possible explanation for the relationship between growth temperature and dhurrin-glucoside accumulation could therefore be that the amount of guttation is dependent on temperature and that dhurrin-glucosides are washed out by mobilization to the exterior inside guttation droplets. However, in this study, it was not possible to detect any dhurrin-glucoside in glucoside extracts of guttation droplets sampled from both etiolated and 'green-grown' seedlings, as had previously been found by Selmar *et al.* (1996) (data not shown). Guttation droplets were extracted essentially in the same manner as that described by Selmar *et al.* (1996) and subjected to reverse phase C18-HPLC. All major and several minor fractions were collected and subjected to cyanide analysis. Only one peak, with the expected retention time of dhurrin, was cyanogenic (data not shown) and, specifically, no peak appeared at the retention time of the putative di-glucoside previously obtained from TLC plates, as described above (5.2.4).

5.3.4 Partial purification of a dhurrin-glucosyltransferase

Based on the results above (see sections 5.3.1 - 5.3.3) young seedlings (3 days growth) grown in a green-house under tropical conditions, were chosen as tissue for glucosyltransferase purification. The extraction protocol employed for sbHMNGT isolation (2.3.2), was tested initially and found to yield active dhurrin-glucosyltransferase activity. All initial steps of the sbHMNGT purification protocol, up to and including Q-Sepharose chromatography, were maintained except for the differential ammonium sulfate precipitation, which was substituted by a single 90% precipitation cut. Q-sepharose chromatography yielded a single peak that eluted at ~300-375 mM NaCl (Figure 5.7). The pooled fractions of dhurrin-glucosyltransferase activity contained only a tenth of the protein content and ~41% of the dhurrin-glucosyltransferase activity of the crude extract, resulting in an approximate 4-fold purification. The substrate specificity of the pooled active ion-exchange fractions was

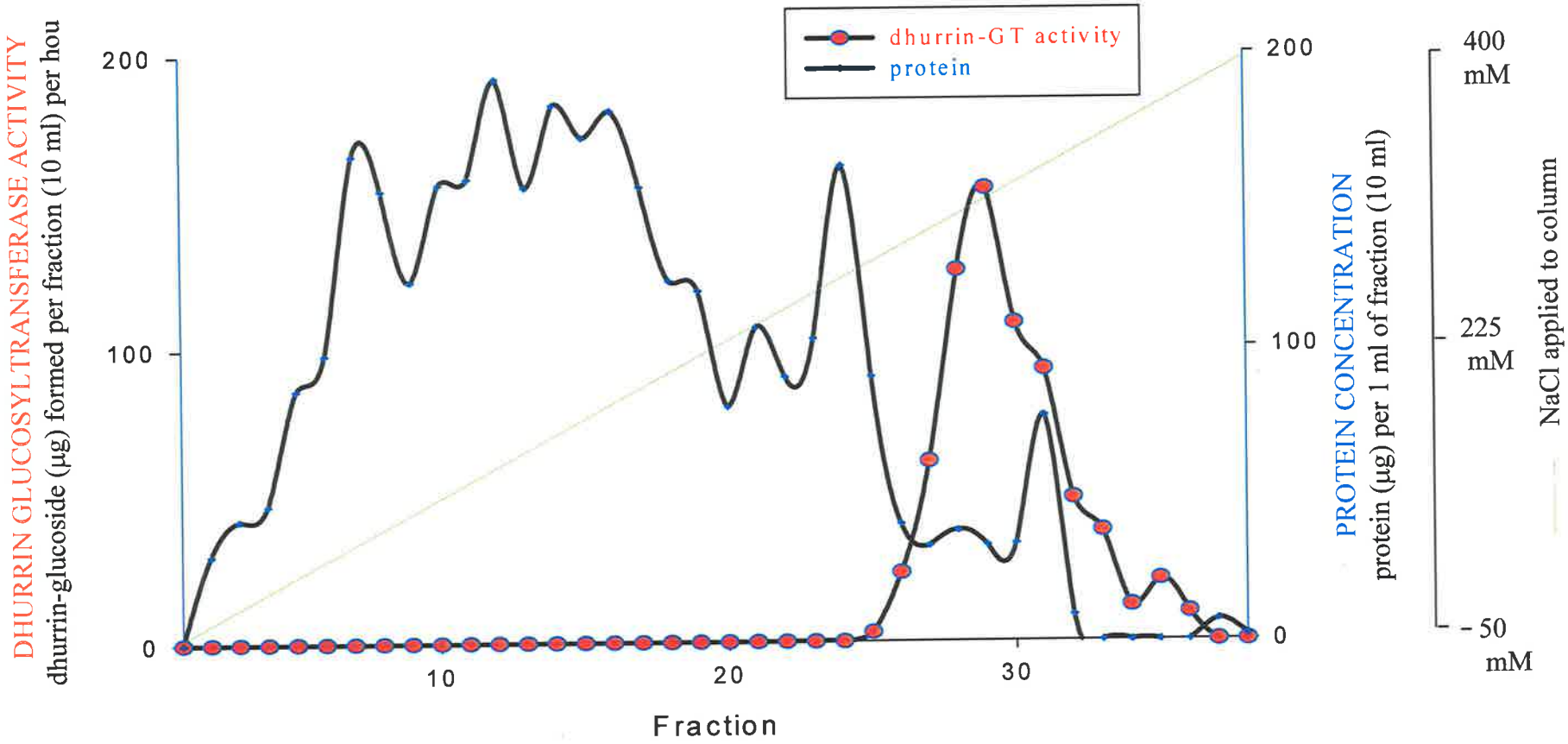


Figure 5.7 Q-Sepharose chromatography of dhurrin-glucosyltransferase. A desalted ammonium sulfate precipitate of 'green-grown' *S. bicolor* extracts was applied to a Q-Sepharose column and eluted with a linear gradient of NaCl (50-400 mM). Aliquots of each fraction were assayed for dhurrin-glucosyltransferase activity and analyzed by LC-MS (m/z 496, RT ~18.4 min) as described above (5.2.3-5.2.4). The dhurrin-glucosyltransferase activity eluted in a single peak with minor shoulders. Aliquots of each fraction were also analyzed for total protein content by staining with Commassie Brilliant Blue G as described by Bradford (1976). The approximate concentration of NaCl in each fraction is indicated by the dotted green line.

not tested due to the heterogeneity of the preparation, as shown by SDS-PAGE (data not shown). Half of the pooled material from the ion-exchange step was thawed and desalted using an Amicon YM10 membrane. The desalted material was split into two fractions, and applied to mini-columns containing Reactive Yellow 3 (Sigma) and Reactive Brown 10 (Sigma), respectively. The dhurrin-glucosyltransferase did not bind to either of the two media and was washed out together with the majority of other polypeptides. However, the activity was essentially retained throughout the trials indicating that further purification is possible if suitable chromatographic steps are found. No further attempts at purification were done due to the time limits imposed on the PhD program. However, it appears that purification to homogeneity of a dhurrin-glucosyltransferase should be feasible.

5.4 Discussion

There are several examples in the literature of internal remobilization of secondary metabolites such as quinolizidine alkaloids (Wink and Witte, 1984), cardenolides (Christmann *et al.*, 1993), glucosinolates (Lykkesfeldt and Møller, 1993) and cyanogenic glucosides (Clegg *et al.*, 1979; Selmar *et al.*, 1988). Whilst quinolizidine alkaloids, cardenolides and glucosinolates are transported in an untransformed state, cyanogenic glucosides are the only example of metabolites, known to the author, that supposedly need to be temporarily converted to a transport form prior to remobilization. Some similarities between cyanogenic glucosides and cardenolides may exist, as only the complex glycosidic forms of cardenolides of *Digitalis lanata* that contain a terminal glucose will be accepted by cardenolide vacuolar transport proteins (Christmann *et al.*, 1993). It has been suggested by Selmar *et al.* (1988) that di-glycosylation is required for the conversion of a storage form of cyanogenic glucosides (the monoglucoside) into a transport form (the diglucoside), due to the distinct substrate specificities of the differently located mono- and di-β-glucosidases in cyanogenic plants. Several components of such a system have been shown to exist in *H. brasiliensis* (Selmar *et al.*, 1988; Selmar, 1993; Gruhnert *et al.*, 1994) and recently also in sorghum (Selmar *et al.*, 1996). There are however, to date, no reports of the description of a glucosyltransferase that catalyzes the conjugation of cyanogenic monoglucosides. It can be concluded that the glucosyltransferase described in this chapter conjugates dhurrin and therefore is potentially the first instance of such a description. However, the involvement of this glucosyltransferase, if any, in dhurrin mobility remains unknown. So far, there is no evidence to suggest that dhurrin is mobilized at all within sorghum, and earlier work by Halkier *et al.*, (1989) concluded that dhurrin accumulated where it was synthesized and that it therefore was immobile in young sorghum seedlings. One possibility is that dhurrin-glucosides represent a terminal degradation-form of dhurrin that may or may not leave the cell of origin. Studies by Bough and Gander (1971) and Adewusi (1990) suggest that there is substantial turnover of dhurrin in sorghum to the extent that the catabolic rate amounts to 30% of the *de novo* synthesis rate in 'green-grown' seedlings. Notably, these authors noted large differences between 'green-grown' and etiolated seedlings. The turnover in etiolated seedlings was up to 10 times higher than in 'green-grown' seedlings (Adewusi, 1990). The higher degree of turnover during darkness could explain the reduced amount of dhurrin-glucoside accumulation in etiolated seedlings, if

dhurrin-glucosides constitute a terminal degradation-form of dhurrin. Similarly, a higher degree of turnover at elevated temperatures (>25 °C) could explain the low amount of dhurrin-glucoside accumulation found under tropical conditions (28 °C day, 25 °C night).

Di-glycosylation is a common event in secondary plant metabolism that appears not to have been studied extensively. For example, it has been estimated that several hundred different di-glycosides accumulate in *V. vinifera* (Sefton *et al.*, 1993; Sefton *et al.*, 1994). It is difficult to believe that all of these glycosides are transported within this plant, and preliminary evidence suggests that this is not the case (Gholami *et al.*, 1995). Di- and tri-glycosylation may have biological roles in plants other than preparation for transport. For example, oligosaccharide-moeities may not affect certain secondary plant metabolites differently to monosaccharide-moeities, apart from a difference in water solubility. The addition of additional sugar moeities may be important for highly hydrophobic compounds such as benzene-derivatives and fatty acids. Certain plants may therefore have evolved glycosyltransferases with a broad substrate specificity in order to ensure that all glycosides have sufficiently high water solubility. Indeed, it is likely that there are di-glucosyltransferases in plants that accept a large amount of different glycosidic substrates. Such an enzyme would not necessarily need a broad specificity in *Vitis vinifera*, for example, given that all of the acceptor substrates contain a glucose-unit (Williams *et al.*, 1995). On the other hand, if the addition of sugar-moeities to monoglucosides prepares secondary metabolites for extracellular transport, one could expect that glucosyltransferases with such a potentially regulatory role for the transport and accumulation of low molecular weight compounds within a plant tissue should exhibit a reasonably narrow substrate specificity.

Only a handful of reports exist on the purification and characterization of plant di-glycosyltransferases to date. All of the glycosides tested against the di-glycosyltransferases from *Tulipa* (Kleinehollenhorst *et al.*, 1982), *Citrus maxima* (Bar-Peled *et al.*, 1991) and *Petunia* (Vogt and Taylor, 1995) were di-glycosylated. However, only a narrow range of substrates, with similarity to the substrates employed in the purification assay, were tested. None of the described di-glycosyltransferases conjugated aglycones to form monoglycosides. Evidence from phenotypic and molecular analysis of mutants of a so-called *Rt* locus suggests that a gene sequence encoding a protein

in *Petunia hybrida* is thought to function as an anthocyanidin-3-glucoside:rhamnosyl-transferase (Kroon *et al.*, 1994; Brugliera *et al.*, 1994). However, the encoded protein has not been biochemically characterized with respect to substrate specificity. Hence, our current knowledge about di-glycosyltransferases is very limited.

Is it possible to conclusively prove that cyanogenic glucosides are mobilized through the 'linustatin pathway' in sorghum? The approach taken in this study was to attempt to isolate the enzyme that catalyzes the conversion of the storage form into the potential transport form. An answer to the question if di-glycosylation plays a part in the mobilization of dhurrin, or if dhurrin is mobilized at all, has not yet been answered. However, subsequent studies of the biological properties of the di-glucosyltransferase and transformation of sorghum with di-glucosyltransferase-encoding cDNAs in 'sense' and 'anti-sense' directions would seem logical to address this issue further. Until the transformation of *S. bicolor* is routinely established there are still interesting discoveries to be made from the continuation of this study. Such discoveries will hopefully shed light on the biology of di-glycosyltransferases in plants and the turnover and transport of dhurrin in sorghum.

Chapter 6

Summary and Future directions

This study was undertaken to increase our current knowledge about glycosyltransferases involved in the biosynthesis of cyanogenic glucosides and to obtain "tools", in the form of cDNAs and antibodies, that will enable even further knowledge to be gained.

In Chapter 2 a protein that catalyzes the glucosylation of *p*-hydroxymandelonitrile, sbHMNGT, was isolated from etiolated seedlings of *S. bicolor*. Selected peptides of digested sbHMNGT were sequenced and one of the peptide sequences indicated that the isolated protein shared similarity with the amino acid sequence of other plant glycosyltransferases. Anti-sbHMNGT antibodies were generated that exhibited high specificity for sbHMNGT in a mixture containing other soluble *Sorghum* proteins. Two isoforms of sbHMNGT were also separated from one cultivar of *S. bicolor*, but attempts to elucidate the source of variation between the two isoforms were unsuccessful.

In Chapter 3 a cDNA encoding for sbHMNGT was isolated using information gained from the peptide sequence of native sbHMNGT. The cDNA was then expressed in an active state in *E. coli* and isolated by the same method as that used for native sbHMNGT. The qualitative and quantitative substrate specificity was then examined by testing isolated recombinant sbHMNGT for the acceptance of 22 different potential substrates. The resultant substrate specificity profile confirmed the identity of sbHMNGT, since maximal rates were obtained towards the believed *in vivo* substrate, *p*-hydroxy-mandelonitrile. Surprisingly, the monoterpenoid geraniol was also glycosylated by sbHMNGT, together with four other substrates with structural similarity to *p*-hydroxymandelonitrile. This suggests that sbHMNGT exhibits limited but extended plasticity towards metabolites with structures similar to the endogenous substrate.

In Chapter 4, isolated recombinant sbHMNGT was employed, together with isolated recombinant CYP79A1, recombinant CYP71E1 and native NADPH-cytochrome P450 oxidoreductase, to reconstitute the entire cyanogenic glucoside biosynthetic pathway *in vitro* with a defined composition. Although dhurrin could be synthesized, the reconstitution was not perfect, since intermediates of the pathway accumulated despite attempts to optimize the relative abundance of the three enzymes of the pathway. In another reconstitution experiment, transgenic *A. thaliana* functionally expressing CYP79A1 and CYP71E1 were transformed with *sbHMNGT*. The transgenic 3x-plants

accumulated dhurrin to similar levels as that found in young green seedlings of *S. bicolor*. Hence, the complete pathway had been transferred to an acyanogenic plant and this is the first reported transfer of an entire biosynthetic pathway from one plant species to another. Furthermore, the importance of glycosylation for the accumulation of stable secondary metabolites was highlighted by the drastic impact of sbHMNGT on the metabolic profile of transgenic *Arabidopsis* expressing the two cytochrome P450s.

In the fifth, and final chapter, a dhurrin-glucoside and a dhurrin glucosyltransferase was identified and partially purified from *S. bicolor*. The identity of the dhurrin-glucoside in the cultivar used in this study was not confirmed, although it may differ from that reported by Selmar *et al.* (1996). Interestingly, there were large variations in the accumulation of the dhurrin-glucoside grown under different conditions, although the real cause of this difference was not elucidated. The establishment of an effective assay for the di-glucosyltransferase using LC-MS, and the stability of the enzyme after several purification steps, suggests that the responsible protein may be purified to a stage that allows peptide sequence to be obtained, if additional purification steps are identified.

In summary, the main aim of this study, the isolation and characterization of sbHMNGT, was accomplished. Furthermore, the entire biosynthetic pathway of cyanogenic glucosides was transferred to a non-native plant species.

These studies open up several possibilities for future studies into cyanogenic glucosides and glycosyltransferase involved in plant secondary metabolism.

Most importantly, the ecological significance of cyanogenic glucosides can now be tested with proper controls, and the importance of cyanogenesis can also be tested if an effective dhurrin-hydrolyzing β-glucosidase can also be inserted into 3x-*Arabidopsis*. Dhurrin may also have an ecological role in the absence of dhurrin hydrolysis, since pests and microbes themselves may contain dhurrin hydrolyzing enzymes (Lechtenberg and Nahrstedt, 1999). A hint towards the ecological importance of the speed of cyanogenic glucoside hydrolysis is given by the presence of enzymes in cyanogenic plants which facilitate the hydrolysis of cyanohydrins, α-hydroxynitrilases (Møller and Seigler, 1998). The importance of the speed of the generation of defensive compounds has been demonstrated for other secondary plant metabolites such as stilbenes (Hain *et*

al., 1993). Hence, transformation of 4x-*Arabidopsis* (3x-*Arabidopsis* with dhurrin β-glucosidase) with a construct containing an α-hydroxynitrilase encoding cDNA, may also provide insights into the importance of the speed of cyanide liberation from cyanogenic plants in a defensive situation. These studies will hopefully be complemented by CYP79A1 "knock-out" or anti-sense studies with *Sorghum*, which together may shed some light on the ecological impact of cyanogenic glucosides and cyanide on both generalist and specialist pests as well as the proposed allelopathic properties of *Sorghum* (Weston *et al.*, 1994).

Another proposed "role" of cyanogenic glucosides is that of a mobilizable nitrogen storage form. One method of investigating this further involves modulating the transport of cyanogenic glucosides. This may possibly be achieved through modulation of the expression level of cyanogenic glucoside glycosyltransferases, if di-glycosides of cyanogenic glucosides constitute a major transport form in cyanogenic plants. If such a di-glycosyltransferase can be isolated and the corresponding cDNA cloned, similar glycosyltransferase-encoding cDNAs may be obtained from other cyanogenic plants by a search for homologous cDNA sequences. Further studies into the dhurrin glucosyltransferase of *S. bicolor* described in Chapter 5 will therefore be interesting to observe. Such studies may also provide additional information about the biology of poly-glycosidic forms of secondary plant metabolites, a subject that to date remains seriously understudied.

Although there were no observable differences in the extracted glycoside profile of *A. thaliana* as a result of the insertion of a functionally expressed sbHMNGT gene into wild-type *A. thaliana* (data not shown), the modulation of glycosyltransferase expression levels in plants will be crucial for increasing our understanding about the exact role of any individual glycosyltransferase *in planta*. For example, if sbHMNGT expression is lowered or eliminated from *Sorghum* it will be interesting to examine if metabolites other than dhurrin also are reduced or eliminated. Will aglycone-precursors of such metabolites be stored in the vacuole or other compartments without glycosylation, or will they be metabolized further?

The finding that sbHMNGT efficiently glucosylates the monoterpenoid geraniol, a highly active flavour compound for humans (Stahl-Biskup *et al.*, 1993), opens up

another unexpected possibility as a result of this study. Further studies into the substrate specificity of sbHMNGT has revealed that a range of other potential flavour compounds also can be glucosylated although at lower maximal rates compared with *p*-hydroxymandelonitrile (Kristensen and Hansen, 2000). For example, one potential application includes the transformation of grapevines with sbHMNGT. Monoterpenoids constitute important flavour compounds in wines made from certain cultivars of grapevine, which accumulate both as glycosides and as aglycones (Wilson *et al.*, 1984). The proportion of glycosylated monoterpenoids in berries may therefore be increased if sbHMNGT is functionally expressed in grapevine berries of these cultivars. Processing of wine involves vigorous alcoholic fermentation, during which the wine is essentially continually sparged with CO_2 released from yeasts. It is most likely that glycosylated monoterpenoids will be retained to a higher degree in fermenting wine than their unglycosylated equivalents, due to the greater water solubility of glycosylated monoterpenoids. Thus, the amount of potential flavour compounds in the resultant wines can therefore be increased as a result of sbHMNGT activity in the grapevine. Since glycosylated flavour compounds are known to by hydrolyzed during wine storage, as a result of the low pH of wine, the total pool of flavour compounds in aged wine may therefore be increased (Skouroumounis and Sefton, 2000).

However, research into the role of particular amino acid residues of sbHMNGT in determining the quantitative and qualitative substrate specificity should most likely commence prior to such applications. Such studies will also be important for other plant glycosyltransferases, and may result in a greater ability to functionally categorize putative glycosyltransferases based on their amino acid sequences.

Finally, perhaps the most exciting possibility of all is the ability to investigate the putative co-localization of sbHMNGT with CYP71E1 and CYP79A1, as has been done for some proteins of the phenylpropanoid pathway (Burbulis and Winkel-Shirley, 1999). With the availability of all three gene sequences and their successful transfer into another plant species, non-destructive markers such as GFP-encoding cDNAs should now be fused to the three genes of the cyanogenic glucoside biosynthetic pathway. The transformation of *A. thaliana* and *S. bicolor* with such GFP-fusions may not only allow the intracellular position of the enzymes to be determined. By choosing different combinations of GFP-encoding cDNA forms, the direct physical association between

different members of the pathway can also be tested using a phenomenon called fluorescence resonance energy transfer (FRET; Pollock and Heim, 1999). Such studies should also be verified by other means of intracellular localization, such as immunolocalization, to validate any conclusions.

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