Phytoremediation of Lindane in Transgenic *Arabidopsis thaliana* Expressing a Bacterial HCH-dehydrochlorinase (LinA) Protein

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Declaration

This is a declaration to state that this thesis is an account of the author's work which was conducted at the University of Sheffield, UK. This work has not been submitted for any other degree of qualification.

Acknowledgements

Completing my PhD was definitely a balancing act. Sometimes I managed to keep my feet planted on the ground, and the rest of the time I was banging my head against it. I figured out what I can and can't do; what I should and shouldn't expect (from myself and from others); and last but not least, what's important and what's not. It was definitely a real test of will-power, courage, strength and endurance; what some might say was "a character-building and learning experience". And I can honestly say I learned a lot. . . About chemical engineering; microbiology; molecular biology; synthetic biology; bioinformatics; plant science; and me of course! It turns out I'm slightly neurotic but not quite mad! Like everyone else doing a PhD....

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“You can't stay in your corner of the Forest waiting for others to come to you. You have to go to them sometimes.”

— A.A. Milne, Winnie-the-Pooh

Abstract

Gamma-hexachlorocyclohexane (ɣ-HCH), commonly referred to as lindane, is one of the most widely studied and ubiquitously detected organochlorinated contaminants within the environment. Mainly used as a pesticide, it is highly resistant to chemical, biological and natural photolytic light degradation which enables it to remain intact for long periods of time. Concerns over its toxicity, persistence and long-range transport have necessitated an environmentally appropriate and cost-efficient remediation strategy to remove it from the ecosystem, especially in developing countries where consumption and production of Lindane can have a serious effect on health, economics and arable land use. Phytoremediation, using plants and their respective enzymes, is an eco-friendly, practical and cost efficient biotechnology for the treatment and removal of lindane. This thesis aims to develop a detailed understanding of transgenic *Arabidopsis thaliana* expressing an HCH-dehydrochlorinase (LinA) protein, from *Sphingobium japonicum* UT26, and its ability to take up, dechlorinate and mineralize the persistent organic pollutant.

This investigation of transgenic phytoremediation utilizes Gateway® cloning technology, *Agrobacterium*-mediated transformation, enzymatic activity assays, along with metabolomic and proteomic techniques to show that transgenic *A. thaliana* can express the bacterial protein and is capable of removing lindane from its environment to either sequester it or metabolize it *in vitro*. However, this work also establishes that *in vivo*, the transgenic plant displays similar growth characteristics to the wild type and is unable to survive on any of the lindane concentrations previously estabilished as being toxic to *A. thaliana*.. Therefore, additional investigations into the metabolome and interactome of transgenic *A. thaliana*, and other plants exhibiting an innate ability to uptake lindane, as well as the controlled expression of engineered proteins, need to be studied before confirming the effectiveness of phytoremediation as a suitable technology for the removal of lindane from the environment.

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Chapter 1.

Introduction

Persistent organic pollutants (POPs) are natural or anthropogenic compounds that are resistant to chemical, biological and natural photolytic light degradation allowing them to remain intact within the environment for long periods of time (Wang, *et al*., 2005). These ubiquitous contaminants and their products are of considerable interest due to their toxicity, their ability to accumulate in the fatty tissues of living organisms, and their subsequent negative impact on the environment and human health.

ɣ-Hexachlorocyclohexane (ɣ-HCH) is one of the most studied and frequently detected POPs within the environment. Also known as Lindane, and henceforth referred to as such, it is a highly chlorinated organic compound which has mainly functioned as a pesticide with insecticidal properties (Bhatt, *et al*., 2009). Additionally, it has been used in agricultural, livestock and human treatments and as a component in industrial solvents (Safe, 1998). It is estimated that global lindane usage from 1950 to 2000 was approximately 1.75 million tonnes (Li, 1998; U.S. Environmental Protection Agency , 2006a). Usage as a pesticide continues in a few third world nations and it is still used for pest and vector control in several developed countries (Kumari, *et al*., 2002).

There are several pathways for lindane to enter the environment. Air releases can occur during manufacturing, application and disposal as well as through volatilization after application (Shen, *et al*., 2004). Further releases into the air, soil and water are also possible from production sites, expired stockpiles and open dump sites, which may not be properly controlled or maintained (Loibner, *et al.*, 1998). Due to its high lipid solubility and hydrophobicity, lindane can bioaccumulate easily in the food chain and rapidly bioconcentrate starting in microorganisms and working up through insects, fish, birds and mammals (IPCS, 1991). Concerns have been raised over its persistence and long-range transport as residues have been reported in both human samples; human blood, breast milk and adipose tissue; and environmental samples; soil, vegetation samples, drinking water, food products and bottled water from North America, The Arctic, Southern Asia, the Western Pacific, and Antarctica (Joint WHO Convention Task Force on the Health Aspects of Air Pollution, 2003; United Nations Environment Programme, 2006).

Lindane works as an ingested stomach poison by inhibiting γ-aminobutyric acid (GABA) neurotransmission; the main inhibitory neurotransmitter in mammalian and insect central nervous systems (Waffard, *et al*., 1989). Respiratory; cardiovascular; haematological; hepatic; endocrine; carcinogenic and reproductive effects, along with tumours and death have also been reported following exposure and inhalation (Willett, *et al*., 1998).

As the toxicity, distribution and persistence of lindane is well established, it is imperative to develop a method by which it can be safely and quickly removed from the environment. Although expensive and potentially dangerous, the standard practice typically used in its remediation involves physical removal or chemical and thermal transformation (Sutton & Hunter, 1989). Hydrolysis is the most common and important abiotic method. Climate conditions, water content, pH, oxygen levels and bacterial presence can also influence the degradation of the otherwise persistent compound (International Programme on Chemical Safety, 1992). Bioremediation is a low-cost, low-technology, and relatively low-disturbance alternative technique that uses microorganisms (indigenous or foreign); fungi; and / or plants (phytoremediation) and their enzymes to biologically degrade organic waste to a non-toxic state, or to levels below threshold concentration limits (Vidali, 2001).

Microbial bioremediation of lindane has been observed under both aerobic and anaerobic conditions (Pal, *et al*., 2005). Despite positive results being reported in laboratory studies, few reports are available for *in situ* treatment of contaminated sites. In recent studies where bacteria have been used for decontamination in field studies, bioaugmentation occurred at relatively low concentrations, requiring both long-term inoculations and nutrient application (Raina, *et al*., 2008).

Increasing attention has been given to phytoremediation; an aesthetically, environmentally and economically-friendly bioremediation technology with additional environmental advantages including biofuel production and carbon sequestration. This green technology is *in situ,* solar driven with no requirement for external carbon, nitrogen or energy sources, immoboilizes hazardous compounds and contributes to soil stabilisation (Susarla, *et al*., 2002). However, as plants often lack the catabolic

enzymes necessary for complete degradation and mineralization of these compounds when compared to microorganisms, there is potential for accumulated toxins to be released back into the environment or food chain.

Thus far, owing to the limitations of natural microbial bioremediation and phytoremediation, no such method for the clean-up of lindane on a global scale has been realized. Improving plants for phytoremediation will likely result from transferring genes known to be involved in xenobiotic degradation from other plants, microbes and eukaryotes, specifically those that can be used to improve the uptake and degradation of lindane.

The catabolic genes and Lin enzymes involved in lindane degradation have been extensively studied in the Gram-negative soil bacterium, *Sphingobium japonicum* UT26 (Nagata, *et al*., 1999). HCH dehydrochlorinase (LinA) mediates the initial transformation of lindane in a specialized pathway by catalysing the first two dehydrochlorination steps of lindane to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN), followed by its spontaneous conversion to 1,2,4-trichlorobenzene (TCB) (Nagata, *et al*., 1999).

Preliminary results from a single proof of concept study have shown that the model plant *Arabidopsis thaliana* modified with the *linA* gene from *S. japonicum* UT26 is able to grow in the presence of normally toxic lindane concentrations, and remove it from the medium whereas the wild type plant is not (De Lorenzo Prieto & Gonzalez Pastor, 2007). However, this cause and effect study does not consider the effects of varying concentration on lindane uptake and degradation, the amount of lindane that is being accumulated within the transgenic plants following its removal is not quantified, nor does it investigate LinA enzyme activity and changes to the global proteome.

This research proposes to advance on the above feasibility study and develop a mechanistic understanding of the transgenic *A. thaliana_linA* plant degradation pathway with a goal in mind to improve environmental remediation efficiency, degradation and mineralization of lindane to CO_2 and H_2O; to remove the potential

accumulation of lindane and its toxic intermediates and their subsequent re-release into the environment and entry into the food chain; as well as eliminating the need for post-phytoremediation clean-up technologies in future commercial applications.

The hypothesis of this thesis is that the development of a single gene-modified plant for the phytoremediation of lindane is enhanced by naturally occurring enzymes present in the native plant xenobiotic degradation pathway, such as Cytochrome P450s, which are capable of breaking down the less toxic intermediate, 1,2,4-TCB, for subsequent metabolization.

The aims of this work were:

1. To transform *A. thaliana* with the *linA* gene from *S. japonicum* UT26 and develop a single gene-modified plant for further investigating the phytoremediation of lindane;

2. To observe the growth and characteristics of *A. thaliana,* modified with *linA,* on control, sub-threshold and toxic concentrations of lindane and 1,2,4-TCB (relative to wildtype *A. thaliana*);

3. To investigate the removal and accumulation of Lindane, 1,2,4-TCB and other potential intermediates from growth media and in *A. thaliana*, modified with *linA,* respectively; and

4. To measure quantitative changes in the global proteome of *A. thaliana*, modified with *linA,* ascertaining whether protein regulation has been affected at the individual level within the plant xenobiotic degradation pathway or within any other metabolic pathway(s).

Background literature, research methodology, experimental results, conclusions and recommendations from this investigation are presented as follows:

Chapter 2 – describes the properties of the POP, lindane; its abiotic environmental remediation; the role of bacterial genes in its bioremediation; and the alternative green method of phytoremediation.

Chapter 3 – addresses aims 1 and 2 and explores the general phenotypic characteristics of wild type *A. thaliana;* its suitability for transformation with *linA*, its subsequent transformation with *linA*; the ability of wild type and transgenic plants to grow under control and lindane stress conditions; and LinA enzyme activity under control and lindane stress conditions.

Chapter 4 – examines the suitability of plant tissue cultures compared to whole plant systems for *in situ* laboratory studies; and the removal and accumulation of lindane, 1,2,4-TCB and other potential intermediates from growth media in wild type and transgenic *A. thaliana* using Liquid/Liquid separation and Gas Chromatography (GC) to achieve aim 3.

Chapter 5 – focuses on aim 4 and investigates changes to the global proteome and metabolic pathways by comparing wild type and transgenic plant proteins in response to control and lindane stress conditions using techniques such as isobaric tagging for relative and absolute quantitation (iTRAQ); Hydrophilic Interaction Liquid Chromatography (HILIC) separation; and Mass Spectrophotometric (MS) analysis.

The methods relevant to each chapter's experimental results are located within the relevant chapter. Major conclusions and recommendations for future work are discussed in Chapter 6.

Chapter 2.

Literature Review

2.1. Introduction to Persistent Organic Pollutants

POPs are carbon-based compounds that are resistant to chemical, biological, and natural photolytic light degradation (Wang, *et al*., 2005). They are deemed persistent as they remain intact for long periods and over time become widely dispersed geographically; accumulate in the fatty tissue of micro- and macro-organisms; and biomagnify as they move higher up the food chain. Although there are a few naturally occurring sources of POPs, most are anthropogenic; created by humans in industrial processes, either intentionally or unintentionally as by-products (Alcock, *et al*., 2004). The majority of POPs were originally designed as long-lasting pesticides while others were created for use in the production of solvents, polyvinyl chloride (PVC) and pharmaceuticals (Ritter, *et al*., 2001).

As a result of their indiscriminate use within the environment over the last several decades and long-range transport through air and water and owing to their lipophilic and semi-volatile characteristics, POPs are now widely distributed throughout substantial parts of the globe including remote areas where they have never been used (Macdonald, *et al*., 2000). POPs are generally hydrophobic and can bioaccumulate in the fatty tissues of living organisms, including fish, birds and humans. At higher levels of the food chain, POPs are found at elevated concentrations as a result of biomagnification posing a significant risk to the environment and human health (Vassilev & Kambourova, 2006). Exposure to POPs can take place through dietary intake, environment or from industrial accidents. It can have developmental and carcinogenic effects; can cause death and illness including neurobehavioural disorders and cancers; and can disrupt the endocrine, reproductive and immune systems (Wania & Mackay, 1996).

Initially, twelve POPs, referred to as the ‘Dirty Dozen’, were recognized by the Stockholm Convention on Persistent Organic Pollutants as causing adverse effects to humans and the ecosystem (Table 2.1) (Stockholm Convention, 2008a). The Convention, which came into force on 17 May 2004, requires its Parties to take measures to eliminate or reduce the release of POPs into the environment. As of

2014, ten additional compounds have been added to the Convention with a further five under consideration (Table 2.2) (Stockholm Convention, 2008b).

Table 2.1 Stockholm Convention on Persistent Organic Pollutants (17 May 2004).

Pesticides	Industrial Chemicals	Industrial By-products
DDT	Polychlorinated biphenyls	Polychlorinated benzodioxins (dioxins)
Aldrin		Polychlorinated dibenzofurans (furans)
Chlordane		
Dieldrin		
Endrin		
Heptachlor		
Hexachlorobenzene		
Mirex		
Toxaphene.		

Information compiled from Stockholm Convention Website (Stockholm Convention, 2008a)

Table 2.2 New Persistent Organic Pollutants and those proposed for listing.

Ammeded 8 May 2009	Ammended 29 May 2011	Proposed for Listing (as of April 2014)
Hexachlorocyclohexane (α-HCH)	Endosulfan	Short chained chlorinated paraffins
β- HCH		Hexabromocyclododecane
Lindane		Hexachlorobutadiene
Hexa- and hepta-bromodiphenyl ether		Pentachlorophenol
Tetra- and penta-bromodiphenyl ether		Chlorinated naphthalenes
Chlordecone		
Hexabromobiphenyl		
Pentachlorobenzene (PCB)		
Perfluorooctane sulfonic acid*		

*including its salts and perfluorooctane sulfonyl fluoride

Information compiled from Stockholm Convention Website (Stockholm Convention, 2008b)

2.2. Hexachlorocyclohexane

1, 2, 3, 4, 5, 6 - Hexachlorocyclohexane (HCH) is one of the most studied and frequently detected contaminants within the environment. HCH does not occur as a natural substance and it exists only through the photochlorination of benzene (IARC, 1979). This reaction is catalyzed by visual or ultraviolet light, X-rays or γ-rays to yield a mixture of isomers consisting of α-HCH, β- HCH, ɣ-HCH (lindane), δ-HCH, ε-HCH and other inerts (Kirk-Othmer, 1985). First prepared in 1825, HCH is a highly chlorinated organic compound with insecticidal properties as discovered by Imperial Chemical Industries Ltd. (ICI) in 1942 (Li, *et al*., 1998). Since its inception it has been used for soil protection, agricultural applications, household pest control, lumber preservation, treatment of lice and scabies, rodent baits, as an element of rocket fuel and as a fire retardant (Safe, 1998).

HCH (also known as benzene hexachloride) is the collective name for the eight isomers of 1,2,3,4,5,6-hexachlorocyclohexane (Willett, *et al.*, 1998). Figure 2.1 shows the structure of its five important and stable isomers which differ in their axial-equatorial positions around the benzene ring. The orientation of the chlorine atoms around the ring decides the differences in their physico-chemical properties and of these, lindane is the only isomer that confers insecticidal properties (Table 2.3) (Bhatt, *et al*., 2009).

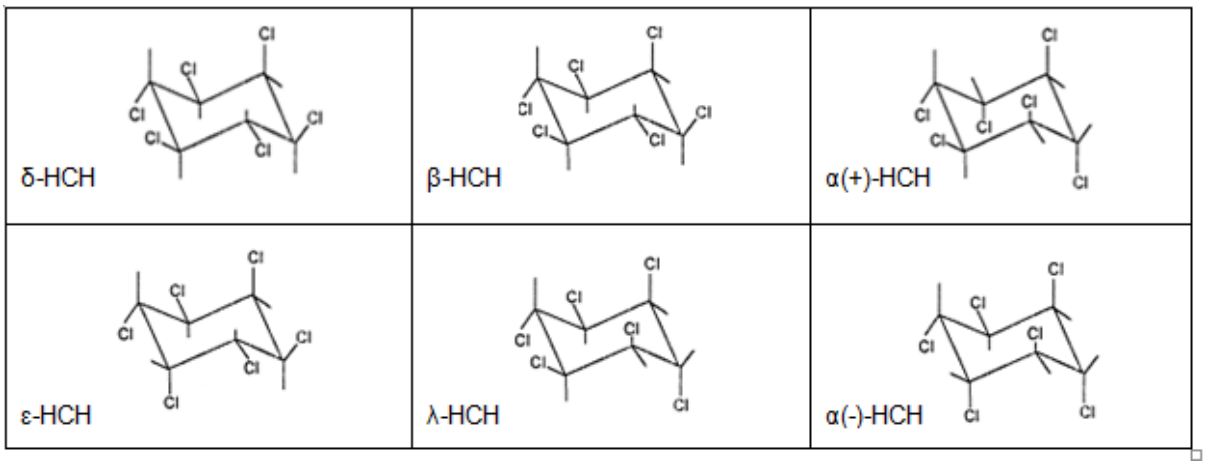

Figure 2.1 Structure of 1, 2, 3, 4, 5, 6–hexachlorocyclohexane (HCH) isomers

Reproduced from Bhatt, *et al.* (2009)

Table 2.3 The physical and chemical properties of various hexachlorocyclohexane isomers

Properties	**α-HCH**	**β-HCH**	**ɣ-HCH (Lindane)**	**δ-HCH**	**ε-HCH**
Molecular Weight (g/mol)	290.83	290.83	290.83	290.83	290.83
Physical State	Crystalline solid, monoclinic prisms	Crystalline Solid	Crystalline solid, monoclinic prisms	Fine plates	no data
Melting Point °C	159-160	309-315	112-113	138-142	219-220
Boiling Point °C	288*	60**	323*	60***	no data
Vapour Pressure (mm Hga)	$4.2x10^{-5}$ *	$3.6x10^{-7}$ ****	$4.2x10^{-5}$ ****	$3.5x10^{-5}$	no data
Solubility in Water (mg/l)	10	5	10****	5-10	no data
Henry's Law Constant	$6.86x10^{-6}$	$4.5x10^{-7}$	$3.5x10^{-6}$	$2.1x10^{-7}$	no data
Log K_{ow}	3.8	3.78	3.72	4.14	no data
Log K_{oc}	3.57	3.57	3.0-3.57	3.8	no data

All data is given for isomers at standard state (25 °C, 100 kPa) unless otherwise noted.
* At 760 mm Hga ** At 0.5 mm Hga *** At 0.355 mm Hga **** At 20 °C

Information compiled from Bhatt, *et al.* (2009)*;* Philips, *et al.* (2005)*;* and Kutz, *et al*. (1991)

HCH is commercially available as either technical HCH or lindane. Technical HCH is predominantly made up of the isomers α, β, γ and δ and relatively small quantities of the ε-isomer. Table 2.4 gives the composition of technical HCH according to different sources as presented by Breivik, *et al*. (1999). The varying proportions of the different isomers may be attributed to technical differences in the production process. Lindane is a formulation containing more than 90% of the γ-isomer, with several countries using it in its pure form at almost 100% (International Register of Potentially Toxic Chemicals, 1983).

Table 2.4 Relative comparison of isomers in technical hexachlorocyclohexane (HCH)

Isomer	%
α	55-80
β	5-14
γ	8-15
δ	2-16
ε	1-5

Commercially, both formulations were used as pesticides, with technical HCH being more widely used as an inexpensive alternative to the controversial and subsequently banned organochlorine insecticide, dichlorodiphenyltrichloroethane (DDT) (International HCH & Pesticides Association, 2006). Between 1948 and 1997, roughly 10 million tonnes of technical HCH were released into the environment (Li, 1998). However, due to its decreasing effectiveness and banned use in most western countries by the 1970s, it was gradually replaced by lindane (Baumann, *et al*., 1980).

2.3. Lindane

Lindane is a chemical variant of HCH and is also commonly known as gamma-hexachlorocyclohexane (γ-HCH), gammaxene, and Gammallin. Treatment of HCH with acetic acid or methanol and subsequent fractional crystallization, concentrates the γ-isomer to the requisite percentage found in lindane (IARC, 1979). The subsequent use of nitric acid is employed to remove odour (SRI, 1987). The manufacture of lindane, which took place in a number of countries, including the United States, India, Japan, China, Brazil, and several European nations, is a relatively inefficient process with only a 10-15% yield (United Nations Environment Programme, 2006). For each ton of γ-isomer extracted from crude HCH, approximately 6-10 tonnes of residual α-, β-, δ-, and ε-isomers remain and are disposed of as waste (International Programme on Chemical Safety, 1992). Lindane was supplied in a number of different formulations including emulsifiable concentrates, soluble concentrates, dusts, ready-to-use liquids, pressurized liquids,

aerosol sprays, granules, and as a smoke generator (Meister, *et al.,* 1998). Although exact information regarding production, sales and usage is difficult to obtain due to a lack of official reporting in manufacturing, it is estimated that global lindane usage from 1950 to 2000 amounted to approximately 600 000 tonnes, with India and possibly Russia being the only countries to still produce it (U.S. Environmental Protection Agency, 2006a).

The majority of the lindane produced was sold separately or jointly with other insecticides, fungicides, fertilizers, or wood preservatives and used in agriculture as a pesticide to treat food crops and forestry products (Haye, 1982). It also had uses in seed, soil, livestock and pet treatment, as well as pharmaceutical applications for the management of lice and scabies (Dutta & Schafer, 2003). In November 2006, the use of lindane was banned outright in 52 countries and severely restricted in 33 others and by 2009, the Stockholm Convention on Persistent Organic Pollutants implemented an international ban on lindane used in agriculture treatments (Commission for Environmental Cooperation, 2006). A specific exemption remains in place and allows for its continued use in alternative remedies for head lice and scabies until 2014 (Stockholm Convention on Persistent Organic Pollutants, 2009). However, its use as a pesticide continues in a few third world nations. As a long-established pesticide with no patents remaining, it is comparatively inexpensive and its environmental persistence is often seen as advantage (in pest control) rather than a hindrance (Weinberg, 2008).

2.4. Environmental Fate and Persistence of Lindane

Considering the global usage of 10 million tonnes of technical HCH represents approximately 1.15 million tonnes of the lindane isomer (mean value), and combining this figure with the 600 000 tonnes of pure lindane released equates to a severe case of global contamination at 1.75 million tonnes. Depending on the target, 12 - 90% of lindane used in agriculture volatilizes into the atmosphere, and is then susceptible to long-range transport. Its subsequent removal from the air by wet or dry deposition contaminates new areas containing water or soil where it can re-enter the food chain (USEPA, 2006b). Due to its high lipid solubility and hydrophobicity,

lindane isomers can bioaccumulate easily in the food chain and rapidly bioconcentrate in microorganisms, invertebrates, fish, birds and mammals (IPCS, 1991). Residues of lindane have been recorded in soil, vegetation samples, drinking water, food products and bottled water from North America, the Arctic, Southern Asia, the Western Pacific and Antarctica raising concerns over its persistence (United Nations Environment Programme, 2006). Table 2.5 highlights the occurrence of HCH isomers in environmental samples from various parts of the world.

Table 2.5 Hexachlorocyclohexane (HCH) residuals reported in countries across the globe.

Country	Location / Type / Site	Hexachlorocyclohexane isomers (mg / kg)					Total HCH
		α-HCH	β-HCH	Lindane	δ-HCH	ε-HCH	
Brazil	Rio de Janeiro	6200	7320	140	530	n/d	14190
Canada	Disposal site	18000	1800	4000	1300	n/d	25100
Germany	Contaminated soil	1.33	15.43	0.02	0.24	n/d	17.02
	Disposal Site	no data	no data	no data	no data	n/d	182000*
	Contaminated soil	no data	no data	no data	no data	n/d	3.8*
	Disposal Site	no data	no data	no data	no data	n/d	9140*
Spain	Accident / Spill	25	15	2.2	0.5	n/d	42.7
	Industrial area	45815	34830	47.6	343	n/d	81036
		15550	140	447	73.7	77.2	16238
	Industrial area	13375	6512.5	11.3	9.9	n/d	19904.6
India	Disposal site	77940	44850	990	no data	n/d	125280
	Hiranki, Dehli	0.0009	0.0111	0.1878	no data	n/d	0.1998
	Barkhalsha	0.00098	0.0084	0.2018	0.0010	n/d	0.2122
	Production Area	38.1	463.4	3.5	7.79	n/d	508.70
USA	Manufacturing Area	no data	no data	no data	no data	n/d	83628*

n/d = No Data (the isomer was not individually analyzed and does not imply absence)
*Total HCH was directly measured and not calculated by summing the individual isomer data.

Information compiled from Abhilash and Singh (2009a) and Lal, *et al.* (2008)

Over time, lindane may be transformed into a variety of other volatile chemicals including ɣ -pentachlorocyclohex-1-ene, g-3,4,5,6-tetrachlorocyclohex-1-ene, α-HCH, β-HCH and δ-HCH (Bintein & Devillers, 1996; Cornacoffet, *et al*., 1988). It may also be broken down into less toxic substances by microorganisms existing within soil,

sediment and water. However, for all practical purposes, the process is slow and dependent on uncontrollable environmental conditions (Agency for Toxic Substances and Disease Registry, 2005). Climatic conditions such as temperature; organic and inorganic matter; soil texture; moisture levels; pH; oxygen levels and bacterial presence influence the biotic and abiotic degradation and half-life rates of the otherwise persistent lindane isomer (IPCS, 1991). However, the rates of biotransformation and elimination increase rapidly when lindane exposure is discontinued (Commission for Environmental Cooperation, 2006). Table 2.6 shows the half-life of lindane under several distinct conditions.

Table 2.6 Lindane half-life under distinct conditions

Condition	Half-life	Reference
Soil (hydrolysis half-life)	330 - 5765 hours	Dorfler, *et al*. (1991)
Sediment	90 days	Bintein & Devillers (1996)
River	3-30 days	ATSDR (2005)
Lake	30-300 days	ATSDR (2005)
Groundwater	< 151 days	Mackay & Leinonen (1975)
Aerobic biodegradation (unacclimated soil die-away study)	744 - 9912 hours	Howard, *et al*. (1991)
Anaerobic biodegradation (unacclimated flooded soil die-away study)	142 - 734 hours	Howard, *et al*. (1991)

2.5. Releases to the Environment

There are several pathways for lindane to enter the environment. Traditionally, most releases are related to its production and its subsequent use as pesticide.

2.5.a. Atmospheric Residues

The largest source of lindane released into the air occurs during agricultural use and aerial application; during manufacturing and disposal; and through volatilization from soil and plant foliage after application (Shen, *et al*., 2004). Volatilsation of lindane from contaminated soil subjected to wind erosion may inadvertently see it distributed

back into the atmosphere (Lewis & Lee, 1976). In addition, it is possible that releases into the atmosphere may have occurred during production as is suggested by its presence from samples taken in the proximity of manufacturing plants (Agency for Toxic Substances and Disease Registry, 2005; USEPA, 1998). Levels of lindane in the atmosphere are sensitive to environmental and weather conditions. Under drought conditions, the volatilization rate of lindane from soil is reduced, resulting in lower atmospheric concentrations (Sang, *et al*., 1999). Although it has a high vapour pressure, evaporation of lindane from water sources is highly dependent on warmer temperatures and is therefore not considered to significantly contribute to atmospheric levels (Mackay & Leinonen, 1975).

2.5.b. Water Residues

When compared to most other organochlorine compounds, lindane is more water-soluble and has a propensity to persist in the water table. Surface water can become contaminated as dissolved chemicals on land are absorbed to particulates and atmospheric depositions (Tanabe, *et al*., 1982). The major atmospheric contributors to surface waters are wet and dry deposition, as well as gas exchange across the air-water interface.

Agricultural run-off is the principal route of lindane to surface water. However, it can also be released to groundwater through soil leachate. Despite its low mobility in soils, studies suggest that lindane does have the ability to migrate to groundwater (Sandhu, *et al*., 1978). While in the water column, lindane may be adsorbed and desorbed to and from sediment and other soil particles at rates dependent on the physical characteristics of the sediment as well as its organic carbon content. It can also be recycled back into the water column due to bacterial activity in sediment samples (Fendinger, *et al*., 1992).

Alongside surface run-off and atmospheric deposition, single identifiable localized source discharges also contribute significantly to surface water contamination. Although not considered to be a major contributor, lindane, as a second line pharmaceutical, may also enter the water system when it is applied as a shampoo or

lotion and subsequently washed off in the shower to be treated with the sewage at a wastewater treatment facility.

2.5.c. Soil Residues

Releases of lindane to the soil can occur by direct application or by direct and indirect releases during production, storage, and / or disposal. It generally tends to remain on the upper layer of the soil with very little movement to the lower soil layers (Martijn, *et al*., 1993). Lindane can then be adsorbed to soil particles, volatilized to the atmosphere, taken up by biological organisms or leached into surface and groundwater. Concentration levels are highly variable and dependant on crop type, agricultural usage patterns, exposure time, moisture levels, temperature, and seasonal variation (Samuel & Pillai, 1990).

In soils and sediments, biotransformation is the primary degradation method, whereas the major mechanism of lindane removal from soil is volatilization. High temperatures and flood-like conditions are considered to be instrumental to increase the volatilization rate of lindane from soil surfaces (Rudel, 1997; Bintein & Devillers, 1996). Studies simulating municipal landfills indicated that lindane does not volatilize or leach from its surface after application, suggesting that co-disposal of lindane with municipal waste should result in negligible releases (Reinhart & Pohland, 1991).

Further releases into the air, soil and water are also possible from date-expired stockpiles, production sites and open dump sites which may not be properly controlled or maintained (IPCS, 1991).

2.5.d. Plant Residues

There are several pathways through which lindane may enter plants. These include partitioning from contaminated soil to the roots, shoots and other arial parts, through atmospheric deposition onto the leaf surface and directly via uptake through the stomata. Metabolism of lindane in plants is not well understood and is dependent on the individual plant and its lipid content as well as the surrounding environmental

conditions (Ullman, 1972). Lindane residues have been found in bark samples and numerous and common edible plants, including carrots, lettuce, cauliflower and spinach. Plants with higher lipid contents, such as carrots, tend to also have higher quantities (Simonich & Hites, 1995).

2.5.e. Residues in Laboratory Animals and Wildlife

After direct experimental exposure, lindane and pentachlorobenzene (PeCB) residues have been observed in liver, adipose, blood, brain and muscle tissue of mice, rats and rabbits, with the highest concentrations in the liver (DeJongh & Blaauboer, 1997; Srivastava & Raizada, 2000). In foetuses and newborns, lindane and PeCB were found to be concentrated in the brain, along with a number of different organs throughout the body (Cerón, *et al*., 1995; Khanna, *et al*., 1991).

After exposure, lindane accumulates in organ tissues and is then excreted over time, accounting for decreasing concentrations after the source of exposure has been removed. In rats exposed to 60 parts per million (ppm) body weight (bw), starting concentrations were 8.64 and 437 parts per billion (ppb) in the liver and adipose, respectively, and had decreased to 0.56 and 11 ppb after seven days (Junqueira, *et al*., 1997). Similar results have been observed in rabbits (Cerón, *et al*., 1995). Alternatively, continuous exposure studies over three generations in mink showed that exposure to lindane at 1 ppm bw/day resulted in higher lindane concentrations of 4.42 ppm in the adipose tissue of third-generation females (Beard & Rawlings, 1998).

Deer exposed to lindane in the natural environment through pesticide use on crops and plants were also found to accumulate lindane in their fatty tissues and in higher concentrations than those from forest habitats (Bro-Rasmussen, 1996; Krynski, *et al*., 1982). Lindane has also been detected in the fat tissue of Japanese northern female fur seals, as well as in the breast muscle tissue of double-crested cormorants in the North American Great Lakes (Iwata, *et al*., 1998). Along the Danube River delta, lindane has also been found to increase in concentration in birds (and their eggs) that are higher up the food chain (Walker & Livingstone, 1992).

2.5.f. Arctic Residues

Lindane is the most frequently found pesticide in the arctic land and water, resulting from its volatilization after application in other countries and subsequent long-range transport by air masses and water currents (Takeoka, *et al*., 1991). Following atmospheric transport, lindane partitions from air into water at low temperatures due to its low Henry's Law constant of 0.03 (Gilman, *et al*., 1997). Lindane is also one of the most abundant organochlorines found in arctic air as shown in measurements taken from Canadian, Russian, Norwegian and Icelandic arctic locations from 1992-1995 (de March, *et al.*, 1998). A summary of lindane concentrations present in various components of the Arctic environment is presented in Table 2.7.

Table 2.7 Concentration of lindane in the arctic environment

Sample Type	Lindane Concentration (parts per trillion)
Air Vapour	$3.7x10^{-15}$
Snow Melt	0.428
Ice	0.186
Sea Water	0.610
Sediment	15.000

Lindane has been detected in all of the levels of the Arctic food web. Higher concentrations have been found at higher trophic levels as found in the lichen, caribou and wolf food chain as observed in several different Canadian Arctic herds (Elkin & Bethke, 1995). Lindane has also been found in high levels in marine food chains throughout the Arctic region, including ringed seals from the Kara Sea, Larga seals from the Sea of Okhotsk and northern fur seals (Nakata, *et al*., 1998; Tanabe, *et al*., 1994).

2.6. Human Exposure

Most of the adverse human health effects associated with lindane are linked to agricultural applications and chronic, occupational exposure (Persistent Organic Pollutant Review Committee, 2007). Besides exposure of workers and environmental contact through residues which persist in the environment, new data reveal that people may be exposed to lindane at unexpectedly high levels. As determined by the Codex Alimentarius, the Acceptable Daily Intake (ADI) for lindane is 0.001 mg/kg of body weight. Published data show that consuming an average local diet anywhere in the world, will cause a person to exceed the ADI for lindane between 3.8 and 12 times. Europe has the highest consumption of lindane through food, with a theoretical maximum daily intake reaching 0.742 mg, or 1237% of the ADI (Codex Alimentarius Commission, 1998). Persons living in arctic regions who depend on traditional and available foods such as fish and other marine mammals are also at a higher risk of exposure (Table 2.8).

Table 2.8 Lindane residues found in various countries and foods

Country	Food	Residue ppm	MRL ppm	Reference
China	Cereals	0.013	0.3	Zhang, *et al*. (1997)
	Vegetables and Fruit	0.005	0.2	
	Meat and Poultry	0.008	0.4	
	Aquatic Products	0.011	2.0	
	Eggs and Egg Products	0.045	1.0	
	Milk and Milk Products	0.018	0.1	
Hong Kong	Milk	0.068	n/d	Wong & Lee (1997)
Nigeria	Cereals	0.008-0.017	0.5	Osibanjo & Adeyeye (1995)
Ireland	Milk and Milk Products	<0.1	n/d	Downey, *et al*. (1975)
	Animal Feed	0.0001	n/d	
France	Fish	0.011-0.029	n/d	Bintein & Devillers (1996)
Greece	Milk and Milk Products	0.0008-0.007	0.01	Mallatou, *et al*. (1997)
Canadian Arctic	Marine Animal Blubber	0.145-0.163	n/d	Kuhnlein, *et al*. (1995)
	Polar Bear Fat	0.144	n/d	
	Fish Flesh, Liver and Eggs	0.005-0.012	n/d	
	Caribou	0.003	n/d	

n/d = No data given by authors

Aside from environmental samples, lindane has been found in human blood, fatty tissues and breast milk and in higher concentrations in people who have been occupationally exposed through spraying (Dua, *et al*., 1998). The presence of lindane in human breast milk has been reported in a number of countries throughout the world, including Australia, Canada, France and the US (United Nations Environment Programme, 2006). Lindane residues are also detectable in cow's milk, suggesting that susceptible children are exposeded to it right from birth. Surveys of cow's milk carried out by the UK-MAFF Working Party on Pesticide Residues (WPPR) have detected lindane in all of the 216 samples tested with more than 4% exceeding the WHO Maximum Residue Limit (MRL) of 0.01 ppm (EPTISA, 2007).

2.7. Mode of Action and Toxic Effects of Lindane

Lindane is a neurotoxin that works as an ingested stomach poison with some fumigant actions, killing insects that consume it or inhale its vapours. The pesticidal mode of action is attributed to its ability to bind the γ-aminobutyric acid (GABA) receptor-chloride channel complex thereby inhibiting GABA neurotransmission (Waffard, *et al.*, 1989). GABA is the main inhibitory neurotransmitter in mammalian and insect central nervous systems and is also responsible for the regulation of muscle tone in humans (Watanabe, *et al*., 2002). This binding activates calcium channels resulting in elevated intracellular calcium levels. The increased calcium concentration induces the protooncogene c-fos, which is associated with epileptic and seizure activity (Agency for Toxic Substances and Disease Registry, 2005). Although a specific organ or body system of toxicity has not been well defined, the main target of acute exposure appears to be the nervous system as identified by experimental animal studies and human exposure reports (Agency for Toxic Substances and Disease Registry, 2005).

The acute toxicity of HCH has been investigated in a diverse range of species and via numerous routes of application. Reports have shown that toxicity levels and symptoms are dependent on a multitude of factors. Younger and elderly respondents are generally more sensitive than healthy adult subjects. In children, a dose equivalent to 62.5 mg/kg has proved fatal whereas in adults, fatality occurred at a

much higher dose of 300 mg/kg when ingested orally (Chen, 1968). At lower, single, ingested doses of 17 mg/kg, human subjects start to display severe symptoms of toxicity (Brooks, 1990). Neurological symptoms of acute exposure and toxicity include behavioural disorders, numbness, tingling, headaches, nausea, dizziness, vomiting, diarrhoea, muscular weakness and convulsions (Willet, *et al.*, 1998). Effects of lindane poisoning in animals include restlessness, increased respiratory rate and frequency of urination, intermittent muscular spasms, salivation, grinding of teeth, loss of balance, convulsions, gasping, biting, collapse and death (Stringer & Johnston, 2001).

As lindane has been widely used for over 50 years, its long-term health effects have been extensively studied. Following exposure and inhalation, the reported chronic effects include nervous disorders, renal and liver damage. Chronic effects also include respiratory, cardiovascular, haematological, hepatic, endocrine, carcinogenic and reproductive effects, shortened lifespan, lowered fertility, changes in appearance and behaviour; along with tumours and death (Stringer & Johnston, 2001). While lindane is generally not considered to be genotoxic, animal studies have shown that lindane can accumulate in their reproductive organs, resulting in changes to foetal immune system development (Watterson & Watterson, 2003).

Although adverse reactions resulting from second-line lindane pharmaceuticals often occur due to misuse, undesirable side effects have also been reported when used properly (Food and Drug Administration, 2003a; FDA, 2003b) Findings range from skin irritation with burning sensations, itching, dryness and rash being the most common symptoms to seizures and in rare cases, death. Warning labels strongly advise that such treatments should be used with caution in infants, those weighing less than 110 lbs (50 kg), the elderly, and those with other skin diseases such as dermatitis and psoriasis due to the elevated risk of neurotoxicity (FDA, 2003c).

2.8. Detection of Hexachlorocyclohexane

2.8.a. Chromatography

Gas-Liquid Chromatography (GC) is the most common and effective method for measuring lindane in environmental samples with efficiency dependent on the solvent extraction techniques. Recovery rates range from 40% (vapour phase extraction) to 100% (solid phase extraction), with sensitivity in the low ppb and parts per trillion (ppt) ranges (Noegrohat & Hammers, 1992; Czuczwa & Stevens, 1989; Lopez-Vila, *et al.*, 1990).

In chromatography, a mixture of substances is dissolved in a mobile phase of gas or liquid and is passed through a stationary phase in order to separate the analyte of interest from other compounds based on their differential partitioning between the phases. The most common form of chromatography used for separation is column chromatography. Specifically, in GC, the mobile phase is a gas, like helium, and the stationary phase is a high boiling point liquid absorbed onto a solid. A compound will travel at a speed dependent on how much of its time is spent moving with the gas as opposed to the liquid (Clark, 2007). Small quantities of the analyte are automatically injected into the machine and carried into the column as a carrier gas (Figure 2.2). The gaseous mixture is passed down a column packed with an inert substance impregnated with a non-volatile solvent. Within the column a molecule may remain in the gas phase, may dissolve in the liquid on the surface of the stationary phase or it may remain in stationary phase (Roberts & Caserio, 1977).

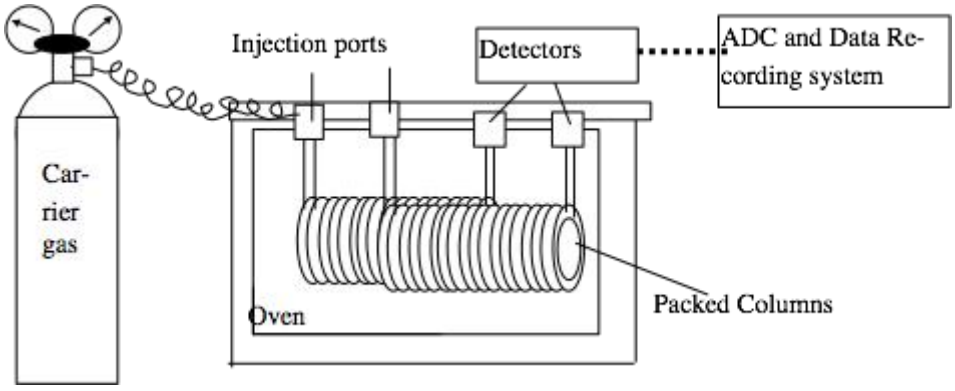

Figure 2.2 A flow scheme for gas-liquid chromatography
Reproduced from Columbia University (2007)

Different substances dissolve in the solvent to differing degrees. Those that are most soluble pass down the column most slowly as they spend more of their time absorbed into the stationary phase. As described by Leathard and Shurlock (1970), the retention time of a particular compound will vary depending on:

- a higher boiling point of the compound which will result in a long retention time;
- a higher solubility in the liquid phase which will result in a high retention time; and
- a higher column temperature which will shorten the retention times for everything in the column meaning better separation is achieved the lower the temperature of the column.

2.8.b. Separation by Solvent Extraction

Before being subjected to GC, a substance of interest must be separated from the rest of the mixture in which it was originally found. A separation process transforms a mixture of substances into distinct products which differ in their physico-chemical properties such as charge, size or structure. Separation processes may be classified as either mechanical or chemical and any combination of processes may be employed to achieve separation depending on the raw mixture as well as its physical state(s). Solvent extraction is also referred to as liquid-liquid extraction or partitioning. It is used to separate compounds from one liquid phase into another liquid phase based on the relative solubility in two different liquids incapable of mixing, such as water and an organic solvent (Figure 2.3) (Cusack, *et al*., 1991).

A substance of interest is preferentially separated from a mixture by dissolving it in an appropriate solvent (Lau, *et al*., 2010). Polar solutes dissolve more efficiently in more polar solvents, and less polar solutes in less polar solvents. Lindane is relatively insoluble in water (7 ppm) but very soluble in the organic solvent chloroform (2400 ppm) (Hazardous Substances Data Bank, 2001).

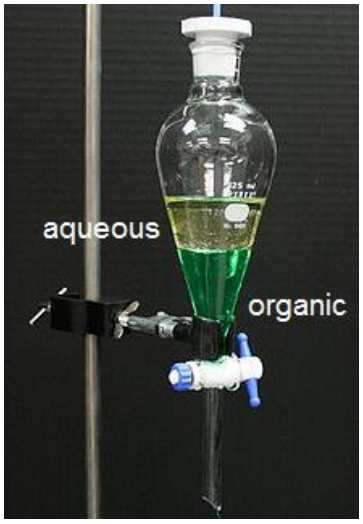

Figure 2.3 Pear-shaped funnel used in solvent extraction

The distribution ratio (D) or efficiency of extraction is equal to the concentration of a solute in the organic phase divided by its concentration in the aqueous phase and is dependent on the temperature, the chemical concentrations, and a large number of other parameters within the system. A value greater than 1 indicates preferential solubility in the organic phase and the larger the value, the less solvent is then required for a given degree of extraction (Abdel-Latif, 2003). In the case of chloroform and water, the distribution ratio (D) would then be equal to 342.86 (2400 ppm / 7 ppm). When considering the suitability of chloroform and water to extract lindane, the efficiency of extraction (D) is much greater than 1, indicating a preference for the chloroform phase, with reasonably small amounts of the solvent needed for extraction.

The extraction capabilities of a solvent are dependent on the chemical structure of itself and the solute. Factors affecting solvent efficiency are selectivity, boiling point, density, interfacial tension, corrosiveness, flammability, viscosity toxicity, stability, availability, compatibility, and cost (Robbins, 1980). Disadvantages of the process may include emulsion formation, loss of compounds and low efficiency. However, in general, the whole process is relatively simple with controlled recovery, large selectivity and flexibility (Abdel-Latif, 2003). The effectiveness of liquid-liquid extraction to separate lindane using different organic solvents was investigated by

Patil, *et al*. (2013). Results are summarized in Table 2.9 and support the validity of using the organic solvent chloroform to extract lindane.

Table 2.9 The efficiency of different organic solvents to extract lindane

Organic Solvent	Recovery Efficiency (%)
n-hexane	14
Ethylene dichloride	7
Chloroform	96

2.9. Abiotic Transformation of Hexachlorocyclohexane

Hydrolysis, catalyzed by hydroxide and hydrogen ions, is the most common and important method in the abiotic transformation of lindane to innocuous intermediates (Agency for Toxic Substances and Disease Registry, 2005). In basic aqueous solutions, lindane undergoes trans-dehydrochlorination of its axial chlorines to give the intermediate 1,3,4,5,6-pentachlorocyclohexane. This compound further reacts with water resulting in 1,2,4-trichlorobenzene, 1,2,3-trichlorobenzene and 3 molecules of hydrochloric acid (Kollig, 1993). In the presence of a catalyst such as palladium, the reaction may result in the final products of benzene and chlorine (Schuth & Reinhard, 1998). Water solubility, hydrolysis and photolysis rate constants of lindane were determined by Saleh, *et al*. (1982) in three different natural water-sediment systems, as well as in a Milli-Q water system. The aqueous solubility of lindane ranged from 7.9 to 8.4 mg/L in surface waters, while in the Milli-Q water, solubility was 9.2 mg/L. Milli-Q hydrolysis data followed first-order kinetics with kh values of $74x10^{-4}$ h^{-1} (pH 9) and $7.4x\ 10^{-1}$ h^{-1} (pH 5) (Saleh, *et al*., 1982).

At a temperature of 25 ± 1 °C, the hydrolysis reactions determined in surface water samples from a eutrophic pond, a dystrophic reservoir and an oligotrophic rock quarry followed first-order kinetics with rate constants of $7.5x10^{-3}$ hr^{-1} (pH 9.3), $8.99x10^{-4}$ hr^{-1} (pH 7.3) and $1.07x10^{-3}$ hr^{-1} (pH 7.8) and corresponding hydrolysis half-lives of 92, 771, and 648 hours, respectively. Hydrolysis experiments in Milli-Q water at pH values of 5, 7 and 9 yielded half-lives of 936, 4331 and 95 hours, respectively (Saleh, *et al*., 1982). The experimental hydrolysis half-life in the natural eutrophic

pond (pH 9.3) was comparable to the experimental value of 95 hours from Milli-Q samples (pH 9). It is therefore theorized that hydrolysis at alkaline pH values is expected to be important in the fate of lindane, while hydrolysis at acidic and neutral pH values is not.

The same natural water samples demonstrated first order aqueous photolysis of lindane in direct sunlight experiments. The rate constants were $4.1x10^{-3}$, $3.9x10^{-4}$, and $4.5x10^{-4}$ h^{-1} with corresponding half-life values of 169, 791 and 1540 hours, respectively (Saleh, *et al*., 1982). Improvements in the photolysis rate were recorded in natural water at pH 9.3, perhaps due to an addition alkaline hydrolysis reaction. After 50 days exposure to sunlight, the concentration of lindane in purified Milli-Q water dropped from 1480 to 1130 pg/mg with a rate constant of $2.24x10^{-4}$ h^{-1} (Malaiyandi, *et al*., 1982).

2.10. Biodegradation of Hexachlorocyclohexane

Microbial biodegradation of lindane has been observed under both aerobic as well as anaerobic conditions by several microorganisms (Bhatt, *et al*., 2009; Lal, *et al*., 2008) (Table 2.10). Aerobic degradation of α- and γ-isomers was originally detected in *Pseudomonas* as observed by Matsumura, *et al.* (1976). However, in 1990, *Sphingomonas indicum B90A* was the first species shown to degrade all of the isomers (Dogra, *et al*., 2004). The catabolic genes and enzymes involved in lindane degradation have been extensively studied in *Sphingomonas paucimobilis* UT26, *Sphingomonas paucimobilis* B90A and *Sphingomonas paucimobilis* Sp+ (reclassified as *Sphingobium japonicum* UT26, *Sphingobium indicum* B90A and *Sphingobium francense* Sp+ respectively) (Pal, *et al*., 2005). The *lin* genes for HCH degradation were first characterized in *Sphingomonas paucimobilis* UT26, a soil-dwelling bacterium which uses lindane as its sole carbon and energy source under aerobic conditions (Imai, *et al*., 1991). The sequential degradation of lindane occurs through several reactions catalyzed by LinA, LinB, LinC, LinD, LinE and LinF enzymes, the first 4 of which can be seen in Figure 2.4 (Nagata, *et al.*, 2007).

Table 2.10 Hexachlorocyclohexane (HCH) degrading microbes

Ability to Degrade	Microbe
α-,β-,γ- and δ- HCH	*Sphingobium japonicum UT26*
	Sphingobium indicum B90A
	Sphingobium francense Sp+
	Pseudomonas aeruginosa sp. ITRC-5
	Microbacterium sp. ITRC-1
	Sphingomonas sp. BHC-A, MI1205, γ^{A-2} and γ$^{1-1}$
	Sphingomonas sp. DS2, DS2-2 and DS3-1
	Pseudomonas fluorescens biovar, biovar I and biovar II
	Pseudomonas sp.
	Vibrio aginolyticus
	Flavobacterium sp.
	Burkholderia pseudomallei
	Bacillus sp.
α- and Lindane	*Rhondanobacter lindaniclasticus*
	Pandoraea sp.
	Pseudomonas sp.
	Sphingomonas sp.
Lindane	*Steptomyces sp. M7*
	E. coli
	Pseudomonas sp.
	Trametes hirsutus (white rot fungi)
	Phanerochaete chrysosprium
	Cyanthus bulleri
	Phanerochaete sordid

Information compiled from Lal, *et al.,* (2008) and Bhatt, *et al.* (2009)

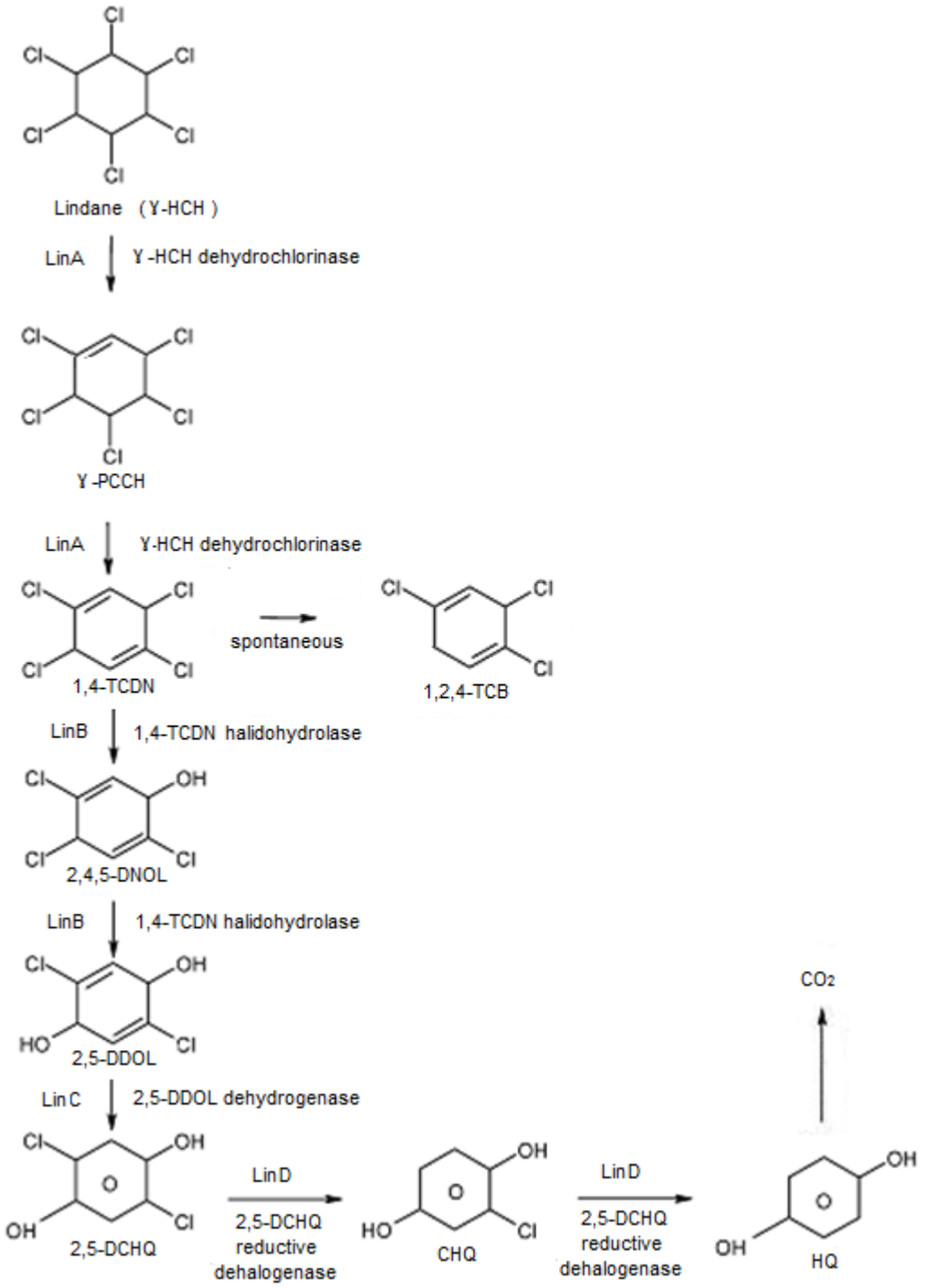

Figure 2.4 The enzymes and intermediates in the degradation pathway of lindane in *Sphingomonas paucimobilis* UT26.

Reproduced from Bhatt *et al.* (2009)

Initially, γ-pentachlorocyclohexane (ɣ-PCCH) is formed via a dechlorination step and is then converted to 1,3,4,6-tetrachloro1,4-cyclohexadiene (1,4-TCDN) by LinA. This is then converted by the hydrolytic dehalogenase, LinB, to 2,5-dichloro-2,5-cyclohexadiene-1-ol (2,5-DDOL) via 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL). The dehydrogenase enzyme, LinC, then converts 2,4,5-DNOL to 2,5-dichlorohydroquinone (2,4 DCHQ), which is subsequently transformed to hydroquinone (HQ) via the dechlorination of chlorohydroquinone (CHQ) under LinD. Mineralization of HQ occurs via two pathways: 1) The nitrophenol pathway or 2) by the dioxygenase enzyme LinE to form maleylacetate (MA) (Nagata, *et al*., 1999). MA is then converted to β-ketoadipate by MA reductase, LinF and metabolized by the β-ketoadipate degradation pathway found in many soil bacteria and fungi (Harwood & Parales, 1996).

The *linA, B* and *C* genes, found in the UT26 genome, are constitutively expressed whereas *linD* and *linF,* which form an operon, are regulated and induced by a LysR-type transcriptional regulator, called LinR, in the presence of CHQ, HQ, and 2,5-DCHQ compounds (Nagata, *et al.*, 1999). The *LinA* enzyme does not exhibit homology to any known proteins whereas LinB, a chlorohydrolase, is similar to the haloalkane dehalogenases present in soil bacteria strains found in environments contaminated with halogenated compounds (Imai, *et al*., 1991; Marek, *et al*., 2000). LinC is homologous to members of the short-chain alcohol dehydrogenases while LinD has similarities with, and is also enhanced in the presence of glutathione S-transferases (GSTs) (Neidle, *et al*., 1992; Miyauchi, *et al*., 1998).

2.11. Remediation of Lindane in the Environment

As the toxicity, distribution and persistence of lindane is well established, it is imperative to develop a method by which it can be safely and quickly removed from the environment.

2.11.a. Physical and Chemical Remediation

Although potentially hazardous and expensive, the standard practice typically used in the remediation of lindane involves the excavation and removal of contaminated soils to a landfill (Baczynski & Pleissner, 2010). Workers are at a high risk of exposure via direct contact or vapours on site. Care must be taken to avoid the accidental movement of lindane from the contaminated site to uncontained areas off-site either through volatilization of the excavated soil (to be treated) during excavation and transport; or from the tyres of transport vehicles in contact with contaminated soil on-site moving off-site for processing. Off-site physical treatments, such as thermal desorption and incineration offer sufficient degradation of lindane but the transport of hazardous materials, large infrastructure of treatment plant, high processing costs and toxic by-products, including the asphyxiant phosgene gas, render it impractical (Nagpal & Paknikar, 2006). More efficient chemical methods, including microwave-induced degradation with NaOH-modified sepiolite and hydrogen peroxide, may also be used to bring about the complete destruction or transformation of lindane (Salvador, *et al*., 2002). However, these energy-intensive and complex engineering processes are highly corrosive, may result in incomplete pollutant removal and are environmentally unfriendly with the emission of greenhouse gases, destruction of soil structure and / or severe landscape alteration (Campos, *et al*., 2008).

2.11.b. Bioremediation

Bioremediation, which uses indigenous or foreign microorganisms, fungi, plants (phytoremediation) and their enzymes to biologically degrade organic waste to an non-toxic state, or to sub-threshold concentration levels, is a low-cost, low-technology, and relatively low-disturbance alternative technique (Vidali, 2001). Substantial work on the bioremediation of lindane has been successfully reported in laboratory studies using microbes and fungi, although few reports are available for *in situ* treatment of contaminated sites (Salaam & Das, 2012). In recent studies where bacteria have been used for decontamination in field studies, high mortality rates were observed, requiring long-term inoculations and nutrient application (Mertens, *et*

al., 2006). Moreover, bioaugmentation using single species only occurred at relatively low concentrations to a maximum of 34 µM (10 mg/kg) (Raina, *et al*., 2008). The survival and degradation abilities of lindane-degrading strains is dependent on several parameters including the depth and extent of contamination; the concentration of the contaminants, nutrients, oxygen and toxic substrates; soil type and properties; and presence of other soil microbes (Federal Remediation Technologies Roundtable, 1997).

Based on the disadvantages of microbial degradation, increasing attention has been given to phytoremediation; an aesthetically-, environmentally- and economically-friendly bioremediation technology with other environmental advantages including biofuel production and carbon sequestration. This green technology is *in situ;* solar driven with no requirement for external carbon, nitrogen or energy sources; immoboilizes hazardous compounds; and contributes to soil stabilization (Pilon-Smits, 2005). Phytoremediation is a highly efficient process and is approximately ten fold cheaper than alternative physical methods (National Risk National Risk Management Research Laboratory (USEPA), 2000). However, as plants often lack the catabolic enzymes necessary for complete degradation and mineralization of these compounds when compared to microorganisms, there is potential for accumulated toxins to be released back into the environment or food chain. Consequently, disposal of these plants is often necessary, requiring further physical or chemical treatment (Abhilash, *et al*., 2009).

2.12. Phytotechnologies

The action of plants on POPs is diverse; they may be immobilized, stored, volatilized, transformed and mineralized or any combination of the above. As such, various phytotechnologies have been devised to remediate POPs.

2.12.a. Phytoextraction

Phytoextraction (also known as phytoaccumulation or phytosequestration) is the uptake of contaminants by plant roots and subsequent movement of the

contaminants to above-ground plant parts (Figure 2.5). The removal of contaminants is generally achieved by harvesting the plant but importantly, when compared to the excavation of soil or sediment, phytoextraction reduces the amount of material to be disposed of. Harvested biomass is analyzed, processed and disposed of either by landfilling or incinerating (Vishnoi & Srivastava, 2008).

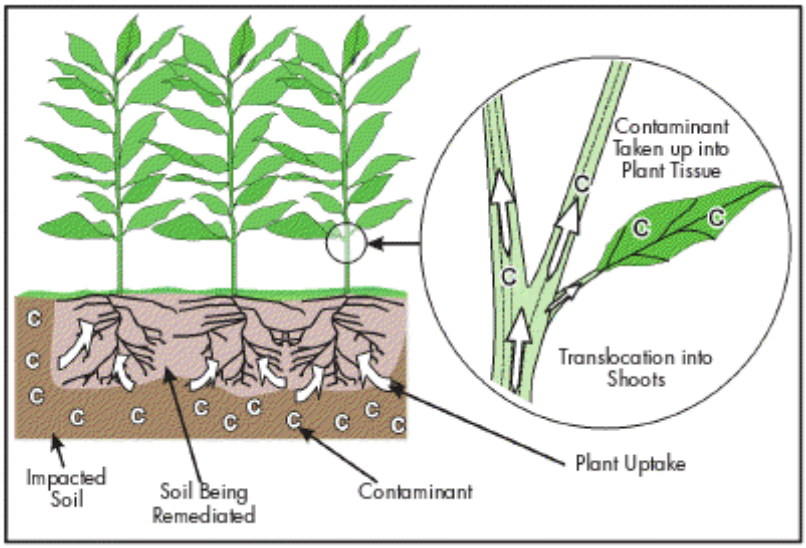

Figure 2.5 Phytoextraction
Reproduced from Interstate Technology & Regulatory Council (2001)

Natural phytoextraction uses plants that naturally take up and accumulate extremely elevated level of contaminants in their stems and leaves and is usually conducted by planting (or transplanting) selected plant species in the contaminated soil. Induced phytoextraction uses fast-growing plants in the contaminated soil that have had amendments added to the soil to increase bioavailability of the toxins to the plants. Another approach is the use of plants that trap the contaminants in their root systems and are then harvested whole (including the roots) using methods similar to those employed for below-ground crops such as potatoes, beets, carrots and peanuts (Greger & Landberg, 1999).

Accumulation of contaminants in the above-ground parts of the plants may pose a risk to animals eating these plants and the environment. Although this technology takes longer than other remediation methods and several crop cycles are usually required to remove all the contaminants to the desired levels, removal rates are

highly dependent on the specific soil chemistry at the site. Phytoextraction is typically less costly than excavation with an estimated cost ranging from $16 to $62 USD per cubic yard of soil treated (Purakayastha & Chhonkar, 2010).

With regards to lindane, phytoextraction is generally not favoured as it is hydrophobic and will, in theory, bind to soil particles and plant roots preventing its uptake. The majority of reports concerning lindane contamination of above ground-plant parts state that this accumulation is mainly due to atmospheric deposition, with little contribution of translocation from roots to shoots (Burken, 2003). However, several species of plant grown in lindane-contaminated soil, including *Capsicum annuum* (chilli) and *Coriandrum sativum* (coriander) were reported to contain lindane in their arial parts, accounting for 23 and 30% loss, respectively, of the initial soil concentration (Barriada-Pereira, *et al*., 2004). These findings support potential remediation of contaminated sites by careful selection of those plants capable of enhanced hydrolysis and increased xenobiotic uptake via the transpiration flux.

2.12.b. Phytostabilization

Phytostabilization is the immobilization of a contaminant in soil through absorption and accumulation by roots, adsorption onto roots, or precipitation within the root zone of plants (Figure 2.6). It is also used to prevent movement of toxins through leaching, erosion, and soil dispersion (Abhilash, *et al.*, 2009) During phytostabilization, proteins and enzymes are released by the roots into the adjacent soil, resulting in changes to the chemistry of the contaminants, which become insoluble; immobilized in the soil or on the root surface; and less toxic (Kuang, *et al*., 2003).

No process residuals are expected and the above-ground biomass is not expected to have any significant concentrations of contaminants or secondary products to dispose of. However, as the contaminants are left in place, a long-term monitoring plan has to be implemented to ensure continuing effectiveness of the stabilizing conditions.

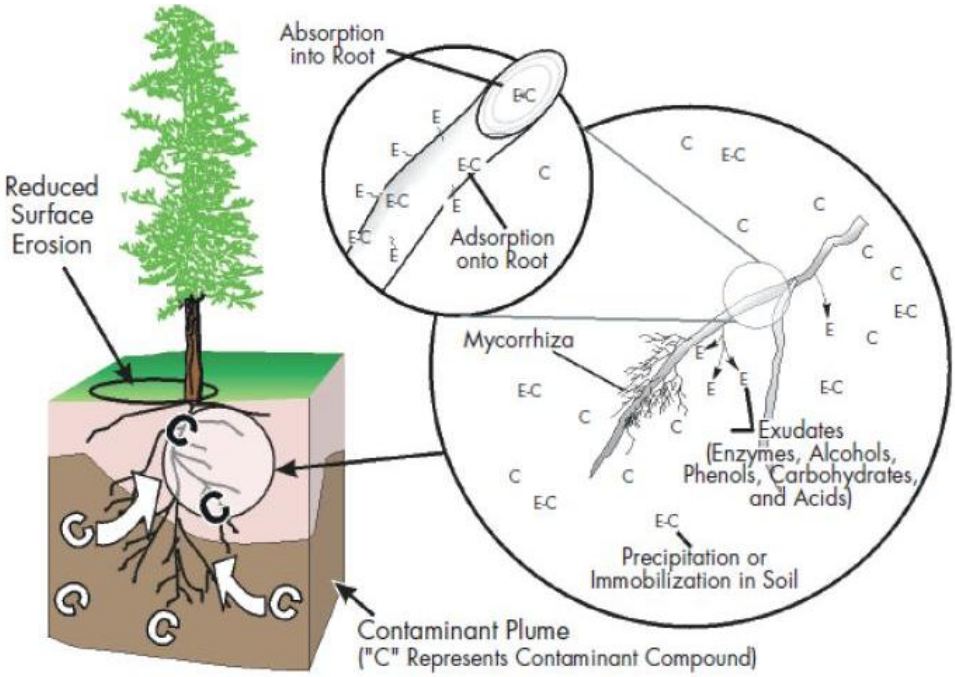

Figure 2.6 Phytostabilization
Reproduced from Biology Online (2008a)

Phytostabilization typically costs less than excavation, although actual costs depend on site-specific conditions such as depth of contamination, soil condition, need for soil conditioning and tilling. Costs are estimated at about $3,000 (USD) per acre (Brookhaven National Laboratory, n.d.)

2.12.c. Phytodegradation

Phytodegradation, or phytotransformation, occurs when contaminants external to the plant or those that are taken up by plants are broken down, or degraded, using naturally occurring metabolic processes within the plant, or via enzymes released by the plants. (Figure 2.7) Additionally, the release of any compounds, or exudates, that cause transformation within the rhizosphere, may result in degradation occurring outside of the plant. However, any degradation within the rhizosphere caused by microorganisms is considered rhizodegradation. Phytodegradation is usually limited to the root zone, or sometime below when the root exudates are soluble, non-sorbed, and transported below the root zone (Pivetz, 2001).

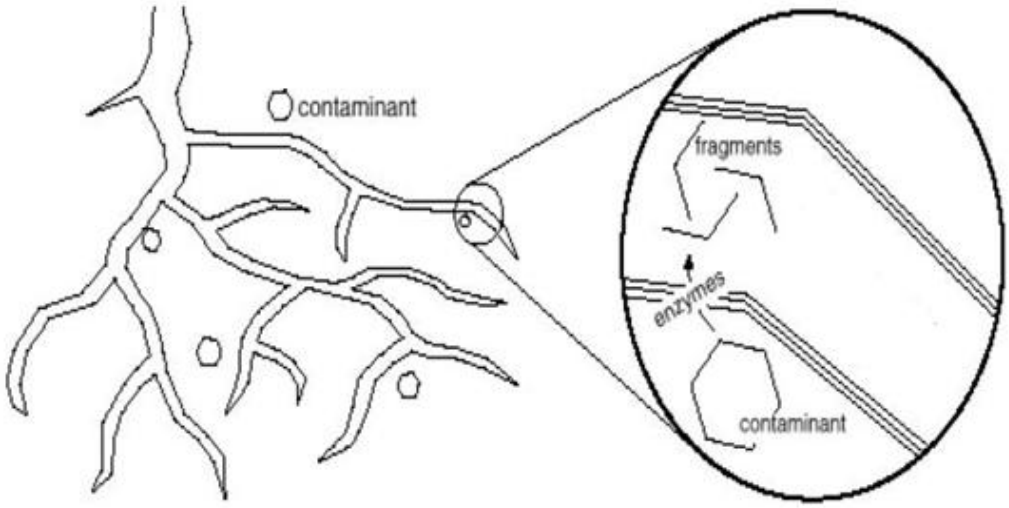

Figure 2.7 Phytodegradation
Reproduced from Biology Online (2008b)

Rhizospheric degradation of contaminants by way of plant exudates can take place in microorganism-free environments meaning that phytodegradation can happen in soils where biodegradation cannot. Uptake is dependent on solubility, hydrophobicity, and polarity but can also be influenced by the age of contaminant, the type of plant along with a number of other physical and chemical soil characteristics. In general, organic compounds with a log K_{ow} ranging from 0.5 up to 3.5 can undergo phytodegradation within the plant, whereas external phytodegradation does not rely on uptake, and so the log K_{ow} is of no consequence (Newman, *et al*., 1998). As lindane has a K_{ow} above 3.0, degradation is more likely to occur outside of most plants.

The disadvantages associated with phytodegradation include the formation of toxic intermediates, or degradation products, and difficulties confirming contaminant destruction though the presence or identity of metabolites within a plant.

2.12.d. Phytovolatilization

Phytovolatilization is the release of a contaminant, or a broken down metabolite from plants to the atmospheric environment through uptake, transpiration, plant metabolism, and plant transpiration (Figure 2.8). Factors such as wind, temperature, isolation, humidity and precipitation can affect the rate of transpiration within a plant.

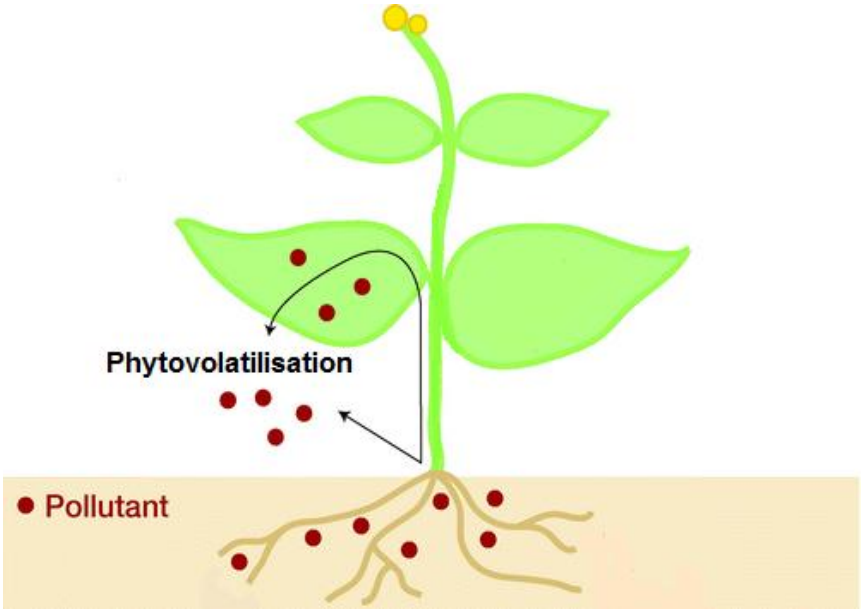

Figure 2.8 Phytovolatilization
Reproduced from Pilon-Smits (2005)

Phytodegradation often occurs alongside phytovolatilization with transformed, less toxic compounds being released to the atmosphere where faster and natural degradation processes such as photodegradation can take place (Suthersan, 2001). Conversely, phytovolatilization may lead to the release of toxins back into the atmosphere where they can accumulate in vegetation and subsequently re-enter the food chain or raw building materials (Biology Online, 2008b).

With respect to lindane, each chlorine atom released from the cyclohexane ring structure reduces the toxicity by several-fold suggesting that its immobilization and degradation using any number of phytotechnologies, individually or in combination, has the potential to remediate and stabilize the surrounding environment.

2.13. Applying Phytoremediation Technologies

A number of issues must be considered before applying phytoremediation technologies to a polluted site: detailed site characterisation, including pollution level; uptake of pollutants by site-suitable plant species (the critical factor in scientific studies and commercial applications); the fate of the pollutant in the plant and

environment; the total cost of cultivation (planting, irrigation, management) and soil amendment; and the total time estimated for remediation. Where phytotechnologies have been employed as a remediation strategy, further considerations regarding the collection and fate of plant biomass (disposal, energy or fibre production) and pollution level of any remaining plant material (specifically underground plant material, which is expensive and difficult to remove) are crucial at the post-harvest stage.

2.13.a. Uptake and Translocation of Organochlorines by Plants

Understanding of the uptake, transport and degradation mechanisms along with the physico-chemical properties of the compounds and enzymes involved is essential to enhance phytotechnologies. The removal of POPs from the soil by plants was first elucidated after observing that organic pollutants vanish faster from vegetative soil samples than from uncultivated soil (Alkorta & Garbisu, 2001). This was further confirmed using plant-mediated degradation of petroleum contaminants in controlled field studies (Banks, *et al*., 2003). As lindane and most other POPs are anthropogenic and thus foreign to natural plant processing systems, passive diffusion and advection are the usual and principal uptake processes from contaminated soil to root surfaces (Trapp, *et al*., 1994). The degree of uptake and distribution appears to be inversely proportional to water solubility or directly proportional to the octanol-water partition co-efficient (K_{ow}) and is dependent on:

- Physico-chemical compound properties such as vapour pressure, water solubility, and molecular weight (Gerhardt, *et al*., 2009);
- Environmental soil characteristics including the water content, temperature as well as the organic and mineral matter content; and
- Plant characteristics such as the type and size of root system, leaf morphology, as well as the lipid, fibre, carbohydrate and water content and quality (Trapp, 2002).

Normally, chemicals in solution (soil or directly from aqueous environments) enter plant roots through cell wells, where the major transport processes of water include

osmosis and bulk flow (Campos, *et al*., 2008). Bulk flow of water in the xylem is driven by a hydrostatic pressure gradient induced by evapo-transpiration of water vapour from the foliage to the atmosphere (Mengel, *et al*., 2001). Movement of chemicals into the xylem is dependent on the chemical polarity and molecular configuration as some chemicals may become attached and / or metabolized in the endodermis before reaching the xylem. Those that do are transported in the transpiration stream or sap, during which time they may degrade, interact with other compounds / enzymes, partition into a number of different plant parts or they may eventually be released through stomatal pores in the leaf back into the atmosphere (Paterson, *et al*., 1990).

Compounds, or toxins, of intermediate solubility are transferred to the shoots more efficiently than others, and as a result, those that are highly hydrophobic (log K_{ow} above 4), such as lindane, tend to bioconcentrate in below-ground plant parts such as roots where they are readily bound to lipids in the cell wall and the lipid bilayer by Hemicellulose (Mengel, *et al*., 2001). Some organics, with a log K_{ow} between 0.5 and 3.5, display both hydrophobic and hydrophilic properties and are able to move through the lipid bilayer for subsequent transportation to the xylem and translocation to the shoot. Others, with a log K_{ow} less than 0.5, are too hydrophilic to pass through membranes and thus, will never enter into the plant. Non-polar molecules with molecular weights less than 500 kDa will bind to the root surfaces, whereas polar molecules, such as lindane, have the potential to enter the root and be translocated (Pilon-Smits, 2005). However, the predicted partitioning of lindane and binding with lipids using the log K_{ow} is markedly lower than its actual measured sorption in roots and shoots (Burken, 2003).

Translocation of POPs from soil to the shoot tissues via the transpiration stream is generally small for hydrophobic compounds like lindane (Burken & Schnoor, 1998). Soil properties such as organic content influence how well the chemicals are sorbed to the soil, making them less available for root uptake and affecting the overall soil/plant (Scheunert, 1985). Chemicals with a high vapour pressure may volatilize into the atmosphere where they may subsequently enter the plant through stomata in above-ground plant parts such as the foliage and stem (Bacci & Gaggi, 1986).

2.13.b. Transformation of Organochlorines by Plants

Plants often lack the catabolic enzymes necessary for complete degradation and mineralization of lindane and other xenobiotics when compared to microorganisms. However, the fate of contaminants entering a plant ultimately depends on their chemical structure, external temperature as well as the plant variety and its vegetative phase (Kvesitadze, *et al*., 2009). Plants demonstrate three main physiological and biochemical processes to defend against the potentially harmful effects of contaminants: excretion, functionalization (followed by conjugation and compartmentalisation) and decomposition or mineralization.

2.13.b.i. Excretion

Excretion, a rare and simple process, involves the translocation and emission of highly mobile and concentrated untransformed xenobiotics through the apoplast (Sandermann, 1992). Alternatively, once a xenobiotic enters plant tissue, it can often be transformed into innocuous products by a variety of biochemical reactions which can be further categorized into three biochemical processes according to the "Green Liver Model": functionalization, conjugation and compartmentalisation (Figure 2.9) (Sandermann, 1994).

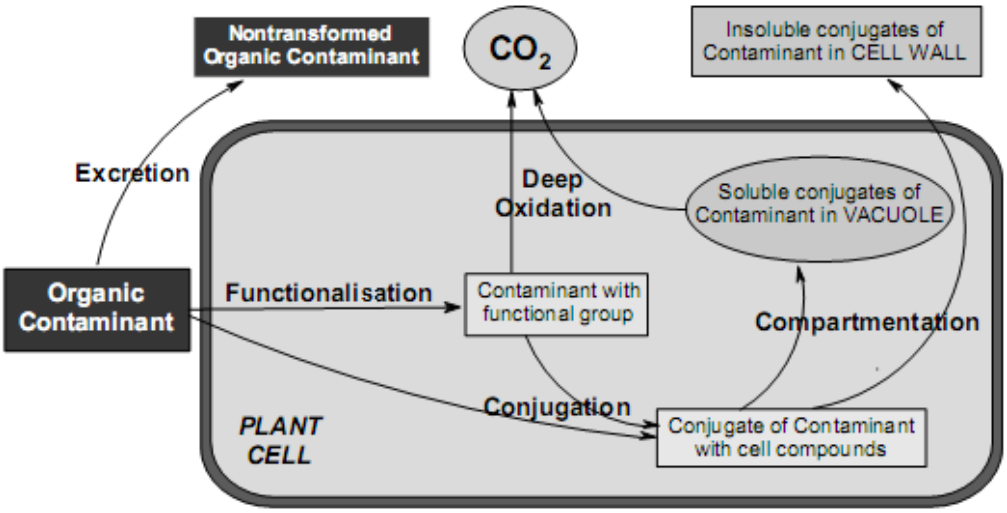

Figure 2.9 The main pathways of contaminant degradation in plant cells in accordance with Sandermann's green liver concept

Reproduced from Sandermann (1994)

2.13.b.ii. Green Liver Model

Functionalization involves the addition of a hydrophilic hydroxyl, carboxyl or amino functional group to a hydrophobic organic xenobiotic molecule following enzymatic oxidation, reduction or hydrolysis (Kvesitadze, *et al*., 2004). The consequential change in reactivity and polarity results in an increased affinity of the molecule to enzymes and further conversion.

Conjugation is a basic phytoremediation course of action and consists of the coupling of a contaminant with a functional group to native cell compounds such as proteins, peptides, amino acids, organic acids, mono-, oligo-, polysaccharides and lignin to form peptide, ether, ester, thioether or other types of covalent bonds (Kvesitadze, *et al*., 2004). The formation of less toxic conjugates bound with non-toxic cellular compounds allows them to remain in the plant cell without instigating damage to cell homeostasis. Conjugate formation also promotes internal mobilization and activation of enzymes responsible for additional transformation.

Although conjugation is a well-used pathway in plant self-defence, it is energetically and physiologically detrimental to other plant processes. Conjugate formation may result in the depletion of cellular compounds important to life and as basic molecular structure of xenobiotics is maintained, toxicity is only partially reduced (Dearing, *et al*., 2005).

Compartmentalization is generally the final phase of conjugate processing. Soluble conjugates are primarily accumulated in vacuoles, while insoluble conjugates are shuffled out of the cell via exocytosis in the apoplast and accumulate in the cell well (Sandermann, 1994). In a process known as storage excretion, plants cells are able to remove contaminant conjugates through an ATP-dependent glutathione pump which actively transports toxic residues away from vitally important structures, including nuclei and mitochondria (Coleman, *et al*., 1997).

Transformations of contaminants during functionalization, conjugation and compartmentalization are catalyzed by a number of enzymes also involved in regular

plant cell metabolism. Table 2.11 lists the enzymes that are directly involved in the transformation process of xenobiotic contaminants.

Table 2.11 Degradation pathway and enzymes of xenobiotics in plants

Phase I – Conversion/Transformation	**Phase II – Conjugation**	**Phase III – Compartmentalization**
Oxidation, reduction and hydrolysis to form a more polar, chemically active and water soluble compound, for phase II reactions. Cytochrome P450s containing peroxidases are key in oxidative processes and deciding the plant's tolerance to xenobiotics. Nitroreductase for degradation of nitroaromatics and laccase for breaking aromatic ring structures.	Organic pollutants or Phase I metabolites are directly conjugated with enzymes, sugars or amino acids resulting in less toxic hydrophilic compounds. Enzymes such as glutathione-S-transferases (GSH), glucosyl transferase (GT) and N-malonyl transferases are associated with Phase II conjugation reactions.	Conjugated xenobiotics are removed from cytosol, transported and sequestered in vacuoles or bound to insoluble cellular structures. ATP-driven vacuolar transporters are the main enzymes involved.

Information compiled from Pilon-Smits (2005) and Susarla *et al.* (2002)

Depending on the structure, concentration and exposure time of the contaminant, along with the energy demands placed on the cell, activation of other enzymes participating indirectly in the detoxification of contaminants, may also be induced (Chrikishvili, *et al.*, 2006).

2.13.b.iii. Degradation and Mineralization

The most important process of organic contaminant decomposition or mineralization in plants is oxidative degradation by Cytochrome P450-containing monooxygeneses, peroxidases and phenoloxidases.

Cytochrome P450-containing monooxygenases are mixed-function enzymes located in the membranes of the endoplasmic reticulum and are integral in the hydroxylation of organic compounds (Schuler, 1996). Nicotinamide adenine dinucleotide phosphate (NADPH) and / or nicotinamide adenine dinucleotide (NADH) reductive equivalents are used in the activation of molecular oxygen and subsequent

incorporation of one of its atoms into a non-polar, lipophilic organic compound (XH). This later results in the formation of a hydroxylated product (XOH) and a water molecule (using a second atom of oxygen) (Figure 2.10) (Sharma & Vanden Born, 1970).

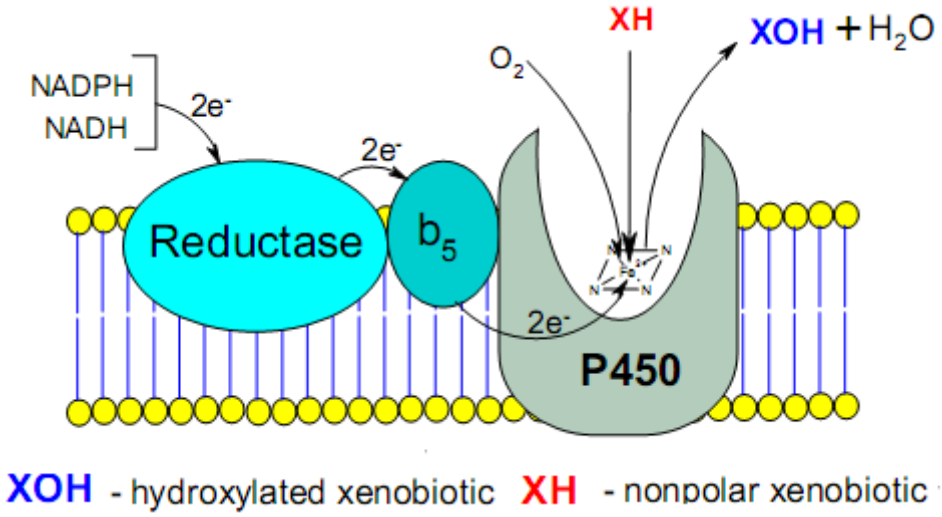

Figure 2.10 Microsomal monooxygenase system
Reproduced from Kvesitadze, *et al*. (2009)

In response to stress, plant peroxidase activity increases and catalyzes a number of free radical reactions. As a consequence, the compound that is directly oxidized by peroxidase goes on to oxidize other compounds, including most organic xenobiotics (Kvesitadze, *et al.*, 2009). Plant peroxidases have a high affinity and wide substrate specificity to organic xenobiotics of different chemical structures and are ubiquitously distributed within plants existing in plasmalemma, cell walls, tonoplasts, cytoplasm, plastids and intracellular membranes of endoplasmic reticulum (Stiborova & Anzenbacher, 1991).

Phenoloxidases are ubiquitous within plant organelles and catalyze both monooxygenase and oxygenase reactions. Phenoloxidases are involved in the oxidation of aromatic xenobiotics and have been found to oxidize benzene and toluene first by their hydroxylation and then by further oxidation to quinine (Martinova, 1993). Alternatively, in situations where it is not a substrate for the phenoloxidase, the xenobiotic may experience co-oxidation whereby a corresponding endogenous phenol is oxidized to form compound(s) with a high redox potential.. This activates the formation of oxygen radicals, such as a super

oxide anion radical (O^{2-}) or a hydroxyl radical (OH) which enables phenoloxidase to indirectly participate in contaminant degradation (Guillén, *et al*., 2000).

Only a few toxic molecules undergo direct degradation. Over 80% are conjugated then compartmentalised in vacuoles and apoplasts followed by the deep oxidation of the toxic molecules (Sandermann, 1994).

2.13.c. Selecting Plants for Phytoremediation

Selecting plants for phytoremediation is a complex task. Factors to consider include soil properties, climate, root type and how effective different species respond to the different types of pollutants. Climate is a major concern as a plant that grows well in one geographical environment and area may perform as well in another. The amount of sun, wind, rain and man-made stress conditions like road salt or vehicle fumes can adversely affect the growth of a specific plant. Likewise, soil type is significant since plants normally prefer a specific type of soil, such as rubble, clay or sand (Xiujin, *et al*., 1987). For phytoremediation to work effectively, plants need to thrive in the designated given environment. Table 2.12 lists the diverse assortment of plants and plant varieties that are often used to remediate different types of contaminants

Root type is another consideration; plants with smaller roots are better at covering a larger surface area within the soil when compared to most trees which typically have much larger roots. However, some plant species with natural remediation capability are too small or have too limited a root system to remove significant amounts of the contaminant. Alternatively, excluder plants able to fix atmospheric Nitrogen are of practical use to aid in restoring contaminated soils allowing the colonization of plants and microbes capable of remediation (Walker, *et al*., 2007)

Table 2.12 Phytoremediative plant species

Pollutant	Phytoremediative Plant Species
Heavy Metals (Zn, Cd, Cr, Cu, Pb)	Alpine Pennycress, cabbage, Pelargonium Geranium, Sunflower.
Aromatics (benzene, toluene, zylene)	Alpine Pennycress, Barley, Cabbage, Crucifer, Dandelions, Grasses (Rye, Fescue, Bermuda, Sorghum), Hop, Indian Mustard, Nettle, Pelargonium, phreatophyte trees (poplar, willow, cottonwood, aspen), Rape Seed, , Sunflowers
Wood Preservatives (arsenic, PAH)	Brake Fern, Indian Mustard, fibrous rooted grass (Rye, Fescue, Bermuda, Sorghum), phreatophyte trees (poplar, willow, cottonwood, aspen)
Leachates (PCBs, pesticides, herbicides)	Grass (Rye, Fescue, Bermuda, Sorghum), legume (Clover, Alfalfa, Cowpea) Mulberry, Osage Orange, phreatophyte trees (poplar, willow, cottonwood, aspen)
Radiation	Indian Mustard, Pigweed, Sunflower
Petroleum	Alfalfa, grasses (Rye, Fescue, Bermuda, Sorghum), Hybrid Poplar, Indian Mustard, Juniper
Explosives	Elodea, grasses (Rye, Fescue, Bermuda, Sorghum), legume (Clover, Alfalfa, Cowpea), parrot feather, phreatophyte trees (poplar, willow, cottonwood, aspen), reed canary grass, water star grass
Nitrates	Grasses (Rye, Fescue, Bermuda, Sorghum), legumes (Clover, Alfalfa, Cowpea), Indian Mustard, phreatophyte trees (poplar, willow, cottonwood, aspen), Bullrush, Cattail, Coontail, Pondweed, Arrowroot, Duckweed; Algae, Stonewort, Parrot Feather, Eurasian Water Milfoil, Hydrilla, Sweet Flag, Water Hyacinths, Water Lilies

Washington State University Extension (2012)

An essential key to the success of phytoremediation is understanding how plants take up water, as this will help develop a better appreciation of how the contaminants in the water will also reach the plant. Plants with many and/or deep roots pump large quantities of water during the growing season, decreasing the flow of contaminated surface toward streams, lakes, groundwater and potable drinking water supply sources. Trees are believed to be more advantageous than annual crops as they have a larger root system and are more amenable to varying soil and climatic conditions. Additionally, their long-term cultivation does not rely on large inputs of fertilizers and/or pesticides and requires no tillage thereby reducing the negative impact on air quality (Mench, *et al*., 2010). Poplars (*Populus* sp.) and willows (*Salix* sp.) are the most common tree species used for phytoremediation as they have

extensive deep reaching roots, grow rapidly, take up large quantities of water alongside a wide variety of contaminants including inorganic and organic compounds, pesticides, ammonia and radio-nuclides (Aronsson, 1996; Hinckley, *et al*., 1994). Additionally, the increased tree cover resulting from phytoremediation can reduce greenhouse gas emissions, urban heat effects and airborne particulates. Poplars and willows have been used extensively in Europe and North America, operationally and demonstratively, as vegetative filters for cleaning polluted drainage water, vegetative landfill caps, wastewater treatment and reuse as well as soil remediation and biomass production for energy use (Aronsson & Perttu, 2001; Elowson, 1999).

A key consideration in selecting the appropriate plant for phytoremediation is whether or not it is native to the geographical area where the clean-up is potentially going to be used. Naturally occurring vegetation found on contaminated sites are often the best candidates (Conesa, *et al*., 2006). This is particularly significant for sites near natural areas that need to be protected as there is the potential for a non-native plant species to break away from a phytoremediation site and take root nearby, threatening the livelihood of native bacterial, animal and plant species of interest.

Monitoring studies have identified several plant species with the ability to remove lindane from the soil. *Solanum torvum* (Turkey berry), *Withania somnifera* (Indian ginseng), *Cynara scolymus L*. (artichoke), *Erica sp.* (Heather), *Cystisus striatus* (Portuguese broom), *Holcus lanatus* (Yorkshire fog), *Capsicum annuum* (chilli) and *Coriandrus sativum* (coriander) can accumulate considerable amounts of lindane isomers in their root, shoot and stem matrix (Abhilash & Singh, 2009a; Pereira, *et al*., 2008; Kidd, *et al*., 2008; Barriada-Pereira, *et al*., 2004).

2.13.d. Economics of Phytoremediation

Phytoremediation is an attractive remediation alternative, commercially and environmentally, due to its low cost, ecological sensitivity and low energy consumption when compared with other treatment methods. Aesthetically, the

plants used in phytoremediation projects also serve to enhance public acceptance and improve the visual landscape architecture in areas that are usually very industrial and barren (Susarla, *et al*., 2002)

In some cases, a plant can chemically change a pollutant into something less toxic leaving no further concerns. In others however, the plants used for phytoremediation absorb and accumulate high levels of contaminants, or metabolites, so that they become toxic to themselves, other organisms and the environment and eventually must be harvested and properly disposed of to prevent revolatilization of pollutants or their entry into the food chain. Still, this approach generates much less waste for disposal than traditional excavation. If further remediation is necessary, the process can be reapplied.

A major obstacle in carrying out of phytoextraction is the disposal of vast quantities of hazardous biomass and / or contaminated plant material. Composting has been shown to generate soluble organic compounds that can enhance metal solubility and significantly reduce the volume of harvested biomass (Hetland, *et al*., 2001). Alternatively, weight loss of contaminated plant biomass by compaction is also a viable option as it will decrease the cost of transportation to hazardous waste disposal facilities. Nonetheless, both methods will still require that any leachate be collected, treated and disposed of appropriately (Blaylock & Huang, 2000).

Another promising method to make use of plant biomass is through a thermochemical conversion process. Combining phytoextraction with biomass combustion and generation could be used commercially as an energy source but only under controlled and confined conditions. The gases and particulates released in the environment are potential hazards and any loss of energy or heat produced in the process should be monitored and prevented. Gasification is a complex process where biomass material undergoes a series of simultaneous chemical changes to yield clean and combustive gas at high thermal efficiencies (Raskin, *et al*., 1997). Pyrolysis, whereby material is decomposed under anaerobic conditions, might also be used for contaminated plant material. As the high cost of installation and operation for the above processes can be a limiting factor for treatment (especially

when employed solely for plant disposal), it is likely to be more advantageous to co-process the plant biomass in existing firing and treatment facilities (Iyer, *et al*., 2002).

Estimating the performance and cost of phytoremediation is a difficult task. Existing data suggest the initial outlay will likely be high due to implementation, monitoring and other regulatory requirements with a rapid decline in cost over time as efficiency and experience are gained. Phytoremediation costs will vary depending on treatment strategy but is often predicted to be less expensive than comparable technologies (Chappell, 1997). Table 2.13 gives estimates of phytoremediation costs in relation to conventional technologies.

Table 2.13 Estimated costs of comparative remediation technologies

Contaminant	Phytoremediation (USD)	Conventional Technologies (USD)
Metals	$80/cubic yard	$250/cubic yard
Petroleum Hydrocarbons	$70000	$85000
Lead (10 acres)	$500000	$12000000
Radionucleotides (water)	$2-$6 / 1000 gallons	None listed
Various (1 hectare/15 cm depth)	$2500-$15000	None listed

Reproduced from Vanek, *et al*. (2010)

Expectations for phytoremediation should also be realistically adjusted as it is considered a lengthy process when compared to traditional engineering approaches. Projects and applications are dependent on rates of plant growth, biological activity, contaminant properties and bioavailability, climatic conditions and are also likely to require multiple growing seasons.

2.13.e. Laboratory in vitro Model Systems

The interactions between plant cells and xenobiotics in the environment may be investigated using several experimental systems (Doran, 2009):

- cell extracts;
- undifferentiated plant cell cultures such callus and cell suspensions;

- differentiated organ cultures such as roots and shoots;
- explants such as leaf disks and excised roots;
- whole plants in hydroponic culture;
- whole plants in potted soil under greenhouse cultivation; and
- whole plants in the field

While each system shares several common features, they also possess important unique properties (Figure 2.11). Plant tissue culture is performed in micoogranism-free media and so entails the growth of plant cells and tissues, *in vitro.* It is a very useful and convenient technique for phytoremediation studies. Established *in vitro* cultures can reproduce indefinitely and only when needed, whereas whole plants have a limited lifespan with each individual plant being replaced after every experiment. Consequently, the length of time necessary to investigate and observe an experimental hypothesis can be greatly decreased using tissue cultures as opposed to intact plants (Doran, 2009).

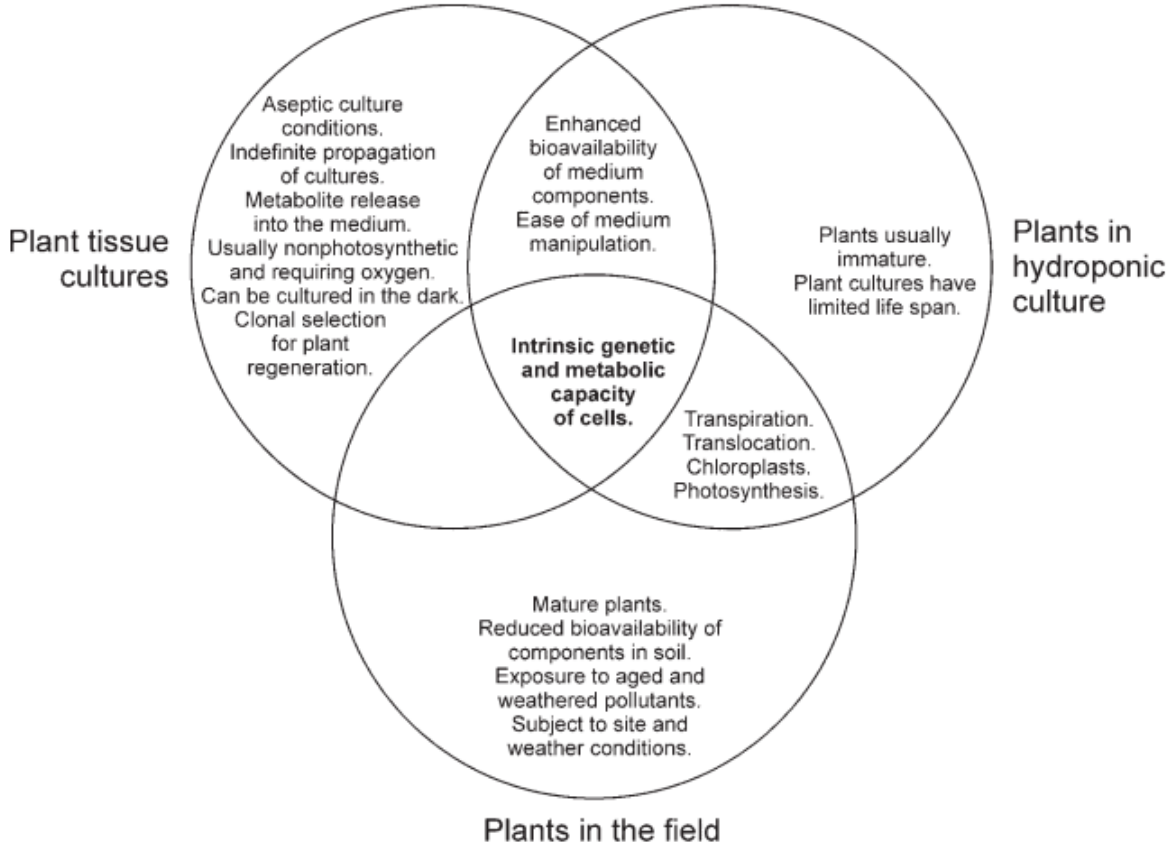

Figure 2.11 Properties of plant tissue cultures, plants in hydroponic culture and plants in the field.

Reproduced from Doran (2009)

Plant tissue cultures also offer the advantage of being able to determine and distinguish the metabolic capabilities of plant enzymes independent of rhizospheric bacteria (Chaudhry, *et al*., 2005). Studies can be carried out under easily manipulated and controlled conditions with respect to medium, nutrients and additives to facilitate substance availability, transport and uptake. Isolation of reaction products may require fewer purification steps and permit the recovery of metabolites and intermediates of higher purity in suitable quantities for further analysis. The relative homogeneity and standardized conditions of plant tissue cultures helps to reduce variability and their ability to propagate without light removes photochemical reactions which may influence substrate metabolism, toxicity and the activity of mineralization in plants, which is particularly important when assessing the capability of plant cells to degrade organic pollutants to water and CO_2 (Van Aken, *et al*., 2004).

Hairy roots, when compared to dedifferentiated tissues, or even shooty teratomas, are of particular importance in phytoremediation research. As they are more closely related in function and structure to the organs of whole plants, they offer a superior level of legitimacy and similarity with respect to their biological behaviour and properties when in direct contact with pollutants. Their simple culture requirements and straight-forward initiation from already transformed plant-material, or direct transformation via the R_i plasmid make hairy roots useful for screening genetic transformants prior to whole plants phytoremediation studies (Aird, *et al*., 1998).

However, as the conditions required by plant tissue cultures are highly aseptic due to bacterial or fungal contamination which can negatively affect cell viability, it is not commercially feasible for time scales over 28 days or large-scale phytoremediation applications (Oksman-Caldentey & Barz, 2002). The necessity for sterile conditions makes phytoremediation of soil or waste containing unidentified microorganisms, as well as growth in bioreactors, realistically impractical and uneconomical. The cost of *in vitro* cell culture production is exponentially higher than whole plant agricultural production and when plant tissue cultures from bioreactors are applied to polluted site, the disruption, excavation and transport of the contaminated soil negates the advantages of using whole plants for *in situ* phytoremediation. Factors such as

culture shaking to encourage oxygen transfer, medium composition, age, plant morphology, timing of pollutant addition and growth inhibitor / inducer levels can all have an effect on the transformation of xenobiotics in plant tissue cultures. However, similar concerns can also arise when using whole plants (Doran, 2009).

In theory, the chemicals metabolized *in vitro* will be subjected to the same enzymatic conditions in the parent plant. Thus, the principal purpose of plant tissue cultures in phytoremediation studies is to understand the enzymatic capacity and reactions that operate in whole plants, independent of microflora influences and compartmentalization of metabolites in other plant structures, and to minimize the initial expense of greenhouse or field trials

2.14. Enhancing Phytoremediation

The future of phytoremediation is in the research and development phase with many technical barriers which still need to be addressed to enhance the clean-up process and make it a commercially viable option.

2.14.a. Supplementation

Phytoremediation works best when supplemented by non-biological remediation technologies. A simple way to improve the process is to fine-tune the growth conditions by supplying the plant and soil with agronomic amendments such fertilizer, water, nutrients and other agents, or by modifying the planting pattern. This can be a very effective tool for enhancing and optimizing plant development and remediation capability.

To provide the most efficient and cost-effective remediation, the applicability of other clean-up techniques in conjunction with phytoremediation must also be assessed. On its own, phytoremediation may prove inadequate due to inherent limitations such as plant survival and proximity to the toxic contaminant. Excavation of polluted soil, combining it with uncontaminated earth and spreading the mixture out over a larger area can then be followed by the use of plant-based remediation over the sites to

provide a successful clean-up (Margaretich, 2003). Endophyte-assisted phytoremediation is an additional and hopeful new field to improve remediation by utilizing microorganisms that exist within plants to provide protection by degrading pollutants, increasing stress tolerance and improving plant growth and development (Weyens, *et al*., 2009). One method of ensuring the overall efficiency and success of microbe-assisted phytoremediation is to inoculate plants with strains of plant growth-promoting bacteria (PGPB) to improve their growth and stress tolerance through the production of phytohormones (such as indoleacetic acid (IAA) and cytokinins) or other plant rhizodeposits (Becerra-Castro, *et al*., 2011).

2.14.b. Pollutant Bioavailability

To optimize phytoremediation, it is imperative that the processes affecting bioavailability are well appreciated and understood. The oxidation state of a molecule can affect its solubility, bioavailability, uptake by plants and toxicity. Soils favour elements in their oxidized forms whereas aquatic environments favour reduced forms. In soils, the bioavailability of cations is generally inversely correlated so that lower pH levels tend to favour cation bioavailability. Other physical conditions such as temperature and moisture can also have an effect. Higher temperatures tend to hasten physical, chemical and biological processes and precipitation will increase plant growth and water-soluble pollutant migration. Ageing of pollutants decreases the bioavailable faction making them more recalcitrant and difficult to phytoremediate (Olson, *et al*., 2003).

2.14.c. Transgenic Phytoremediation

A promising approach to improve the ability of plants to tolerate, accumulate and degrade xenobiotic pollutants is by identifying unique genes from other microbes, fungi, plants and mammals which are capable of those desired actions. Isolated genes can then be transformed into chosen plants using *Agrobacterium*-mediated or direct DNA methods, such as particle gun bombardment, or gene transfer (Eapen, *et al*., 2007). Another option is to modify and regulate the appropriate intrinsic or transgenic enzymes or transporters involved in metabolic rate limiting steps. In 1986,

the first transgenic plants for phytoremediation of heavy metal-contaminated soil were developed using *Nicotiana tabaccum* expressing a yeast metallothionein gene to pass on higher tolerance to cadmium (Hooda, 2007). Tobacco was also the first plant modified to target organic pollutants like explosives and halogens in 1999 (Hooda, 2007).

Historically, most transgenic phytoremediation research has been performed on laboratory model plants and plant tissues such as *Nicotiana tabaccum, A. thaliana, Solanum lycopersicum* and *Solanum tuberosum,* as they have limited space requirements and require only light, air, water along with a few minerals to complete their short life cycle. More importantly, they possess a manageable, sequenced genome that can easily be manipulated through genetic engineering offering the ability to test hypotheses quickly and efficiently enabling rapid research progression (Members of the Multinational Arabidopsis Steering Committee, 2002). However, their relatively small biomass and shorter life span restricts their use for on-site remediation. For this reason, the knowledge gained from laboratory experiments is often then applied to initiate improvements in other plants with increased economic and cultural importance.

Particular interest has focused on the genetic manipulation of trees such as willow and poplar due to their extensive root systems, robust growth, larger biomass and tolerance to both organic and inorganic pollutants (Burken & Schnoor, 1998). Although several reports are available documenting the successful development of transgenic trees, *Agrobacterium*-mediated transformation of forest trees remains a challenging obstacle (Han, *et al*., 2000). Work to date has largely looked at the transformation of constitutively expressed single genes or traits. Table 2.14 gives an example of some of the most likely candidate genes for transgenic phytoremediation. To facilitate advancement in the field of transgenic phytoremediation, a systems-wide approach using multiple genes involved in the complete pathways for metabolism and targeted co-ordinate expression in root, uptake, translocation, and sequestration or under certain conditions needs to be developed (Dowling & Doty, 2009; Dhankher, *et al*., 2002). With government and environmental regulations restricting the use of transgenic plants, future advancements of phytoremediation are

likely to employ genetic use restriction technologies (GURTs) causing second generation seeds to be sterile and prevent the spreading of transgenes and genetically modified organisms (GMO) in the environment (Hills, *et al*., 2007). Genetic engineering of the chloroplast genome to obtain high expression without the risk of GMO dispersal via pollen has also been suggested as a possible solution to aggressive GMOs and interbreeding (Ruiz, *et al*., 2003).

Table 2.14 Candidate genes for transgenic phytoremediation

Gene	Source	Action	Pollutant
Cytochrome P450s	Mammalian	Degradation/ tolerance	Wide range of organics
Pentaerythritol tetranitrate reductaste	Bacteria	Degradation	Explosives, nitroesters and nitoaromatics
Mn-peroxidase	*C. versicolor*	Degradation	PCP
Nitroreductase	*E. coli*	Degradation	TNT
Biphenyl chlorophenyl dioxygenase (BphA)	*B. xenovorans*	Degradation	PCBs
Atrazine chlorohydrolase (atzA)	Bacteria	Degradation	Atrazine
Glutathione synthesases	Endogenous / other plant sources	Tolerance	Wide range of organics
XplA	Bacteria	Degradation	RDX
NaDPH dependent nitroreductase	Bacteria	Degradation	Explosives
HCH dehydrochlorinase (linA)	*S. japonicum UT26*	Degradation	Pesticide HCH
Laccase	Fungus	Degradation	Phenols
Gshl	*E. coli*	Tolerance	Cd
Cup1	*A. thaliana*	Tolerance	Zn, Cu
TaPCSl	Wheat	Tolerance	Cd
merA	Gram –ve bacteria	Tolerance / Volatilization	Hg
APs	*A. thaliana*	Hyperaccumulation	Se
ACC	Bacteria	Tolerance	Cd, Co, Cu, Mg, Ni, PB , Zn
FRE1 / FR2	*S. cerevisiae*	Hyperaccumulation	FRE2
Citrate synthase	*P. aeruginosa*	Chelation	Increase Citrate levels

Compiled from Hooda (2007); Abhilash, *et al.*(2009)*;* Eapen *et al.*(2007) and Campos, *et al.* (2008)

To increase crop production and maximize land use, cultivation in barren areas of high xenobiotic contamination will be necessary. The use of transgenic crops is often curtailed for ethical reasons and it is generally preferable to use transgenic plants that are poisonous or unprofitable (weeds) and are not eaten by insects, animals or humans to prevent any metabolic product reaching the food chain. However, the ethical and environmental risks associated with genetically modified crops will have to be considered and minimized to meet the mounting requirements of the growing global population. The insertion of Cytochrome P450 genes with the ability to catalyze the oxidation and subsequent metabolism of organic substances, has already been shown to improve herbicide resistance, degradation and residual agrochemical clean-up ability while retaining normal morphological and physiological traits in food crops such as rice (Kawahigashi, *et al*., 2008). Transgenic plants utilizing model plant systems that have been engineered to rapidly transform and detoxify POPs could be used in phytoremediation applications to assess feasibility and risk parameters prior to field testing and commercialization if issues such as cost and public acceptability are overcome.

2.14.c.i. *Arabidopsis thaliana as a Model Organism*

A. thaliana is generally preferred in laboratory-based studies for transgenic plant development. It has limited space requirements and requires only light, air, water and a few minerals to complete its fast life cycle which produces numerous self-progeny. Typically, the average growth span of *A. thaliana* from germination to senescence (changes to its biology as it ages after maturing) is approximately 7 to 8 weeks (Table 2.15). More importantly, it possesses a small, sequenced genome that can be easily and rapidly manipulated through genetic engineering compared to most other plants. An extensive toolkit for its manipulation has been developed including efficient mutagenesis; simple transformation technology; DNA, RNA, protein, and metabolite isolation; along with numerous detection methods (National Science Foundation, 2013). Thus, *A. thaliana* offers the ability to test hypotheses quickly and efficiently with the gained knowledge being used to initiate improvements in other plants with increased economic and cultural importance.

Table 2.15 Timeline of *Arabidopsis thaliana* growth stages

Stage Number	Days after sowing	Description
0.0	n/a	Seed germination
0.1	3.0 (on plates)	Seed imbibitions
0.5	4.3 (on plates)	Radicle emerges from seed coat
0.7	5.5 (on plates)	Hypocotyl and cotyledon emerge from seed coat
1		Rosette growth
1.0	6.0 (on plates)	Cotyledons fully open
1.02	10.3 (on plates) 12.5	2 rosette leaves are greater than 1 mm in length
1.03	14.4 (on plates) 15.9	3 rosette leaves are greater than 1 mm in length
1.04	16.5	4 rosette leaves are greater than 1mm in length
1.05	17.7	5 rosette leaves are greater than 1mm
1.06	18.4	6 rosette leaves are greater than 1mm
1.07	19.4	7 rosette leaves are greater than 1mm
1.08	20.0	8 rosette leaves are greater than 1mm
1.09	21.1	9 rosette leaves are greater than 1mm
1.10	21.6	10 rosette leaves are greater than 1mm
1.11	22.2	11 rosette leaves are greater than 1mm
1.12	23.3	12 rosette leaves are greater than 1mm
1.13	24.8	13 rosette leaves are greater than 1mm
1.14	25.5	14 rosette leaves are greater than 1mm
3		Rosette Growth
3.20	18.9	Rosette is 20% of final size
3.50	24.0	Rosette is 50% final size
3.70	27.4	Rosette is 70% final size
3.90	29.3	Rosette growth is complete
5		Inflorescence emergence
5.10	26.0	First flower buds are visible in the rosette, plant has not yet bolted
6		Flower production
6.00	31.8	First flower is open, petals are at 90 degree angle to the pistil
6.10	35.9	10% flowers to be produced are open
6.30	40.1	30% flowers to be produced are open
6.50	43.5	50% flowers to be produced are open
6.90	49.4	Flowering complete, flowers are no longer produced.
8		Silique or fruit ripening. Seed pods become brown and then shatter.
8.00	48.0	First silique or seed pod shatters.
9		Whole plant senescence begins. Plant starts to lose pigment becoming brownish.
9.70		Senescence complete

Reproduced from TAIR (2006)

2.14.c.ii. *Agrobacterium*-mediated Transformation

A number of techniques are available to transfer DNA into plant cells including particle bombardment, electroporation and viral transformation (or transduction). However, *Agrobacterium*-mediated transformation is the easiest and most utilized method (Gelvin, 2003). *Agrobacteria* are soil-borne, bacterial plant pathogens and genetic transformation results from the transfer of a large tumour-inducing (Ti) plasmid and its integration into the plant nuclear genome. Ti plasmids are 200 to 800 Kb in size, with the actual transferred DNA (T-DNA) regions being approximately 10 to 30 Kb in size (Barker, *et al*., 1983).

Processing and export of T-DNA from the Ti plasmid to the plant cell is governed by virulence (*vir*) genes on the Ti plasmid, which are stimulated by wounded plant cells that secrete the low-molecular weight molecules, acetosyringone and hydroxy-acetosyringone (Garfinkel & Nester, 1980). T-DNA contains the oncogenic genes, encoding the enzymes that cause tumour formation and the genes involved in opine synthesis. Once synthesized, opines are excreted by tumour cells and provide a source of carbon and nitrogen for *Agrobacterium* (Hooykaas, *et al*., 1984). Regulation of the *vir* genes involves a series of integrated transcriptional actions. The series begins with the constitutively expressed *virA* gene that encodes a transmembrane protein to detect the presence of acetosyringone. A regulatory protein, VirG, then sends a signal to *virB, C, D and E* when plant inducible factors. The DNA binding protein, VirE2*,* sheaths the T-strand during transfer to the plant cell; cleavages at the 25 bp direct repeats borders of the T-DNA are conducted by VirC and VirD2 endonucleases; and VirB is involved in directing T-DNA transfer at the bacterial cell surface (Gelvin, 2003).

T-DNA is enclosed by highly homologous 25 bp T-DNA border sequences, referred to as TL and TR, flanking the region in a directly repeated orientation. These sequences allow the T-DNA to be processed from the Ti plasmid by the activity of the VirD2 border-specific endonuclease. Deletion of the TL border does not affect transformation. However, manipulation and deletions of the TR border prohibits the transfer of genes required for oncogenic activity and as a result, the transformation phenotype is not detected (de la Riva, *et al*., 1998).

Cleavage of the 25-bp T-DNA border requires VirD1/VirD2 proteins, and occurs mainly by cutting the T-DNA 'lower strand', between nucleotides 3 and 4 of the border sequence. The 5' end of the T-strand relates to the TR border while the TL border relates to the 3' end. This single T-strand is transferred to the plant cell as a DNA/protein complex where it binds to one strand of the plant DNA and a torsional change occurs in the plant DNA resulting in another cut. A homologous strand is produced as each of the T-strands is ligated to the plant DNA (Stachel, *et al*., 1986) (Figure 2.12). Rearrangement and duplication of target DNA is a product from the repair and replication of the staggered nick in the plant DNA. Besides being AT-rich, the target DNA site does not appear to have any other specific characteristics (Gelvin, 2003).

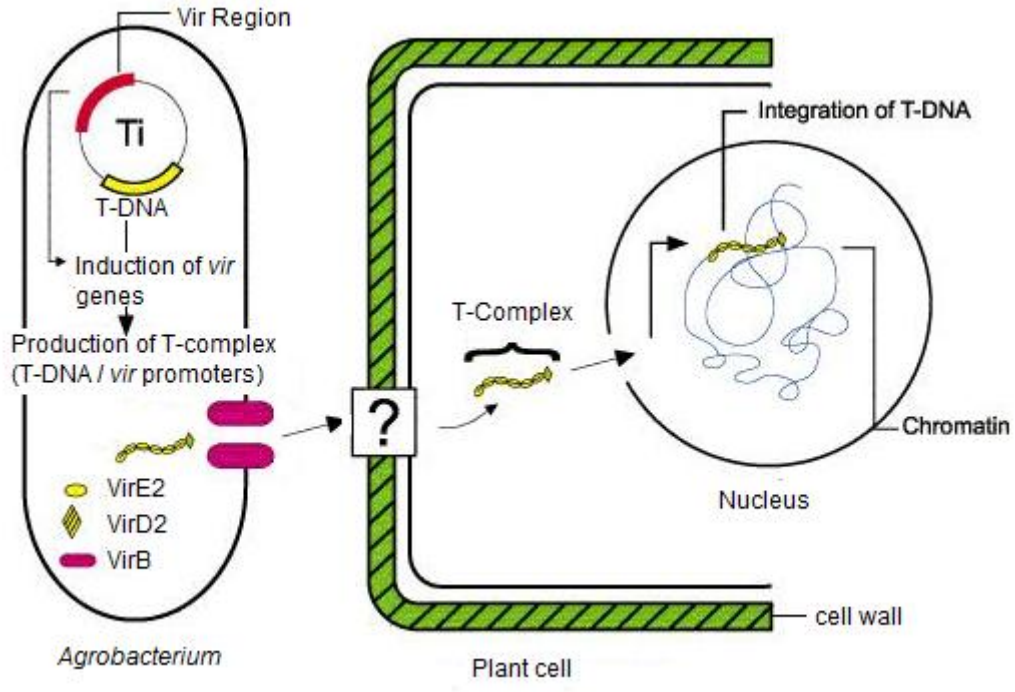

Figure 2.12 *Agrobacterium*-mediated transfer of T-DNA into the plant genome
Adapted from Zupan, *et al*. (2000)

Exploiting the fact that tumour formation in plants is the result of a transformation and T-DNA integration, T-DNA genes are only transcribed in plant cells and that any DNA between the T-DNA borders can be transferred to plant cells has resulted in the construction and use of non-virulent bacterial vector strains for plant transformation (Hooykaas, *et al.*, 1984).

2.14.c.iii. Gateway® Recombination Cloning Technology

Traditional restriction enzyme cloning technology involves a number of steps which can take considerable time and effort. The efficiency is highly variable and dependant on using restriction enzymes that do not cut within the gene of interest, how clean the DNA sample is and the size of the fragment. Gateway® Technology is a rapid, cost-effective and highly efficient universal cloning method that avoids typical cloning limitations by exploiting the site-specific recombination properties of bacteriophage lambda and its ability to integrate and cut itself into and out of the bacterial chromosome (Katzen, 2007). The reaction is 99% efficient, works without the need for restriction enzymes, ligase or subcloning and is able to successfully maintain the fragment orientation and overall reading frame.

The Gateway system proceeds under the direction of the BP and LR clonase reactions. The BP reaction is catalyzed by the phage integrase and the integration host factor. This reaction transfers a DNA or PCR fragment, flanked by two *attB* sites, into a donor vector (pDONR) carrying two *attP* sites. The DNA fragment is subsequently inserted into the donor by matching the *attB* and *attP* sites (Hartley, *et al*., 2000). This recombination results in an entry clone, pENTR, flanked by two *attL* sites (Figure 2.13). Entry clones, as a rule, are generally not used for direct transformations as the *attL* sites are too long (96 bp) to be placed as spacers between sequences, but are instead used as the key substrates in the LR reaction.

The LR reaction is catalyzed the enzyme integrase, the integration host factor, and the phage excisionase. The LR clonase mix shuffles the DNA fragment from pENTR into a destination vector (pDEST) carrying two *attR* sites. The DNA fragment is then inserted into another new expression clone (pEXPR) after recombination of the *attL* and *attR* sites and once again flanked by *attB* sites (Figure 2.14). The resulting expression clones are used to test gene function(s) (Karimi, *et al*., 2007).

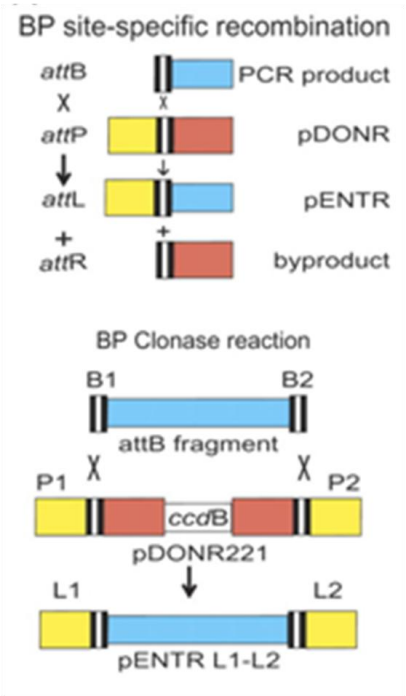

Figure 2.13 Schematic representation of the Gateway® Technology BP reaction. Adapted from Karimi, *et al*. (2007)

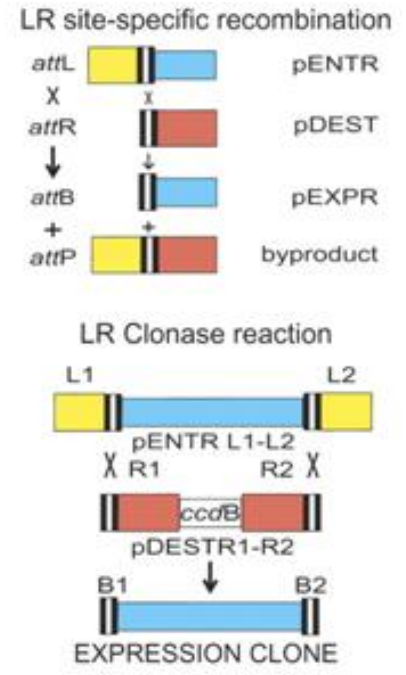

Figure 2.14 Schematic representation of the Gateway® Technology LR reaction. Adapted from Karimi, *et al.* (2007)

2.14.c.iv. Directional Cloning using Gateway® Technology

The pENTR™ Directional TOPO® Cloning Kit allows blunt-end PCR products to be directionally cloned into a Gateway® entry vector at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required. In the Invitrogen system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). An overhang in the cloning entry vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation (Figure 2.15).

The enzyme Topoisomerase I, from *Vaccinia* virus, binds DNA at CCCTT specific sites and cuts the phosphodiester backbone in one strand. Reversing the reaction and releasing topoisomerase is also possible as a covalent bond is formed between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I which can then react with the 5′ hydroxyl of the original cleaved strand (Invitrogen by Life Technologies, 2012).

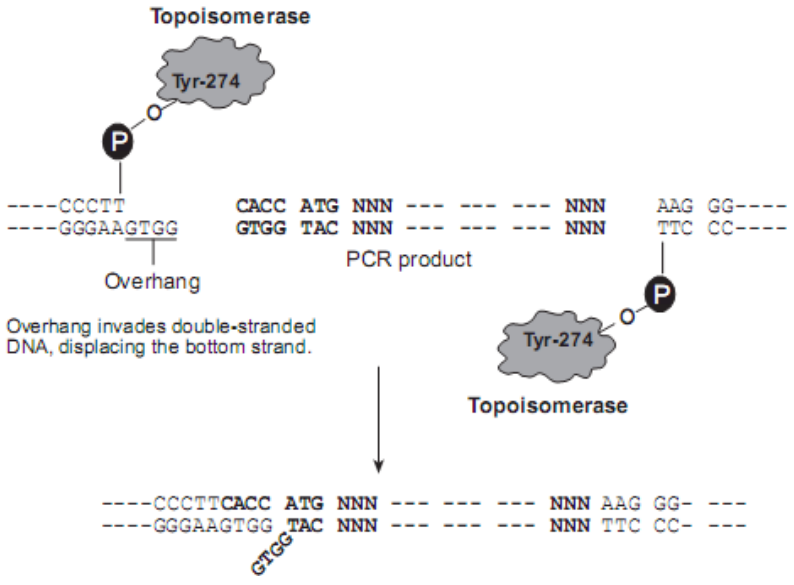

Figure 2.15 Directional cloning using Gateway® Technology.

Reproduced from Invitrogen pENTR™ Directional TOPO® Cloning Kit User Guide (2012)

2.15. Transgenic Phytoremediation of Lindane

Preliminary results from a single proof of concept study have shown that the model plant *A. thaliana* modified with *linA* from *S. japonicum* UT26 is able to grow in the presence of 34 µM (10 mg/kg) lindane, a normally toxic concentration, and remove it from the media, contrary to the wild type plant (which does not contain the *linA* gene) (Figure 2.16) (De Lorenzo Prieto & Gonzalez Pastor, 2007).

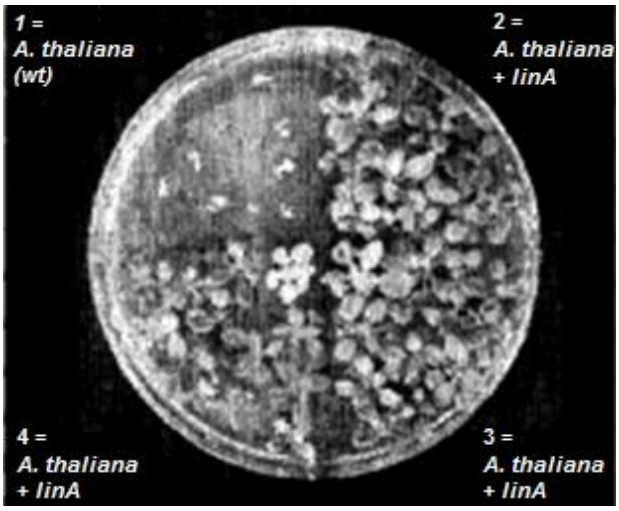

Figure 2.16 Growth of wild type *Arabidopsis thaliana* (1) and *Arabidopsis thaliana* modified with linA (2, 3 and 4) in the presence of 34 µM (10 mg/kg) Lindane

2.15.a. Constructing Transgenic A. thaliana

Transgenic plants for lindane degradation were created by De Lorenzo Prieto & Gonzalez Pastor (2007) as summarized below.

The *linA* gene (Figure 2.17) was amplified by Polymerase Chain Reaction (PCR) and the resulting PCR fragments were digested with the restriction enzymes, BamHI and EcoRI, and cloned into the vector pCAMBIA3500 (Figure 2.18). *Agrobacterium tumefaciens* was then transformed with the above vector and transformants were selected using kanamycin. Wild type plants of *A. thaliana* (Col-0 variety) were transformed by immersing their flowers in a medium containing stationary phase cells from *Agrobacterium tumefaciens* containing the pCAMBIA3500-linA construct.

```
  1 atgagtgatc tagacagact tgcaagccgg gccgcgattc aggacctcta ctctgacaag
 61 ctcattgccg tagacaagcg ccaagagggc cgtctcgctt ctatttggtg ggatgatgca
121 gagtggacca ttgagggaat cggcacctac aagggcccgg aaggcgccct cgatttggcc
181 aataacgtac tctggccaat gtttcacgaa tgtattcatt atggaaccaa tctgcgcttg
241 gaatttgtga gcgcggacaa ggtaaatggt attggcgacg tccttctcct tggaaatctc
301 gtcgaaggta atcagtcgat tcttatcgct gcggtcttca cggatgagta tgagcgccgt
361 gacggggtgt ggaagttctc taagcgcaac gcatgcacga actatttcac cccgctggcc
421 ggcattcatt tcgcaccgcc cggcattcat ttcgcaccgt ccggcgcata a
```

Figure 2.17 Sequence of the *linA* gene (Imai, *et al*., 1991).

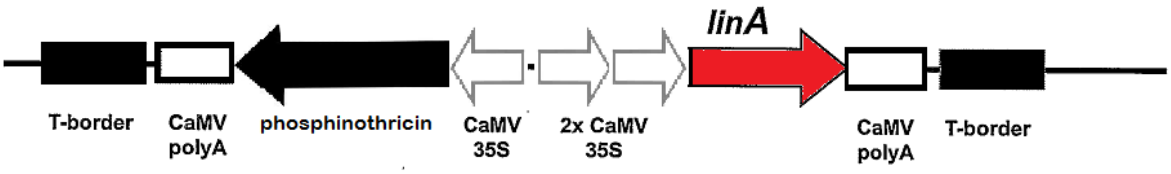

Figure 2.18 pCAMBIA3500-linA construct

Seeds were collected from mature plants and re-sown in soil. Transgenic plants were selected for using the herbicide phosphinothricin, with a success rate of approximately 1%. The surviving transgenic plants were grown until maturity, at which time seeds were collected. Seeds were subsequently planted on lindane-containing Murashige and Skoog (MS) agar media to select for lindane-resistant transformed lines. After several generations of selection, three transgenic lines were obtained.

RNA was extracted from transgenic plants to determine whether or not the *linA* gene was being transcribed correctly using reverse transcriptase-PCR (RT-PCR). Furthermore, the RNA between the three transgenic lines was quantified using realtime-PCR by incorporating a fluorophore into the double strand and comparing the Ct (threshold cycle) value of amplification between the *linA* and *actin* genes by measuring the fluorescence. Differences in the actual quantity of starting cDNA material from each transgenic line were normalized by comparing the ratios of *linA* to *Actin.* The resulting values were very similar and indicated comparable levels of transcription Table 2.16 .

Table 2.16 Relative transcription levels of the *linA* gene

Transgenic Line	Ct-linA/Ct-actin
linA-wt (5.1)	681
linA-CtHIS (2.1)	711
linA-CtHIS (10.1)	766

Information compiled from De Lorenzo Prieto & Gonzalez Pastor (2007)

2.15.b. Degradation Capability of Transgenic A. thaliana

Wild type and transgenic plants were grown on lindane-contaminated MS-agar media for six weeks after which time the amount of lindane remaining was determined (De Lorenzo Prieto & Gonzalez Pastor, 2007). The ability of the transgenic lines to degrade lindane was ascertained following EPA Method 3550 (USEPA, 2007a), which consists of a first phase polar solvent extraction, solvent concentration and subsequent clean-up using a nitrogen flow packed column. The extract obtained was then analyzed using gas chromatography with an electron capture detector (GC / ECD) based on EPA Method 8081B (USEPA, 2007b). Results indicated that the transgenic lines were able to significantly reduce the amount of lindane present in the plates by approximately 98% (Table 2.17). The authors claim that other preliminary studies have confirmed that lindane is not present in the aerial parts of the plant. However, no supporting data is given.

Table 2.17 Reduction of lindane after 6 weeks of growth

	Lindane present after 6 weeks (mg/kg)	Standard Deviation	Reduction (%)
Control (no cultivation)	3.187	0.360	n/d
Wild type	2.910	0.158	n/d
linA-wt (5.1)	0.038	0.022	98.8
linA-CtHis (2.1)	0.044	0.025	98.6
linA-CtHis (10.1)	0.041	0.028	98.7

n/d = No data given by authors

Information compiled from De Lorenzo Prieto & Gonzalez Pastor (2007)

2.15.c. Limitations of Transgenic A. thaliana Study

In the study, the authors concede that a more robust plant, with a larger root system and the ability to resist and/or accumulate higher concentrations of lindane would be better suited for actual on-site field applications. It is also noted that confinement of actual soils to be treated, preventing dispersion of transgenic plants, hybridization with other wild type plants and competition with other plants in the environment is a major concern and problem when developing transgenic phytotechnologies. However, in the areas where these technologies might be applied, the sites of remediative interest are generally already self-contained.

Although the previous study is the only one of its kind to demonstrate proof of concept and that phytoremediation with *linA* is feasible, it is not well characterized. Whereas a mechanistic study analyzes biological or chemical events associated with novel observations and ultimately provides information concerning the molecular, cellular or physiological processes responsible in the cells and organisms, the De Lorenzo Prieto and Gonzalez Pastor study (2007) is cause and effect and therefore lacking in several key principles. The effects of varying the concentration of lindane on the uptake and degradation reactions have not been investigated, nor has the amount of lindane, or its metabolites, accumulating in plant, been measured. In addition, although the literature states that the transgenic plants have the ability to significantly reduce the amount of lindane present in the media by 98%, starting concentration(s) have not been stated and translation of the protein has not yet been demonstrated. Investigating and varying these parameters, along with quantifying changes to the global proteome, will elucidate the role of the LinA enzyme allowing for further developments in the transgenic phytoremediation of lindane.

2.16. Proteomics

Proteomics is the large-scale study of proteins, the molecules that are the main components of an organism's cellular metabolic pathways. The proteome refers to the entire set of proteins and unlike the genome which is relatively static, the global proteome changes constantly in response to different intra- and extracellular

environmental stressors (James, 1997). A typical proteomic workflow may include the following 6 steps:

- Extraction and isolation of protein from cells, without altering the protein sample in its identity or quantitative composition.
- Protein fractionation or separation to reduce the complexity of the protein sample.
- Tryptic digestion of proteins into peptides for easier detection.
- Peptide separation using liquid chromatography.
- Peptide detection using mass spectrometry.
- Data interpretation to determine protein sequence.

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2.16.a. Two-dimensional Liquid Chromatography Separation

Two-dimensional liquid chromatography (2D-LC) is used to reduce the complexity of trypsin digested protein extracts, or peptides, before undergoing tandem mass spectrometry (MS/MS). LC can purify, quantify and identify the distinct compounds within a mixture by utilizing a number of different stationary phases, a pump that moves the analyte and mobile phase(s) through the column, and a detector that gives a unique retention time or ultraviolet-visible spectroscopy (UV-Vis) data (if equipped) for the analyte (Snyder, *et al*., 2009).

There are two variants of LC according to the polarity of the stationary phase and solvent. Normal phase LC (NP-LC) uses a polar stationary phase and a non-polar, non-aqueous mobile phase, whereas reverse phase LC (RP-LC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase which operates on the principle of Van der Waals dispersion forces. Polar molecules are highly soluble in the solvent and do not need to need to break hydrogen bonds as they move in between the molecules of the mobile phase. Therefore, retention time is shorter for polar molecules, while non-polar molecules elute more slowly (Buszewski & Noga, 2012). Although RP-LC is the most commonly used form for separation of peptide samples due to a lack of reproducibility of retention times in NP-LC and its

compatibility with MS, NP-LC methods are currently undergoing a resurgence (Faiers, 2007).

2.16.a.i. Hydrophilic Interaction Liquid Chromatography

Hydrophilic interaction liquid chromatography (HILIC) is a special case of normal phase chromatography that gives another method to separate small polar compounds on polar stationary phases. Like NP-LC, HILIC uses polar stationary phases, while the mobile phase is like those utilized in the RP-LC mode (Guo & Gaiki, 2005). Ion chromatography (IC) is another feature of HILIC that analyzes charged substances. Figure 2.19 shows how HILIC and other areas of chromatography are able to expand and meet the many requirements of separation technology.

HILIC rises above many of the limitations, including poor solubility, often found in NP-LC and is able to analyze compounds that always elute near the void in reverse-phase chromatography; it can be easily coupled to mass spectrometry (MS) in the electrospray ionization (ESI) mode. It is well established as an effective separation mode for uncharged highly hydrophilic and amphiphilic compounds that can not be retained in RP-LC and also do not have enough charge to allow effective retention in IC (Cubbon, *et al*., 2007).

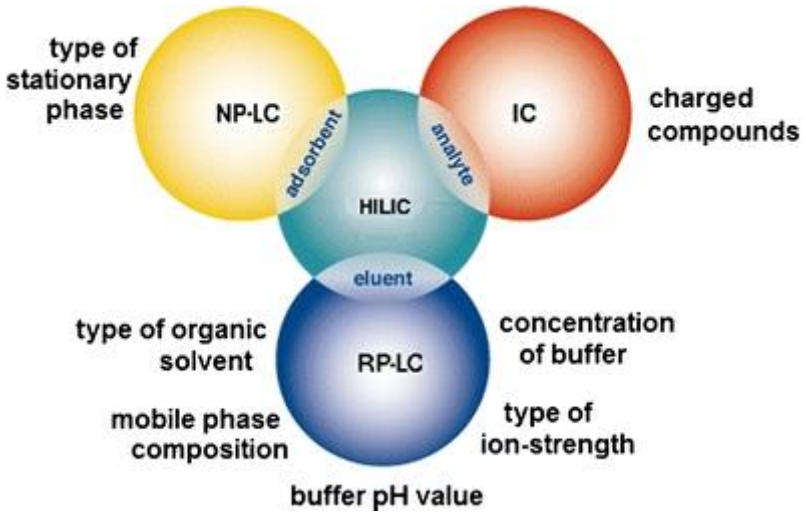


Figure 2.19 HILIC combines the characteristics of the three major methods in liquid chromatography
Reproduced from Buszewski & Noga (2012)

2.16.b. Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio (*m/z*) of charged particles (Sparkman, 2000). It can be used qualitatively to identify unknown compounds and quantitatively to determine the amount of a compound in a sample. A typical MS sequence is as follows (Figure 2.20):

- Sample vaporization
- Sample ionisation to form charged particles (ions)
- Ion acceleration into highly focused beam
- Ion deflection/separation in an analyzer using electromagnetic fields. The lighter and higher charged ions are more highly deflected.
- Ion detection by beam of ions passing through the machine
- Ion signal processed into mass spectra
- Output from a chart recorder represented as a 'stick diagram' and shows the relative current produced by ions of varying mass/charge ratio (*m/z*) (de Hoffman & Stroobant, 2002).

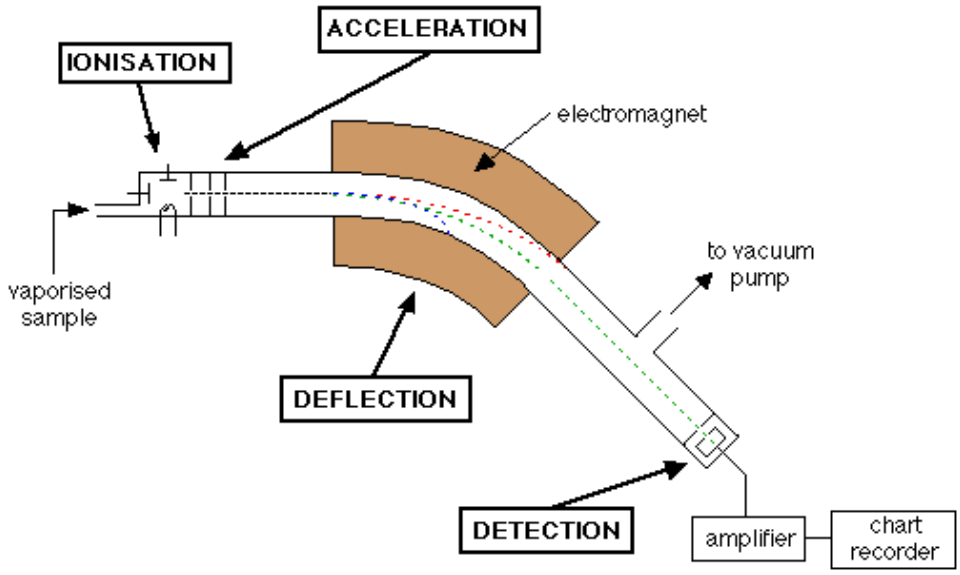


Figure 2.20 A full diagram of a mass spectrometer

Reproduced from Clark (2000)

2.16.b.i. Tandem Mass Spectrometry

Tandem MS or MS/MS employs two stages of mass analysis to selectively examine the fragmentation of particular ions in a mixture of ions. The goal of MS/MS is to obtain more structural information on a particular ion species when fragmentation is obscured by other compounds present in the mixture; introduced either by the ion source or by the matrix; or when the original ionization method yields few structurally diagnostic fragments (De Hoffmann, 1996). Instruments may be made up of two mass spectrometers assembled in tandem or may comprises analyzers capable of storing ions to exploit a sequence of events in time allowing the selection of one ion fragment by ejecting others (Figure 2.21).

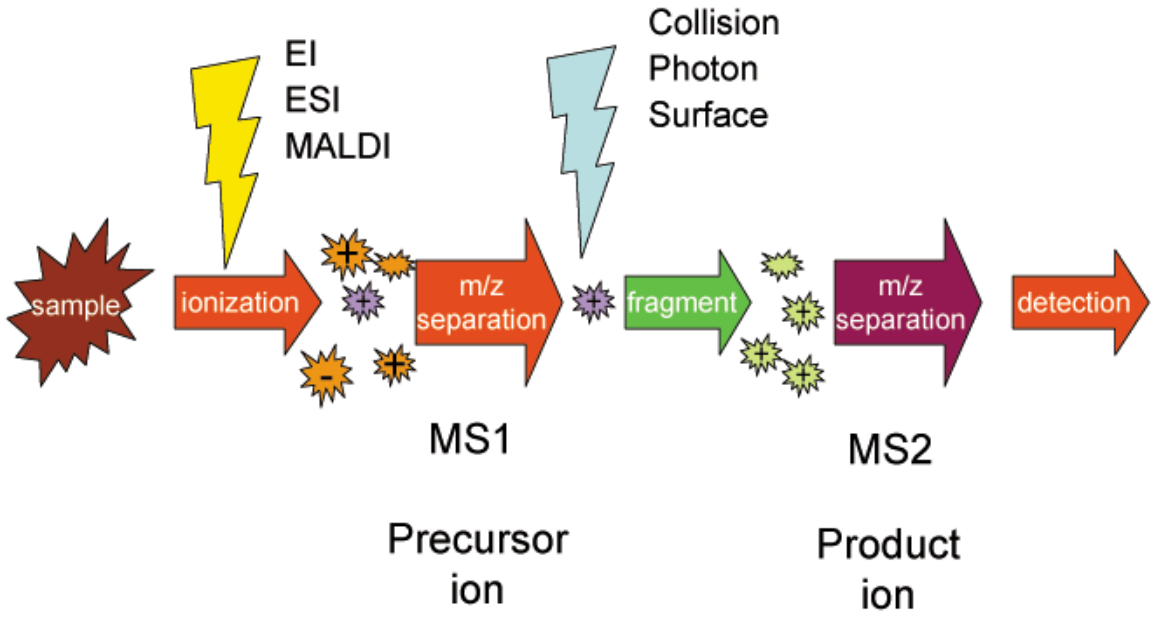


Figure 2.21 Tandem MS/MS schematic
Reproduced from Murray (2006)

Three main scan modes are available using MS/MS. In product scans, ions of a given m/z are selected by the first MS and induced to fragment for analysis by the second MS allowing ion fragments from a specific compound in a mixture to be recorded. Precursor scans select ions in the second MS with a specific *m/z* value after fragmentation in the first MS and neutral loss scans allow the selective recognition of all ions that have lost a neutral fragment after fragmentation using both MS together (Busch, *et al*., 1998). The *m/z* values of the resulting fragment ions are

matched to theoretical information in a database to generate peptide identifications and ultimately protein identifications.

2.16.c. Isobaric Tags for Relative and Absolute Quantitation

The development of *in vitro* peptide labelling techniques, such as isobaric tags for relative and absolute quantitation (iTRAQ), had made it possible to quantify relative changes to the global proteome of a (transgenic) organism under conditions of metabolic stress using two-dimensional liquid chromatography (2D-LC) separation coupled to tandem mass spectrometry (MS/MS) (Leitner & Lindner, 2006).

iTRAQ is a quantitative, non-gel based strategy that works in tandem with MS/MS and has the advantage of allowing up to 8 different conditions to be analyzed in one experiment (Leitner & Lindner, 2006). The protocol follows a collection of up to eight different reagents for quantitative protein analysis whereby isobaric mass labels are placed at the N-termini and lysine side chains in a peptide digest mixture. The entire system consists of a charged N-methylpiperazine-based reporter group, a peptide N-hydroxysuccinimide ester reactive group and a neutral mass balance carbonyl group (Sachon, *et al*., 2006). The total mass of reporter and balance components of the system are kept constant at 145.1 Da using differential isotopic enrichment with13C, 15N, and 18O atoms. The different groups appear in the range of *m/z* 113 to 119 and 121 in tandem mass spectra. A multiplex collection of similar but differentially labelled peptides will appear as one ion signal in MS. The groups will then emerge as distinct ions (m/z 113–119, 121) after collision-induced dissociation (CID) MS/MS analysis of the precursor ion, (Sachon, *et al*., 2006). The relative quantification of the peptides is deduced from the relative intensities of the reporter ions ultimately making it possible to quantify relative changes to the global proteome. Figure 2.22 shows a typical iTRAQ workflow.

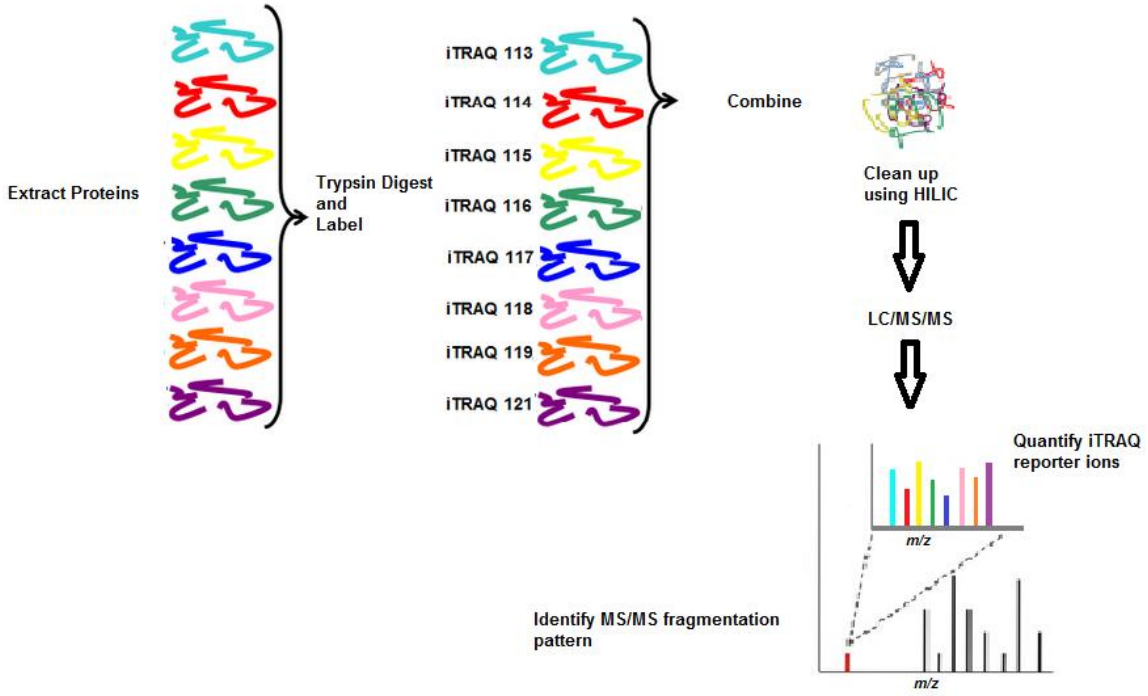


Figure 2.22 iTRAQ workflow

2.16.d. Proteomic Studies in A. thaliana

Proteomic studies are essential to understanding the dynamic and complex features underlying the molecular mechanisms responsible for growth, development, and environmental interactions in *A. thaliana*. Several methods have been successfully used in identifying proteins and their function.

Following the advent of the deoxyribonucleic acid (DNA) chip (or microarray), protein microarray technology emerged. The presence of specific sequences can be determined using probe-target hybridization to characterize protein abundance and function. In one proof of concept study using *A. thaliana* protein microarrays, 173 known and novel potential calmodulin (CaM) and calmodulin-like proteins (CML) targets were identified. This information provided a resource for the scientific community in the form of new testable hypotheses in the area of CaM/Ca^{2+}-regulated processes (Popescu, *et al.*, 2007). The challenges of protein microarrays include generating large or amplified amounts of protein, the wide variety of physic-chemical properties, differences in size, structural stability, functional integrity,

immobilization without affecting function and variability due to post-translational modifications (Chandra, *et al.*, 2011).

Advances in plant proteomics were made using two-dimensional gel electrophoresis (2DE) and MS. 2DE begins with separation using one-dimensional electrophoresis (1DE) followed by a second separation in a direction 90 degrees from the first. The proteins may be separated according to their protein mass, the protein-complex mass in the native state, and the isoelectric point (O'Farrell, 1975). Although 2DE is more effective at separating proteins than 1DE, the resolution of protein spots on a 2D gel is limited by factors such as abundance, size, and other electrophoretic properties (Jung, *et al.*, 2000). In order to improve the sensitivity, the global proteome has to be fractionated into sub-proteomes. Park (2004) summarizes a number of studies investigating the proteomes of *A. thaliana* and its subcellular compartments and organelles.

When compared to traditional 2DE, iTRAQ is less labour intensive, covers a larger percentage of the proteome and is able to identify low abundance proteins. This is a distinct advantage when analysing plant proteomes which are typically over-abundant in Ribulose-1,5-bisphosphate carboxylase oxygenase, (RuBisCO), a ubiquitously found enzyme involved in carbon fixation. iTRAQ has successfully been used to quantify the relative changes in protein abundance in several *A. thaliana* studies in response to toxicity (heavy metals, e.g. zinc) and nutrient deficiency (iron) (Fukao, *et al*., 2011; Lan, *et al*., 2011). To date, there have been no proteomic studies examining the effect of lindane on *A. thaliana.*

2.17. Hypothesis Development

Considering the enzymes known to be involved in bacterial lindane degradation and the plant xenobiotic degradation pathway, the single gene transfection of *linA* into *A. thaliana* should result in the intermediates: γ-PCCT 1,4-TCDN and 1,2,4-TCB when grown on lindane-contaminated medium (Figure 2.4). The direct absence of the LinB enzyme, which is not homologous to any known plant proteins, should hinder subsequent degradation into any other metabolites found in the *S. japonicum* UT26 degradation pathway.

However, Cytochrome P450, well characterized for its part in the oxidative degradation of environmental xenobiotic toxins, has been implicated in 1,2,4-TCB degradation, as seen in a study involving white-rot fungus *Trametes versicolor* (Marco-Urrea, *et al*., 2009). In cell cultures containing the Cytochrome P450 inhibitors, piperonyl butoxide (PB) and 1-aminobenzotriazole (ABT), a decrease in the degradation of 1,2,4-TCB was observed. To confirm that the reduction of 1,2,4-TCB metabolism was not a by-product of non-specific growth inhibition, differences in the fungal dry weight of each treatment were measured (Table 2.18).

Table 2.18 Effect of different 1,2,4-trichlorobenzene (TCB) concentrations and Cytochrome P450 inhibitors (1-aminobenzotriazole (ABT) and piperonyl butoxide (PB)) on 1,2,4-TCB degradation by white-rot fungus *Trametes versicolor*.

	Initial 1,2,4-TCB (µM)	**1,2,4-TCB Degraded (µM)**	**Degradation (%)**	**Fungal Dry Weight (mg)**
Control Medium	0.905±0.108	0.674±0.095	74	34.8 ± 1.2
	1.694±0.042	1.035±0.108	61	n/d
	2.261±0.369	1.306±0.028	58	n/d
	3.194±0.089	1.565±0.078	49	n/d
Medium + ABT	0.905±0.108	0.155±0.015	17	35.5 ± 1.7
Medium + PB	0.905±0.108	0.114±0.012	13	33.5 ± 0.2

n/d = No data given by authors

Information compiled from Marco-Urrea, *et al*. (2009)

PB and ABT did not affect cell yields of *T. versicolor*, suggesting that Cytochrome P450 is responsible for the initial oxidation and degradation of 1,2,4-TCB. In *Bjerkandera adjusta* DSM3375, it has been shown that Cytochrome P450 inhibitors had a negative effect on 2,4,6-trinitrotoluene metabolism but did not affect the mineralization of [14C] glucose (Eilers, *et al*., 1999). This result explains and supports the observation that fungal cell growth, but not 1,2,4-TCB degradation, still proceeded in the presence of the inhibitors.

This protein and Cytochrome P450s from the *A. thaliana* xenobiotic plant degradation pathway, share a conserved core region, the cypX superfamily, which is involved in secondary metabolite biosynthesis, transport, and catabolism. Within the family, sequence conservation is relatively low and only 3 motifs or residues are

found to be absolutely conserved. However, their overall physical characteristics remain highly conserved. A haem-binding loop constitutes the conserved core, which is composed of a four-helix bundle, a meander, helices J and K, and two sets of beta-sheets containing a conserved cysteine (Marchler-Bauer, *et al*., 2013).

It is therefore hypothesized that *A. thaliana* modified with only *linA* will result in the stable intermediate, 1,2,4-TCB, to be subsequently degraded in the plant xenobiotic degradation pathway via Cytochrome P450 and other catabolic enzymes (Figure 2.23).

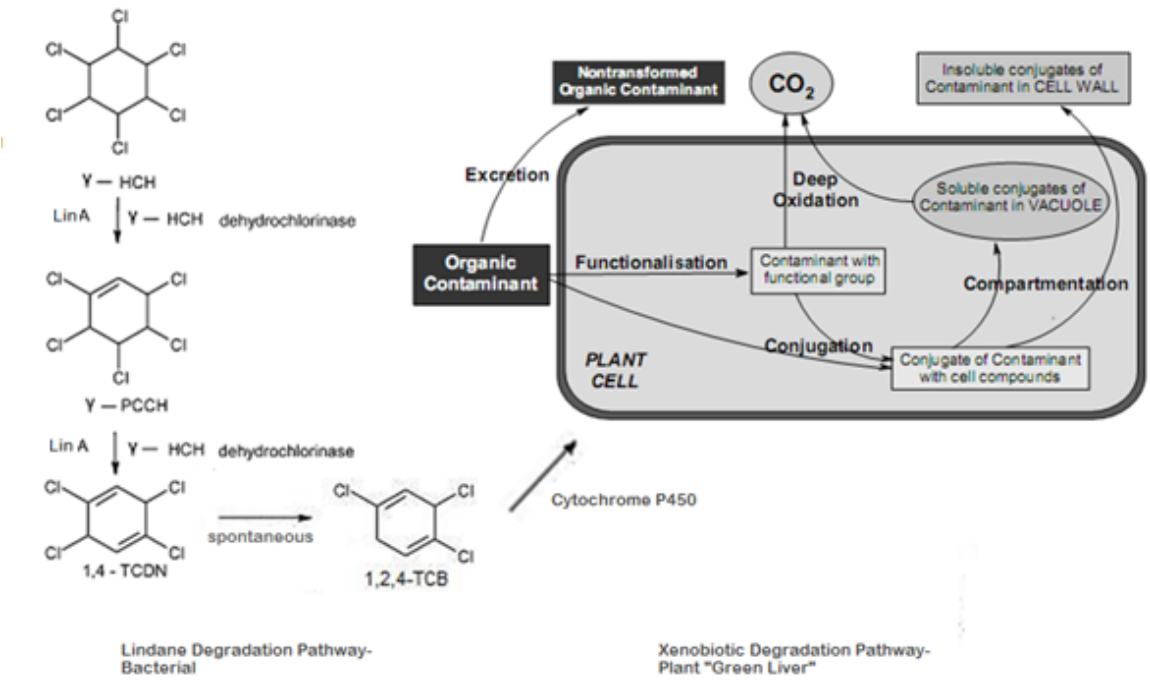


Figure 2.23 The hypothesized metabolism of lindane in *Arabidopsis thaliana* transformed with *linA* from *Sphingobium japonicum* UT26.

Modified from Bhatt, *et al*. (2009) and Sandermann (1994)

This hypothesis will be tested by following and advancing aspects of the study by De Lorenzo Prieto and Gonzalez Pastor (2007), starting with transforming *A. thaliana* with the *linA* gene from *S. japonicum* UT26. Growth will be observed under conditions of lindane; 1,2,4-TCB; and 1,2,4-TCB / P450 Inhibitor stress. The presence of lindane and 1,2,4-TCB will be measured from the growth media and

within plant tissues after pre-determined growth periods. Finally, changes to the global proteome will be quantified and compared using iTRAQ and MS analytic techniques. Methodology, results and discussion will be detailed in the following chapters.

2.18. Conclusion

The development of an effective lindane-remediation strategy, to safely and quickly remove the highly toxic, ubiquitous and persistent organic pollutant from the environment is paramount. Phytoremediation is an aesthetically, environmentally and economically-friendly biotechnology using plants that are able to sequester and transform organic waste to a non-toxic state, or to sub-threshold levels below concentration limits. Efficiency of the natural plant processes can be greatly enhanced by genetic manipulation, exploiting the enzymes of lindane-degrading bacteria. This alternative green technology is *in situ*, solar driven, low-cost, and relatively low-disturbance making it a prime choice for remediation of lindane in both developed and developing countries.

The single gene transfection of *linA,* from the bacterium *S. japonicum* UT26, into the model plant *A. thaliana* should result in a plant expressing the LinA protein for the purpose of lindane dehydrochlorination. Since LinA does not require any cofactors and the removal of every individual chlorine atom from the cyclohexane ring decreases the harmful effects several-fold, it is worth considering its potential in the development of a transgenic phytoremediation technology for lindane.

Chapter 3.

Transformation and Characterization of Arabidopsis thaliana Modified with linA

3.1. Introduction

In Chapter 2, it was established that the persistent organic pollutant, lindane, is ubiquitously found within the physical and geographical environment and negatively impacts a wide range of microorganisms, invertebrates, fish, birds and mammals (IPCS, 1991). As the toxicity, distribution and persistence of lindane is well established, it is imperative to develop a method by which it can be safely and quickly removed from the environment. Current standard remediation practices, including physical removal and chemical transformation, require large infrastructure, are costly and potentially dangerous (Sutton & Hunter, 1989). Hydrolysis is the most common and important abiotic method while bioremediation, which uses indigenous or foreign microorganisms, fungi, plants (phytoremediation) and their enzymes to biologically degrade organic waste to a non-toxic state, or to sub-threshold levels below concentration limits is a low-cost, low-technology and relatively low-disturbance alternative technique (Vidali, 2001).

Thus far, owing to the limitations of natural microbial bioremediation and phytoremediation, no such method for the clean-up of lindane on a global scale has been realized. Despite positive results being reported in laboratory studies, in recent studies where bacteria have been used for decontamination in field studies, bioaugmentation occurred at relatively low concentrations of HCH, requiring both long-term inoculations and nutrient application (Raina, *et al*., 2008). In phytoremediation, the majority of plants do not have the full set of metabolic enzymes for complete degradation and mineralization of these compounds when compared to microorganisms. Improving plants for phytoremediation will likely result from transferring genes known to be involved in xenobiotic degradation from other plants, microbes and eukaryotes, specifically those that can be used to improve the uptake and degradation of lindane.

Preliminary results from a single proof of concept study have shown that the model plant *Arabidopsis thaliana* modified with *linA* from the bacterium *Sphingobium japonicum* UT26 is able to grow in the presence of normally toxic lindane concentrations, and remove it from the media whereas the wild type plant is not (De

Lorenzo Prieto & Gonzalez Pastor, 2007). However the study does not consider the effects of different lindane concentrations with respect to its uptake and degradation within the plant, nor does it account for the mass balance of lindane within the plant and / or media environment. Therefore, in this chapter, the feasibility of transgenic *A. thaliana* to grow on, and ultimately remediate lindane-contaminated environments, was assessed by cloning the *linA* gene from *S. japonicum* UT26, a known lindane-degrader, using Gateway® Technology, and subsequently transforming it into *A. thaliana* by means of *Agrobacterium*-mediated transformation.

A. thaliana was chosen as the host organism, not only to reproduce and build on the initial findings from the previous research, but also because of its limited space requirements, short life cycle and small, fully sequenced genome and associated protein databases (National Science Foundation, 2013). Gateway® Technology was not used by De Lorenzo Prieto & Gonzalez Pastor (2007), but is used in this thesis as it is a rapid, cost-effective and 99% efficient universal cloning method that avoids typical cloning limitations while successfully directing and maintaining PCR fragment orientation and overall reading frame (Katzen, 2007).

By exploiting the known facts pertaining to *Agrobacterium*-mediated transformation; the host properties of *A. thaliana*; the effectiveness of Gateway® Technology Cloning; and the enzymatic potential of LinA, aims 1 and 2 of this thesis are addressed. Transforming *A. thaliana* with *linA* and then observing its growth and characteristics on control, sub-threshold and toxic concentrations of lindane and 1,2,4-TCB examines the hypothesis of this thesis and the development of a single gene-modified plant for the phytoremediation of lindane, and its intermediates, that may be enhanced by naturally occurring enzymes present in the native plant xenobiotic degradation pathway. This work will build upon the current understanding of transgenic phytoremediation in lindane-contaminated environments and will lend itself to advancing the mechanistic understanding in future work.

3.2. Methods

3.2.a. Growth of Wild Type A. thaliana on Agar

3.2.a.i. Seed Sterilization

Approximately 100 µl wild type (w/t) *A. thaliana* Columbia-0 (Col-0) seeds (Professor Andrew Fleming, Department of Animal and Plant Science, University of Sheffield) were placed in a 1.5 ml eppendorf tube. 800 µl Reverse Osmosis (RO) purified water and 200 µl Economy Bleach (Ottimo Supplies) were added to the reaction tube followed by 0.5 µl Tween-20 Ultrapure (Sigma) to give 0.05% (v/v). The mixture was incubated for 10 minutes with occasional vortexing. Seeds were then spun down in a bench-top centrifuge on short spin until it reached 5000 rpm. The liquid was removed from the tube and washed with 1µl RO water and repeated twice more for a total of 3 washes. After the final wash, the liquid was removed and 300 µl of RO water was added to the tube, which was then covered in foil, placed in the fridge at 4 °C and left for 4 days to stratify or break dormancy.

3.2.a.ii. Growth of w/t *A. thaliana* on Lindane

7 square (120/120/17 mm) Petri dishes (Greiner Bio-one) were prepared with 0.8% ½ MS Plant Agar (Duchefa Biochemie) and differing concentrations (0, 4, 34, 68, 102, 136 and 170 µM of 97% lindane (Sigma Aldrich) to analyze the growth capacity of w/t *A. thaliana* on lindane contaminated-media. 0 µM was used as a control, 4 µM was chosen as it is the highest allowable environment limit, 34 µM is the threshold for bacterial degradation and 170 µM is the threshold for degradation by a bacterial consortium (Raina, *et al*., 2008; Ministerie van Volkshuisvesting, 2000; Elcey & Kunhi, 2010). An additional 7 plates were prepared with the same molar concentrations of ≥ 99% - 1,2,4-TCB (Sigma Aldrich). Sterilized seeds were resuspended in 0.11% - ½ MS Plant Agar and using a 6x6 point grid template, 36 sterilized seeds were transferred onto each plate using a 100 µl pipette tip to control distribution. Each plate was sealed with micropore tape and placed in a growth

cabinet at 22 °C with 16 hours of light and 8 hours of dark. Growth was observed after 28 days. All growth experiments were done in triplicate.

3.2.a.iii. Threshold Toxicity Growth of w/t *A. thaliana* on 1,2,4-TCB

To determine the upper threshold of 1,2,4-TCB toxicity on the growth on w/t *A. thaliana*, plates and seeds were prepared as above, with 1,2,4-TCB concentrations ranging from 100 µM up to 1 M (increasing by a factor of 10). Due to the highly toxic and fumigant properties of 1,2,4-TCB, plates were placed in a well-ventilated growth room, where no other experimental procedures were taking place. Growth was observed after 28 days.

3.2.a.iv. Growth of w/t *A. thaliana* on 1,2,4-TCB and 1-aminobenzotriazole

1 mM 1-aminobenzotriazole (ABT), a P450 inhibitor, was added to 1,2,4-TCB-spiked plates to ascertain whether or not inhibition of w/t *A. thaliana* growth occurred. Plates with concentrations that are not normally detrimental to growth were prepared as above and growth was observed after 28 days.

3.2.b. Verification of linA from pUC18-EcoRI-SD-linA-HindIII Plasmid

3.2.b.i. Minipreparation of Plasmid DNA

The *linA* gene was obtained in plasmid form, using the pUC18-EcoRI-SD-linA-HindIII vector (Professor Yuji Nagata, Graduate School of Life Sciences, Tohoku University) (Figure 3.1). The vector was transformed into competent cells to verify its activity based on its ability to confer ampicillin resistance (Figure 3.2). 1 µl of plasmid DNA was added to Library Efficiency® Chemically Competent (*Escherichia coli)* DH5α cells (Invitrogen) and left on ice for 30 minutes. Cells were then heat-shocked at 42 °C for 60 seconds, followed by the addition of 1 ml Lennox Luria-Bertani (LB) Broth (Fischer Scientific). Cells were incubated for 1 hour at 37 °C with shaking at 180 rpm and then centrifuged at 3500 rpm for 1 minute. All but 100 µl of the supernatant

was removed and the pellet resuspended. The suspension was then evenly spread onto 100 µg/ml ampicillin-LB agar (Sigma) selective plates and incubated overnight at 37 °C without shaking.

GAATTCTAAGGAGGATACAAAATGAGTGATCTAGACAGACTTGCAAGCCGGGCC
GCGATTCAGGACCTCTACTCTGACAAGCTCATTGCCGTAGACAAGCGCCAAGAG
GGCCGTCTCGCTTCTATTTGGTGGGATGATGCAGAGTGGACCATTGAGGGAAT
CGGCACCTACAAGGGCCCGGAAGGCGCCCTCGATTTGGCCAATAACGTACTCT
GGCCAATGTTTCACGAATGTATTCATTATGGAACCAATCTGCGCTTGGAATTTGT
GAGCGCGGACAAGGTAAATGGTATTGGCGACGTCCTTCTCCTTGGAAATCTCGT
CGAAGGTAATCAGTCGATTCTTATCGCTGCGGTCTTCACGGATGAGTATGAGCG
CCGTGACGGGGTGTGGAAGTTCTCTAAGCGCAACGCATGCACGAACTATTTCAC
CCCGCTGGCCGGCATTCATTTCGCACCGCCCGGCATTCATTTCGCACCGTCCG
GCGCATAATCTAAGCTT

Figure 3.1 Sequence of EcoRI-SD-linA-HindIII insert from pUC18 plasmid.
The EcoRI site is yellow, SD sequence highlighted grey, *linA* underlined and HindIII pink

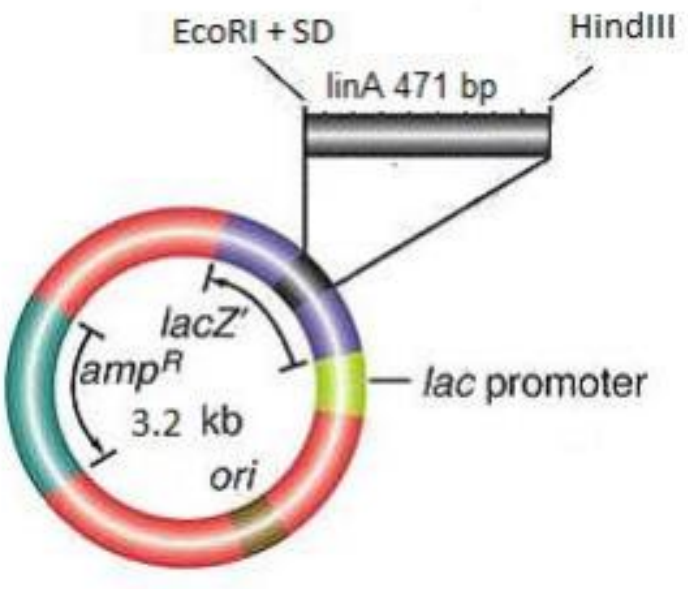


Figure 3.2 The plasmid construct pUC18-EcoRI-SD-*linA*-HindIII
Modified from Invitrogen pENTR™ Directional TOPO® Cloning Kits Manual (2012)

6 colonies were then streaked onto an ampicillin-LB selective sectioned agar plate and incubated overnight at 37 °C without shaking. A single colony from the freshly streaked plate was then inoculated into 5 ml ampicillin-LB medium and incubated

overnight 37 °C with shaking at 180 rpm. Cells were harvested by centrifugation at 8000 rpm for 3 minutes and all traces of supernatant removed. Plasmid mini-preps were prepared using the QIAprep Spin Mini-prep Kit as per the guidelines set out in the QIAprep Spin Mini-prep Kit Protocol.

3.2.b.ii. Verifying the Presence of *linA*

To verify the presence of *linA* in the plasmid construct pUC18-EcoRI-SD-linA-HindIII, restriction analysis was carried out by combining and incubating the following at 37 °C for 1 hour after which it was visualized on a 1% agarose gel:

- 10x EcoRI buffer (BioLabs) 2 µl
- Nuclease Free water (Ambion) 11 µl
- Plasmid DNA (>20 ng / DNA band) 4 µl
- EcoRI (20 u/µl) (BioLabs) 1 µl
- HindIII (10 u/µl) (Promega) 2 µl

3.2.b.iii. PCR of *linA* from pUC18-EcoRI-SD-linA-HindIII Plasmid

linA primers were designed to enable directional cloning using the pENTR™ Directional TOPO® Cloning Kits (Invitrogen). The forward PCR primer contained the sequence, CACC, at the 5′ end of the primer as they are designed to base pair with the overhang sequence, GTGG, in the pENTR™ TOPO® vector. The primers used to amplify the *linA* sequence are shown in Table 3.1.

Table 3.1 Primers used to amplify *linA* sequence from vector pUC18-EcoRI-SD-linA-HindIII

Oligo Name	Sequence (5' – 3')	Tm (°C)	MW (g/mol)	GC Content (%)
For_LinA	CACCATGAGTGATCTAGACAGA	58.4	6752	45.5
Rev_LinA	TTATGCGCCGGACGGTGCGAAATG	66.1	7433	58.3

PCR was carried out using the plasmid DNA obtained from the above mini-prep. The reaction was set up as described below and visualized on a 1% agarose gel:

- 10x Buffer for HotStart Polymerase (TOYOBO/Novagen) 5 µl
- MgSO4 (25 mM) (TOYOBO/Novagen) 3 µl
- dNTPs (2 mM) (TOYOBO/Novagen) 5 µl
- For_linA (10 µM) (Eurofins) 1.5 µl
- Rev_LinA (10µ M) (Eurofins) 1.5 µl
- KOD DNA Polymerase (2.5 u/µl) (TOYOBO/Novagen) 1 µl
- Plasmid DNA (~10 ng) 1/ 2 µl
- Nuclease free Water (Ambion) (up to 50 µl total volume) 31 / 32 µl

The PCR reaction was repeated 2 additional times to increase the yield of PCR product, all of which was loaded onto a 1% agarose gel. The desired band was then isolated and purified from the gel using a microcentrifuge as per the guidelines set out in the QIAquick Gel Extraction Kit Protocol (Qiagen) with the final elution being carried out in 30 µL of buffer to concentrate the sample. Sequencing was carried out (Core Genomics Facility, University of Sheffield) and the results were aligned against the known sequence as given previously in Figure 3.1.

3.2.c. DNA Cloning of linA

3.2.c.i. pENTR/D-TOPO Topoisomerase Reaction

Blunt-end PCR fragments were cloned into the pENTR/D-Topo Gateway entry vector (Invitrogen). Purified PCR product concentrations were verified using gel electrophoresis quantification. The reaction was set up as described below using a 1:1 and 2:1 molar ratio of purified PCR product : TOPO vector

Purified PCR elute products samples 1 and 2, were determined as being approximately 120 ng/5 µl or 24 ng/µl and 200 ng/5 µl or 40 ng/µl, respectively. A 1:1 molar ratio of purified PCR product : pENTR/D-Topo Gateway entry vector was calculated as follows:

- D-Topo Vector ~ 2.5 kb
- purified PCR Product ~ 0.5 kb
- D-Topo Vector (20 ng/µl) is therefore 5 times the size of the PCR Product and a 1:1 molar ratio is 4 ng/µl PCR Product : 20 ng/µl DTopo Vector

Blunt-end cloning of the purified PCR products into the pENTR/D-Topo Gateway entry vector (Invitrogen) was carried out using 2:1 and 1:1 molar ratios of purified PCR product : TOPO vector as follows:

- 2:1 molar ratio
  - 0.5 µl Purified PCR product (sample 1) ~ 12 ng (~ 2 to 3 mol)
  - 1 µl pENTR/D-Topo Gateway entry vector = 20 ng (1 mol)

- 1:1 molar ratio
  - 0.5 µl 5x dilution Purified PCR product (sample 2) ~ 4 ng (1 mol)
  - 1µl pENTR/D-Topo Gateway entry vector = 20 ng (1 mol)

The reaction was carried out as per the guidelines set out in the Invitrogen pENTR™ Directional TOPO® Cloning Kits User Manual.

- Salt Solution (1.2 M NaCl / 0.06 M MgCl2) 1 µl
- Purified PCR Product (4 - 12 ng/µl) 0.5 µl
- Sterile water up to 5 µl
- TOPO vector (20 ng/µl) 1 µl

The 6 µl reaction mixture was gently stirred with a pipette tip and incubated at room temperature for 3 hours. 2 µl of the reaction mixture was used to transform ½ vial (25 µl) of One Shot® TOP10 (Invitrogen) and α-select™ Gold Efficiency (Bioline) chemically competent cells.

The reaction was gently stirred with a pipette tip and incubated on ice for 30 minutes. Cells were then heat-shocked for 30 seconds at 42 °C and immediately transferred to ice. 250 µl of room temperature LB was added to the mixture and incubated 1

hour at 37 °C with shaking at 200 rpm. 50 and 225 µl from each transformation were spread on room temperature kanomycin-LB agar plates and incubated overnight at 37 °C without shaking.

Individual colonies selected on kanamycin-LB plates were subsequently cultured overnight in kanamycin-LB at 37 °C with shaking at 200 rpm. Plasmid mini-preps were prepared using the QIAprep Spin Mini-prep Kit as per the guidelines set out in the QIAprep Spin Mini-prep Kit Protocol. Insertion of the PCR fragment into the TOPO vector was verified by PCR using the For_LinA and Rev_LinA primers, DNA sequencing and single restriction enzyme analysis as follows:

- 10x Multicore buffer (Promega) 1 µl
- Nuclease free water (Ambion) 6 µl
- Plasmid DNA (114.2 ng/µl) 2 µl
- EcoRI (12 u/µl) (Promega) 1 µl

3.2.c.ii. LR Clonase Reaction

Plasmid mini-prep stocks containing the entry clone with verified insertions were used to shuttle the desired *linA* gene insert to the secondary destination plasmid; Ctapi (Dr. Lee Hunt, Animal and Plant Sciences, University of Sheffield). Vector and mini-prep concentrations were determined by biospectrophotmetric DNA quantification (Eppendorf). This reaction was mediated by the Gateway® LR Clonase™ enzyme mixture (Invitrogen) and set up as described below:

- TOPO_linA entry clone (114.2 ng/µl) 3 µl
- Ctapi destination vector (150 ng/µl) 2µl
- TE buffer, pH 8.0 up to 8 µl
- 5x LR Clonase II enzyme mix 2 µl

1 µl of the reaction mixture was used to transform ½ vial (25 µl) α-select™ Gold Efficiency (Bioline) chemically competent cells. Selection was carried out using

spectinomycin-LB, plasmid mini-preps were prepared and insertion of the *linA* gene into the Ctapi destination vector was verified by PCR, DNA sequencing as well as single restriction enzyme analysis.

3.2.d. Electrotransformation of Agrobacterium

50 µl of electrocompetent *Agrobacterium tumefaciens* GV3101::pMP90 RK cells (Professor Andrew Fleming, Department of Animal and Plant Sciences, University of Sheffield) were thawed on ice and 5 µl of plasmid DNA obtained from Ctapi destination vector were added. The DNA and cells were mixed and chilled on ice for 5 minutes before being transferred to a pre-chilled 2 mm electroporation-cuvette (Biorad). The cuvette was transferred to the electroporator which was set to 2.5 kV. Immediately after electrotransporation, 1 ml low-salt LB (Sigma Aldrich) was added to the cuvette. The resulting bacterial suspension was transferred to a culture tube containing 3 ml low-salt LB which was incubated for 2 hours at 28 °C with shaking at 200 rpm. 50 and 200 µL of the transformed cells were spread on low-salt LB agar plates containing kanomycin, spectinomycin, rifampicin and gentamycin (KSRG) which were incubated at 28 °C without shaking for 3 days. Selected colonies were transferred to 3 ml low-salt KSRG-LB and cultured for 2 days at 28 °C with shaking at 200 rpm. A glycerol stock of the positive bacterial suspension was prepared for storage. Plasmid mini-preps were prepared and presence of the *linA* gene was verified by PCR as well as single restriction enzyme analysis.

3.2.e. Transformation of A. thaliana by Floral Dipping

3.2.e.i. Germination and Growth of *A. thaliana* on Soil

A 5 x 7 inch rectangular planting tray with drainage holes was filled above the rim with pot and bedding compost (Levington M3 Scotts), and packed down until flush. The tray was then placed inside a larger 9 x 14 inch tray without holes and both were filled with water to wet the soil. Using a creased piece of paper to control and visual distribution, approximately 60 unsterilized *A. thaliana* seeds were evenly sown on the soil surface. The tray was placed in a transparent plastic bag, sealed with masking

tape to make it air-tight and stored for 5 days at 4 °C to vernalize, or hasten development of the seeds. The tray was then moved to the growth cabinet for 24 hours before opening the plastic bag and leaving it another 24 hours after which time the transparent bag was removed completely. Once the seedlings had 4 leaves, at approximately 7 days after planting (dap), they were replanted into individual pots, segregated and protected using aracon tubes and maintained until flowering. The first flower bolts were removed to promote branching for future use in the floral dip transformation of *A. thaliana*.

3.2.e.ii. Floral Dipping

The *Arabidopsis* floral dipping technique used for *A. thaliana* transformation was performed as described by Clough and Bent (1998). 5 ml of low-salt KSRG-LB broth was inoculated with 50 µl from the 3 ml *Agrobacterium* suspension and grown overnight at 28 °C with shaking at 200 rpm. 500 µl from this new suspension was added to 50 ml low-salt LB (antibiotic free) and grown overnight at 28 °C with shaking at 200 rpm. The 50 ml suspension was then centrifuged in a Beckman centrifuge for 10 minutes at 4000 g to harvest the cells. The resulting pellet was then resuspended in 25 ml - 5% Sucrose (Sigma) solution containing 0.05% Silwet (Lehle Seeds); a non-phytotoxic wetting agent. The optical density (OD) verified to be approximately 0.8 at 600 nm using an Eppendorf Biospectrophotometer and the solution was subsequently aliquotted into 1.5 ml Eppendorf tubes.

Previously grown *A. thaliana* flowers were then individually dipped in the sucrose solution and held for 5 to 10 seconds (Figure 3.3a). Dipped plants were placed in a dark box for 24 hours, to facilitate transfection in conditions of high humidity. Transformation was repeated after 5 days using 200 ml sucrose - *Agrobacterium* solution, into which the entire plant was dipped and held for approximately 1 minute (Figure 3.3b). Plants were maintained until seeds produced.

Figure 3.3 Floral dipping techniques for *Arabidopsis thaliana* transformation with *Agrobacterium*. Panel (a) illustrates the initial single flower dipping method (ensuring all flower bolts are dipped) while (b) shows the subsequent whole plant dipping method (5 days later).

3.2.e.iii. Harvesting of Seeds (T_0)

Each plant was cut at its base, discarding the pot, soil and roots. Shoots from 2 plants were carefully removed from the protective aracon tubes, placed on white sheets of paper and gently pressed to release and capture seeds. The seeds, siliqua, petals and other plant material were passed through a sieve 5 times to separate out the seeds which were stored in a 50 ml falcon tube with pin-sized holes through the lid to avoid fungal infection and decrease humidity. The procedure was repeated with remaining plants, two at a time.

3.2.e.iv. Selection of Transformants (T_1, T_2 and T_3)

Approximately 100 µl of T_0 seeds were sterilized as previously described (Chapter 3.2.a.i). After 4 days, the seeds were divided into 6 – 15 ml falcon tubes and made up to 4 ml with RO water. 8 ml of hot 0.7% - ½ MS Plant Agar was added to each tube. The agar and seeds were quickly mixed and poured onto 0.8% - ½ MS Plant Agar selective plates containing the antibiotic BASTA (20 µg/ml) (Sigma Aldrich) as well as Cefatoxime (CEF) (25 µg/ml) (Mrs. Marian Bauch, Animal and Plant Sciences, University of Sheffield) to suppress *Agrobacterium* growth. The seed mixture was evenly spread on the plates by gently swirling. The selective plates were then sealed with micropore tape and placed in a growth cabinet at 22 °C with

16 hours of light and 8 hours of dark for 7 days. After formation of secondary leaves, transformants were transferred to antibiotic-free soil and maintained until seeds were produced. Harvesting and selection of seedlings was repeated, without the use of CEF on selection plates, until a homozygous line was obtained (T_3).

3.2.f. DNA Extraction

Plant DNA was extracted from plant leaves using the REDExtract-N-AMP™ Plant PCR Kits (Sigma Aldrich) as per the instructions found in the technical bulletin. Plant material was collected into a supplied 2 ml collection tube using the lid of the tube to punch a leaf disk directly into the tube without the use of forceps and eliminating any possible contamination. Briefly, the DNA was extracted in 100 µL of the Extraction Solution at 95 °C for 10 minutes, followed by the addition of 100 µL of the Dilution Solution to neutralize any inhibitory substances.

An aliquot of the PCR-ready, diluted extract was combined with the REDExtract-N-AMP™ PCR ReadyMix (containing buffer, salts, dNTPs and Taq polymerase), For_LinA and Rev_LinA primers. PCR was carried out according the REDExtract-N-AMP™ Plant PCR Kits Protocol (Sigma Aldrich) and amplified DNA was loaded directly onto an agarose gel without the addition of loading buffers. DNA sequencing was used to confirm the *linA* insert from samples with banding at approximately 475 bp.

3.2.g. RNA Isolation

3.2.g.i. RNA Extraction

50-100 mg of leaf tissue was collected in a 1.5 ml eppendorf tube and immediately frozen with liquid nitrogen. The frozen tissue was ground using a small pestle. 500 µL of TRIzol® Reagent (Ambion) was added to the ground tissue and the mixture was homogenized on ice. An additional 500 µL TRIzol® Reagent was added and the solution was incubated at room temperature (RT) for 5 minutes to permit complete dissociation of the nucleoprotein complex. 0.2 ml of chloroform was added to the

tube which was then shaken by hand for 15 seconds. The mixture was incubated at RT for 2 minutes before being placed in a centrifuge for 15 minutes at 12000 rpm. The mixture separated into 3 distinct phases at this point:

- A colourless upper aqueous phase;
- An interphase; and
- A lower red phenol-chloroform phase

The aqueous phase of the sample, containing the RNA, was removed and placed in a new, ice-cold 1.5 ml tube. 500 µL of 100% isopropyl alcohol was added to mixture which was then incubated at RT for 10 minutes and placed in a centrifuge for 15 minutes at 12000 rpm. The supernatant was removed, leaving only the RNA pellet, which was washed with 1 ml of 80% ethanol. The contents were mixed by vortexing and placed in a centrifuge for 5 minutes at 12000 rpm. The ethanol wash was removed and the pellet was air-dried in the tube upside-down at RT for 5 minutes. The RNA pellet was resuspended in 20 µL nuclease-free water (Ambion) and stored at -80 °C.

3.2.g.ii. RNA Quantification and DNase Treatment

The RNA was quantified using an Eppendorf Biospectrophotometer and treated with DNase as follows:

- RNA (2 µg) x µl
- 10x DNase I buffer (Life Technologies) 5 µl
- rDNase I (2 u/µl) (Life Technologies) 1 µl
- Nuclease-free water (Ambion) up to 50 µl

The reaction mixture was incubated at 37 °C for 30 minutes. 5 µl of DNase Inactivation Reagent (Life Technologies) was added to the tube and left to incubate at RT for 2 minutes. The tube was placed in a centrifuge for 90 seconds at 13200 rpm and the RNA supernatant was transferred to a fresh tube (stored at -80 °C).

3.2.g.iii. First Strand cDNA Synthesis

The following reagents were added to a 1.5 ml eppendorf tube:

- DNase treated RNA (~1 µg) x µl
- oligo dT_{18} (0.5 µg/µl) (Life Technologies) 2 µl
- RNase-free water (Ambion) up to 15 µl

The reaction mixture was heated to 70 °C for 5 minutes and transferred immediately to ice. The reaction tube was then placed in a bench-top centrifuge on short spin until it reached 5000 rpm.

3.2.g.iv. Reverse Transcription

The following reagents were added to a 1.5 ml eppendorf tube:

- cDNA mixture 15 µl
- 5x M MLV reaction buffer (Promega) 5 µl
- dNTPs (Promega) (10 mM) 1.25 µl
- M MLV reverse transcriptase (200 u/µl) (Promega) 1 µl
- RNase-free water (Ambion) 2.75 µl

The reaction mixture was incubated at 42 °C for 2 hours and then placed in a bench-top centrifuge on short spin until it reached 5000 rpm. The mixture was stored at -20 °C.

3.2.g.v. Polymerase Chain Reaction

PCR was performed on the template cDNA to determine if the *linA* gene was being transcribed into RNA. The following reagents were combined as follows:

- Template cDNA 2 µl
- 10x High Fidelity Buffer (NEB) 2.5 µl
- $MgSO_4$ (50mM) (NEB) 1 µl
- dNTPs (10mM) (NEB) 0.5 µl
- Platinum® Taq DNA Polymerase (5 u/µl) (NEB) 0.2 µl
- For_LinA primer (10 µM) (Eurofins) 2.5 µl
- Rev_LinA primer (10µM) (Eurofins) 2.5 µl
- Nuclease-free water (Ambion) 13.8 µl

3.2.h. Protein Analysis

3.2.h.i. Protein Extraction

3 discs of 1 cm^2 were excised from the leaves of the original w/t line; two transgenic lines, thus referred to as LinA2 and LinA4; and an untransformed line designated linA(-), grown on both lindane-free and sub-threshold lindane (30 µM). Liquid nitrogen was used to flash freeze the plant material which was then immediately homogenized using a micropestle. 1 ml of extraction buffer was added to each reaction tube which was incubated at 4 °C for 20 minutes with occasional mixing by inverting the tube several times. The suspension was centrifuged at 4 °C at 15000 rpm for 4 minutes and the supernatant was transferred to a new tube. Extraction in 500 µl extraction buffer was repeated on the remaining pellet and the resulting supernatants were combined. Samples were then concentrated using the Amicon® Ultra-0.5 Centrifugal Filter Devices, with an optional buffer exchange using 0.5 M Triethylammonium bicarbonate (TEAB) (Sigma Aldrich), according the manufacturer's protocol. Protein concentration was quantified using the Bradford ULTRA Assay Kit (Westburg) on a Nanodrop 2000 Spectrophotometer (Thermo Scientific).

3.2.h.ii. Western Blotting

Translation of RNA transcripts into protein was determined using The NuPAGE® Novex® Bis-Tris Mini Gel protocol with the following modifications:

- Extracted protein (< 0.5 µg protein load / band) 20 µl
- 4x NuPAGE LDS Sample Buffer 7.5 µl
- Deonized water (Millipore) 2.5 µl

Proteins from the samples grown on lindane-free media were incubated at 70 °C for 10 minutes and cooled to room temperature. 20 µl of the biotinylated protein ladder (Cell Signaling) was incubated at 95 °C for 5 minutes and cooled to room temperature. Two pre-cast 4 - 12% mini-gels (Invitrogen) were identically loaded with 5 µl pre-stained protein ladder (0.05 – 0.1 mg/ml of each protein marker) (Cell Signaling), 10 µl biotinylated ladder, and 20 µl of the sample mixture. Run conditions were 50 minutes in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (1x) (Invitrogen) at a current of 60 mA. Following the run, one gel was placed directly in Instant Blue (Expedeon), a Coomassie based protein staining solution.

The protein bands from the unstained gel were transferred to a nictrocellulose membrane using an iblot® 7-minute blotting system (Life Technologies) as per the manufacturer's protocol. Following the transfer, the membrane was incubated in 25 ml of blocking buffer at RT for 30 minutes and then washed 3 times for 1 minute in 15 ml wash buffer. The membrane and 10 µl of primary LinA antibody (Yugi Nagata, Graduate School of Life Sciences, Tohoku University) were incubated in 10 ml of the primary antibody dilution buffer with gentle agitation overnight at 4°C followed by 3 washes of 1 minute in 15 ml wash buffer. The membrane and 5 µl of secondary HRP-conjugated anti-rabbit antibody (Cell Signaling) and 10 µl of HRP-conjugated anti-biotin antibody (Cell Signaling) were incubated in 10 ml blocking buffer with gentle agitation at RT for 1 hour followed by 3 washes of 1 minute in 15 ml wash buffer. HRP-Bound proteins were detected by incubating the membrane in 10 ml Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) for 5 minutes with gentle agitation.

3.2.h.iii. Enzyme Activity Assay

Enzymatic activity of the translated protein, LinA, indicated by a release of chloride ions (Cl^-), was assessed by colorimetric determination using Mercuric(II) Thiocynate

($Hg(SCN)_2$) (Sigma Aldrich). When $Hg(SCN)_2$ and Cl^- ion are combined, an absorption at 250 - 280 nm is observed that rises proportionately with chloride concentration (Cirello-Egamino & Brindle, 1995).

A 20 ppm stock of Cl^- from Sodium Chloride (NaCl) was prepared and diluted, using 0.5 M TEAB Buffer. 5 µl from a 0.3% solution of $Hg(SCN)_2$ (dissolved in 100% acetonitrile) was added to 50 µl of each NaCl concentration and the absorbance was recorded to produce a standard curve, at 280 nm, using a Tecan GENios Microplate Reader.

5 µl of each protein extract, from samples grown under lindane-free and sub-threshold, 30 µM, lindane conditions, was diluted 1:10 to a final volume of 50 µl using the 0.5 M TEAB Buffer. 5 µl from a 0.3% solution of $Hg(SCN)_2$ (dissolved in 100% acetonitrile) was added to each sample which was then incubated with 30 uM lindane for 1 minute and the absorbance was recorded at 280 nm, using a Tecan GENios Microplate Reader, to determine the amount of chorine released.

3.2.i. Growth of A. thaliana Modified with linA

7 square (120/120/17 mm) Petri dishes (Greiner Bio-one) were prepared with 0.8% ½MS Plant Agar and differing concentrations (0, 4, 34, 68, 102, 136 and 170 µM of 97% lindane (Sigma Aldrich) to analyze the growth capacity of w/t and transgenic *A. thaliana* on lindane contaminated-media. Sterilized seeds were resuspended in 0.11% - ½ MS Plant Agar and using a 6 x 6 point grid template, 9 seeds from the wild type (w/t), transgenic (LinA2 and LinA4) and the negative control (linA(-)) lines were transferred onto the same plate in a 3 x 3 pattern (to give a 4 square comparison plate) using a 100 µl pipette tip to control distribution. Each plate was sealed with micropore tape and placed in a growth cabinet at 22 °C with 16 hours of light and 8 hours of dark. Growth was observed after 28 days.

3.3. Results

3.3.a. Growth of w/t A. thaliana on Lindane

At t = 28 days after planting (dap), growth was limited to the plates with 0 and 1 μM lindane. Further experimentation examined growth concentrations between 1 μM and 34 μM (at 5 μM increments) to determine if 34 μM was the toxic threshold. Equal and robust growth was seen for all concentrations up to 30 μM suggesting that 34 μM lindane is the threshold for toxicity (Figure 3.4). Equal and robust growth was seen for all concentrations of 1,2,4-TCB at t = 28 dap (Figure 3.5).

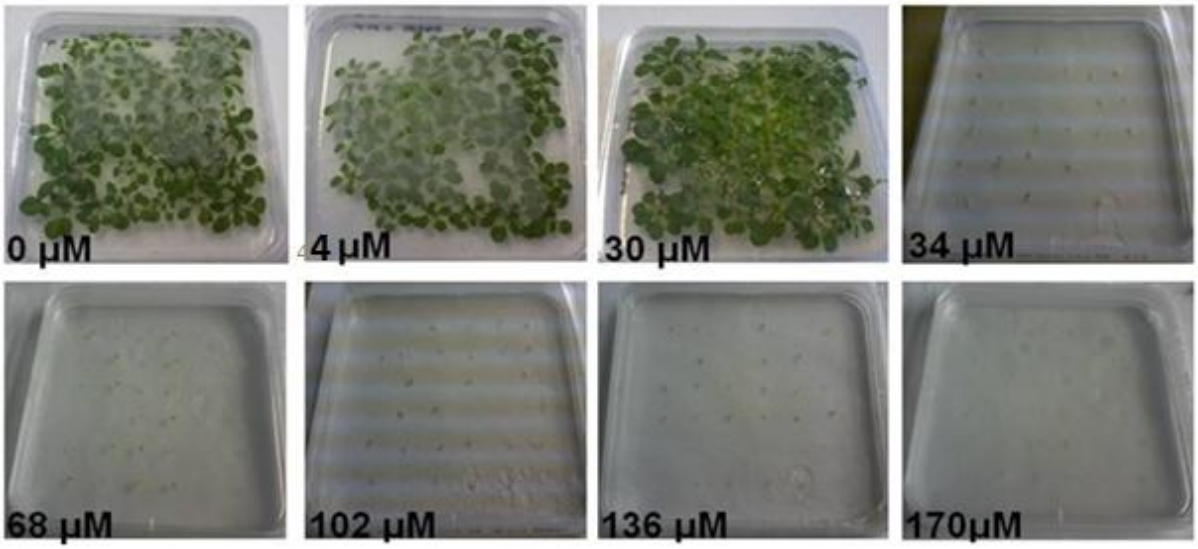


Figure 3.4 Growth of wild type *Arabidopsis thaliana* on increasing concentrations of lindane

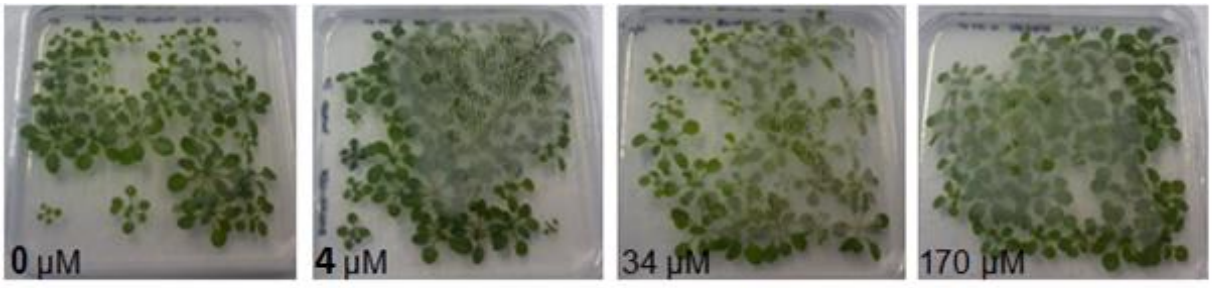


Figure 3.5 Growth of wild type *Arabidopsis thaliana* on increasing concentrations of 1,2,4-trichlorobenze (TCB)

3.3.b. Threshold Toxicity Growth of w/t A. thaliana on 1,2,4-TCB

At t = 28 dap, equal and robust growth was seen for all concentrations up to 1.5 mM 1,2,4-TCB suggesting a very high toxicity threshold (Figure 3.6).

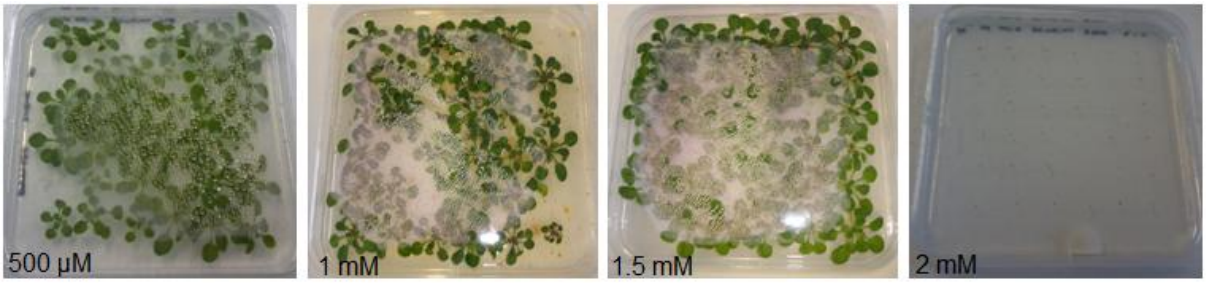


Figure 3.6 Threshold toxicity growth of wild type *Arabidopsis thaliana* on increasing concentrations of 1,2,4-trichlorobenzene (TCB)

3.3.c. Growth of w/t A. thaliana on 1,2,4-TCB and ABT

At t = 28 dap, growth was limited to the plates with 0 and 1 µM 1,2,4-TCB when in the presence of the P450 inhibitor, 1 mM ABT. As growth has previously been shown to occur at concentrations up to 1.5 mM 1,2,4-TCB (in ATB-free media), this inhibited growth suggests that Cytochrome P450s may be involved in the metabolism of 1,2,4-TCB in *A. thaliana* (Figure 3.7).

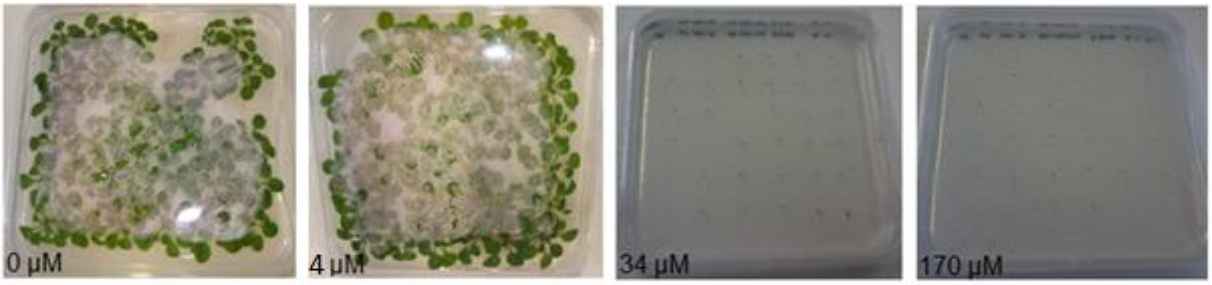


Figure 3.7 Growth of wild type *Arabidopsis thaliana* on 1,2,4-trichlorobenzene (TCB) and 1-aminobenzotriazole (ABT)

3.3.d. Verification of linA from pUC18-EcoRI-SD-linA-HindIII Plasmid

3.3.d.i. Minipreparation of Plasmid DNA

Vector activity of pUC18-EcoRI-SD-linA-HindIII was confirmed based on its ability to confer ampicillin resistance. Successfully transformed competent DH5α cells were grown on ampicillin/LB selective plates (Figure 3.8).

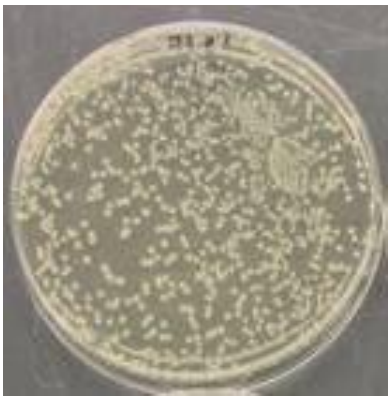


Figure 3.8 Growth of competent DH5α cells transformed with pUC18-EcoRI-SD-linA-HindIII

The presence of an insert in accordance with the *linA* gene in the plasmid construct, pUC18-EcoRI-SD-linA-HindIII, was verified through restriction analysis using EcoRI and HindIII. As the length of the circular construct is approximately 3.2kb, and the *linA* gene is 471bp, with flanking EcoRI and HindIII restriction sites (Figure 3.2), it was expected that the double restriction digest of the construct would result in two bands at approximately 471bp and 2.7kb. Gel electrophoresis revealed two bands of expected length in lane 2, confirming the presence of an insert in accordance with *linA* (Figure 3.9).

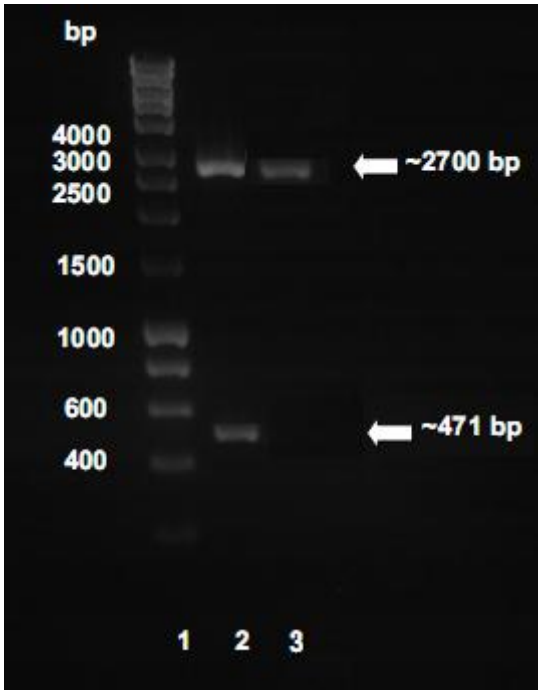


Figure 3.9 Restriction digest analysis of the construct pUC18-EcoRI-SD-linA-HindIII. Lane2 containing the plasmid DNA shows a bright band at approximately 2.7 kb and a faint band at approximately 471bp when compared against the DNA Ladder in lane 1 and negative control, pUC18 in lane 3.

3.3.d.ii. PCR of *linA* from pUC18-EcoRI-SD-linA-HindIII Plasmid

PCR using the *linA* primers (Table 3.1) gave amplification products of approximately 475bp; which was the expected and desired size considering the length of the *linA* gene (471 bp) plus the CACC overhang created from the forward primer (Figure 3.10). However, non-specific bands appeared at approximately 850 bp, 1200 bp and 3200 bp.

To ensure purity, bands of approximately 475bp from all PCR reactions were excised and purified from the agarose gel in 2 separate 1.5 ml Eppendorf tubes. Gel electrophoresis confirmed the presence of the desired 475bp product and the concentrations were determined as being approximately 120 ng / 5 µl and 200 ng / 5 µl in samples 1 and 2, respectively, when compared against the DNA Ladder (5 µl) and pUC18 negative control (Figure 3.11). The known concentrations were subsequently used to determine the molar ratios for future DNA cloning.

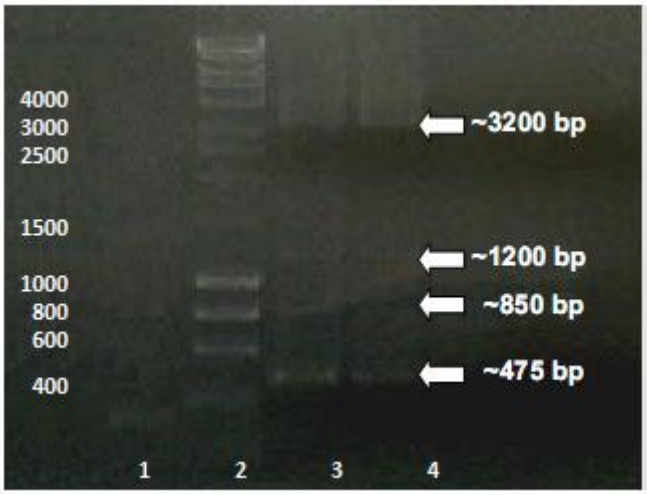


Figure 3.10 PCR analysis of the minipreparation plasmid DNA from pUC18-EcoRI-SD-linA-HindIII. Lane 3 (1 µl) and lane 4 (2 µl) show similar results with the desired band at approximately 475bp as well as non-specific bands at 850bp, 1200bp and 3200bp when compared against the DNA Ladder in lane 2 and the negative control pUC18 in lane 1.

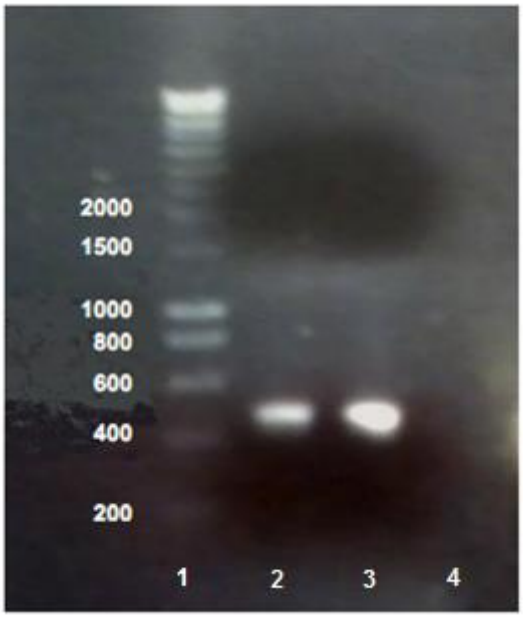


Figure 3.11 Gel electrophoresis of extracted and purified 475 bp bands from all PCR reactions (lanes 2 and 3) and the negative control pUC18 (lane 4).

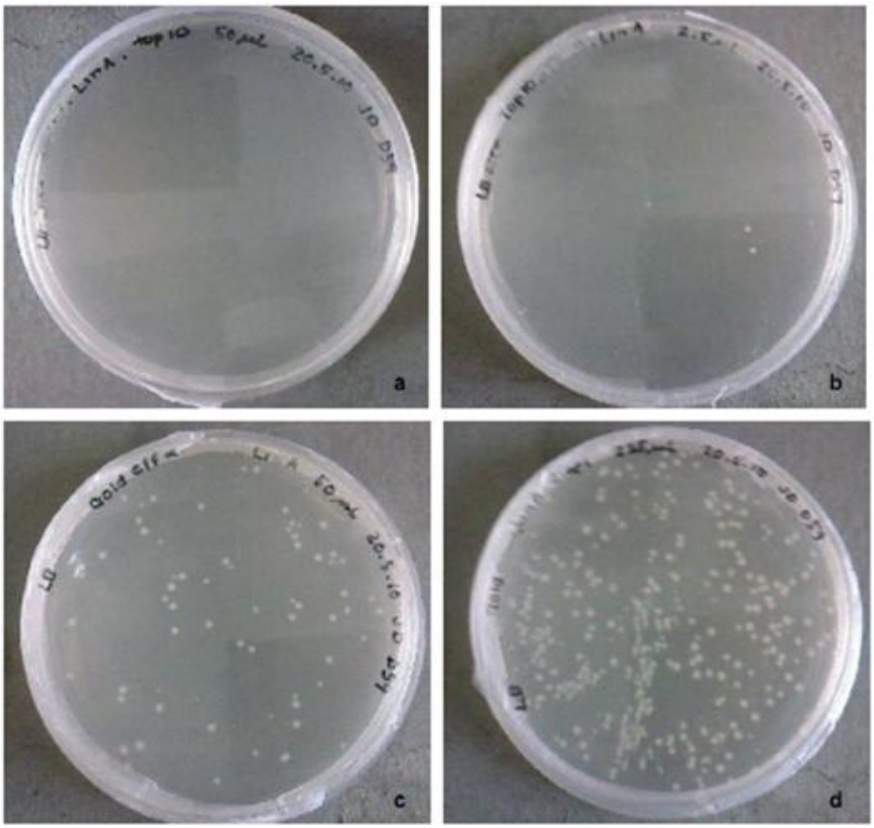
Sequencing of the purified PCR product was carried out and the results confirmed the identity of the *linA* gene sequence when compared against the expected CACC_LinA sequence with 98.8% homology (Appendix Figure A1).

3.3.e. DNA Cloning of linA

3.3.e.i. pENTR/D-TOPO Topoisomerase Reaction

Blunt-end cloning of the purified PCR products into the pENTR/D-Topo Gateway entry vector (Invitrogen) was carried out using 2:1 and 1:1 molar ratios of purified PCR product : TOPO vector. Transformations using the 1:1 molar ratio reaction product and α-select™ Gold Efficiency (Bioline) chemically competent cells resulted in many well-spaced colonies when 50 µl and 225 µl volumes were plated onto kanomycin/LB selective plates (Figure 3.12 c and d). When the same reaction was used to transform One Shot ® TOP10 (Invitrogen) cells, 0 and 3 colonies were present on the 50 µl and 225 µl plates, respectively (Figure 3.12 a and b). The 2:1 molar reaction did not result in any colonies using TOP10 cells or α-select cells at any plated volume (not shown). The theorised resulting plasmid construct, pENTR/D-TOPO_LinA, is shown in Figure 3.13.

Figure 3.12 Transformations of 1:1 molar ratio pENTR/D-TOPO Topoisomerase reaction using competent One Shot ® TOP10 (Invitrogen) (a and b) and α-select™ Gold Efficiency (Bioline) (c and d) at different plating volumes of 50 µl (a and c) and 225 µl (b and d).



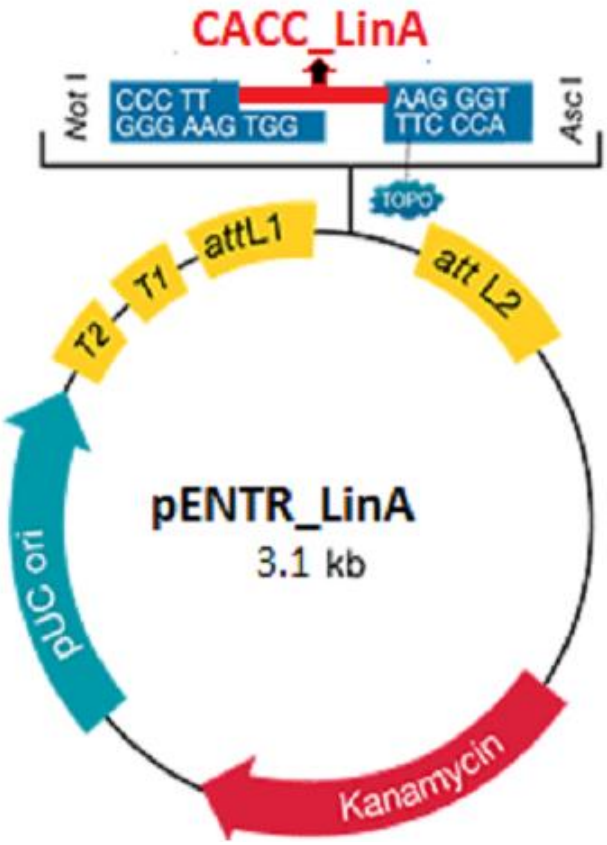


Figure 3.13 Theoretical and predicted construct, pENTR/D-Topo_LinA, resulting from the Invitrogen pENTR Directional TOPO Cloning reaction using the blunt-ended and purified PCR fragment.

Modified from Carr (2009)

Mini-preps were carried out on 2 positive colonies obtained from the 50 µl plate using α-select™ Gold Efficiency competent cells (Figure 3.12c). Insertion of the PCR fragment into the pENTR/D-TOPO vector was confirmed using PCR and a single restriction enzyme digest (Figure 3.14). PCR analysis using the For_LinA and Rev_LinA primers revealed bands at approximately 475 bp for both samples 1 and 2 in lanes 4 and 5, respectively. A single restriction digest of the predicted 3.1 kb pENTR/D-TOPO construct, using EcoRI would linearise the plasmid to give one distinct band. Sample 1 in lane 4 reveals a band at approximately 3.5 kb whereas Sample 2 in lane 7 shows a band at approximately 3.1 kb; the desired size. Sequencing was carried out confirming the presence of the *linA* gene on sample 2 which was used for subsequent cloning reactions (Appendix Figure A2).

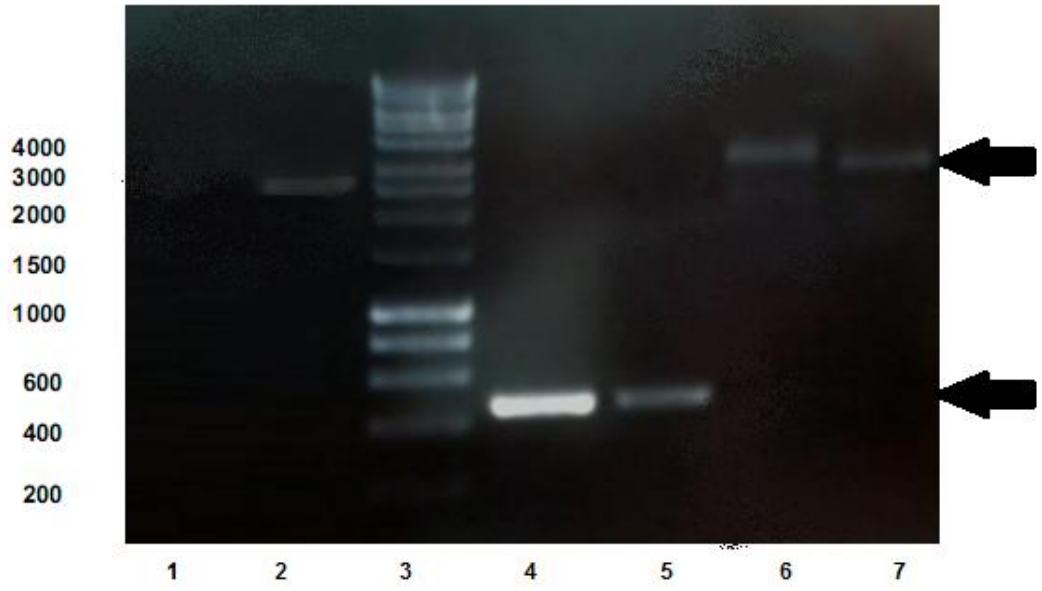


Figure 3.14 Analysis of *Escherichia coli* D-Topo_LinA transformants.
PCR from Samples 1 and 2 gave a desired band at approximately 475bp (lanes 4 and 5) whereas only Sample 2 gives the desired band length of 3.1kb after restriction digest (lane 7). Lanes 1 and 2 represent PCR and restriction digest, respectively, of the negative control, pUC18

3.3.e.ii. LR Clonase Reaction

Plasmid mini-prep stocks containing the entry clone D-Topo_LinA were used to shuttle the desired *linA* gene insert to the secondary destination plasmid; Ctapi. The Ctapi destination vector and pENTR/D-Topo_LinA plasmid concentrations were determined by biospectrophotometric DNA quantification to be 785 ng/µl and 41.6 ng/µl, respectively. The protocol requires:

- 50-150 ng/µl of pENTR/D-TOPO_LinA entry clone in a volume of 1-7 µl
- 150 ng/µl Ctapi destination vector in a volume of 1 µl

The Ctapi destination vector was diluted 5x to give 157 ng/µl and 3µl from the pENTR/D-Topo_LinA plasmid were used in the LR clonase reaction. The theorized and resulting plasmid construct, Ctapi_LinA, is shown in Figure 3.15.

α-select™ Gold Efficiency (Bioline) chemically competent cells were used for transformation and selection was carried out using spectinomycin-LB. Both plating volumes of 50 µl and 225 µl gave well-spaced colonies (not shown).

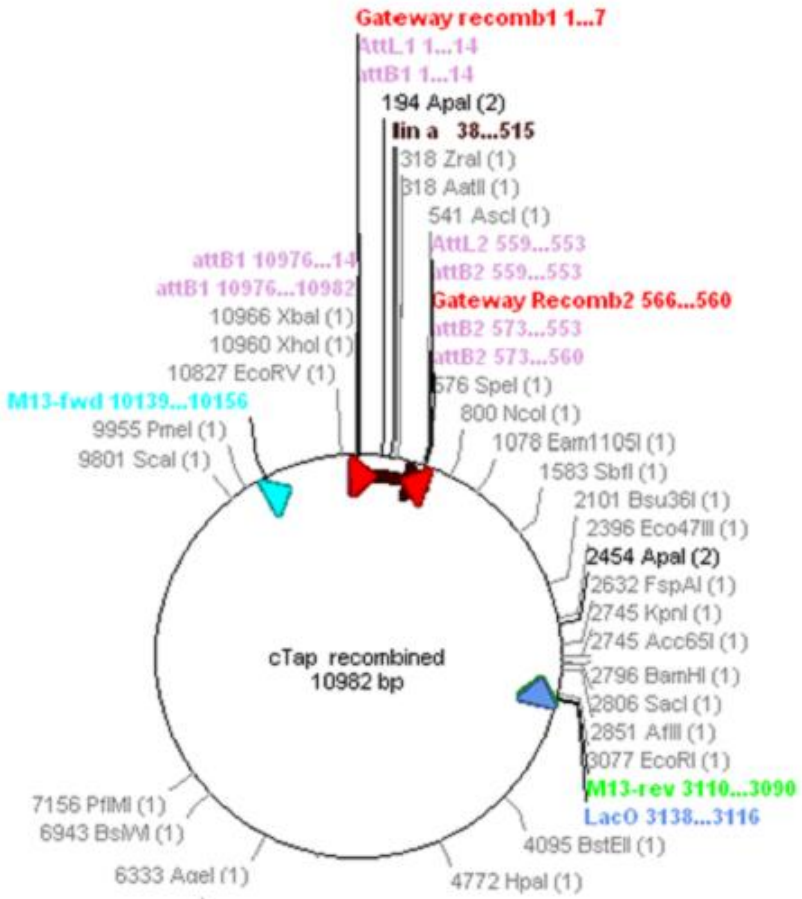


Figure 3.15 Theoretical and predicted vector construct, Ctapi_LinA

(created using ApE Software - A Plasmid Editor)
(http://www.biology.utah.edu/jorgensen/wayned/ape/)

Plasmid minipreparations were carried out on positive colonies and the shuffling of the *linA* PCR fragment from the pENTR/D-TOPO entry vector to the Ctapi destination vector was confirmed using PCR and a double restriction enzyme digest. PCR analysis using the For_LinA and Rev_LinA primers revealed bands at approximately 475 bp for both samples 1 and 2 in lanes 2 and 3, respectively (Figure 3.16). As the predicted construct is approximately 10.9 kb, a double restriction digest using EcoRI and EcoRV would linearize the plasmid to give 2 distinct bands at approximately 3.2 kb and 6.7 kb. Samples 1 and 2, in lanes 2 and 3, respectively, both show bands of the desired sizes suggesting the presence and correct orientation (reading frame) of the of the *linA* insert (Figure 3.17) which was confirmed by DNA sequence analysis using the For_LinA primer on Sample 1 (Appendix Figure A3).

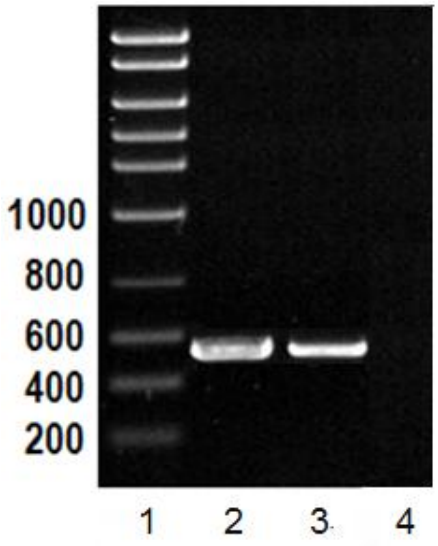


Figure 3.16 PCR analysis of *Escherichia coli* Ctapi_LinA transformants. Samples 1 and 2 gave a desired band at approximately 475 bp (lanes 2 and 3) compared to the negative control, pUC18 in lane 4.

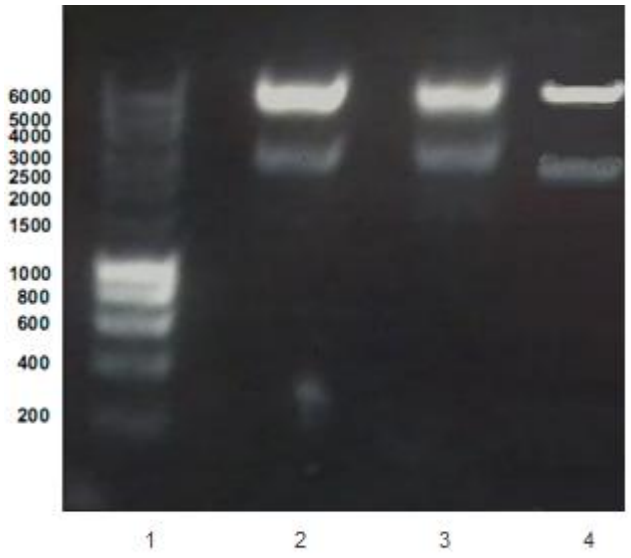


Figure 3.17 Double restriction digest analysis of *Escherichia coli* Ctapi_LinA transformants. Samples 1 and 2 give desired bands at approximately 3.2 kb and 6.7 kb (lanes 2 and 3) when compared to the negative control empty Ctapi plasmid in lane 4.

3.3.f. Electrotransformation of Agrobacterium

Ctapi_LinA minipreparation plasmids from sample 1 were used to transform *Agrobacterium*. 3 days after spreading 50 and 200 µL of the transformed cells onto low-salt LB agar plates containing kanomycin, spectinomycin, rifampicin and

gentamycin (KSRG) for selection, all plates showed growth (not shown). Ten colonies were selected to be transferred to 3 ml low-salt KSRG-LB broth to be cultured. After 2 days, all culture tubes showed growth. Plasmid mini-preps were prepared from 3 of the culture tubes and the presence of the *linA* gene was verified in all 3 samples by PCR (Figure 3.18).

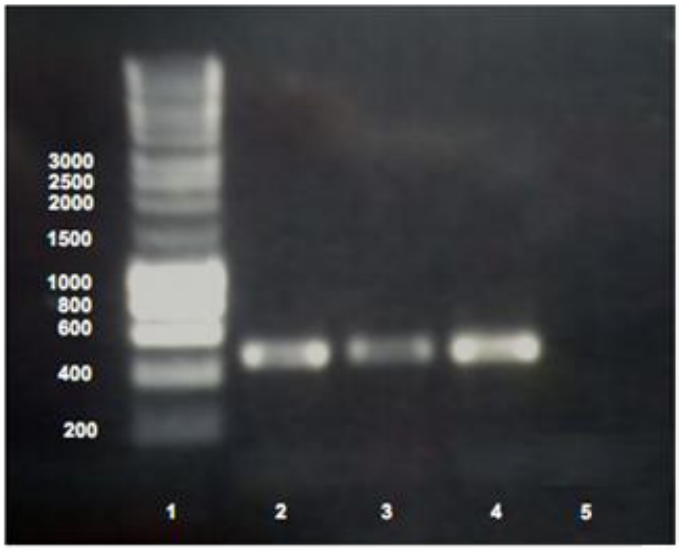


Figure 3.18 PCR analysis of *Agrobacterium* Ctapi_LinA transformants.
Samples 1, 2 and 3 give a desired band at approximately 475bp (lanes 2, 3 and 4) when compared to the negative control Ctapi plasmid in lane 5.

3.3.g. Transformation of A. thaliana by Floral Dipping

3.3.g.i. Germination and Growth of *A. thaliana* on Soil

Approximately 60 seedlings were sown on blank soil containing no lindane, which exhibited satisfactory growth at t = 7 dap. At t = 21 dap, plants had between 10 and 14 rosette leaves greater than 1 mm and by t = 49 dap, rosette growth was complete, plant had bolted and produced flowers with no further flower production taking place (Figure 3.19).

Figure 3.19 Growth of wild type *Arabidopsis thaliana* (col-0) on soil at t = 7, 21 and 49 dap (days after planting)

3.3.g.ii. Floral Dipping

After transformation, plants were maintained until seeds produced. 14 days after initial transformation (dat), seed pods become brown, then shattered, and whole plant senescence began. Plants continued to lose pigment, becoming brownish until senescence was complete.

3.3.g.iii. Harvesting of Seeds (T_0)

At 21 dat, seeds were harvested from each plant. A total volume of approximately 200 µl of seeds was collected from each plant (Figure 3.20).

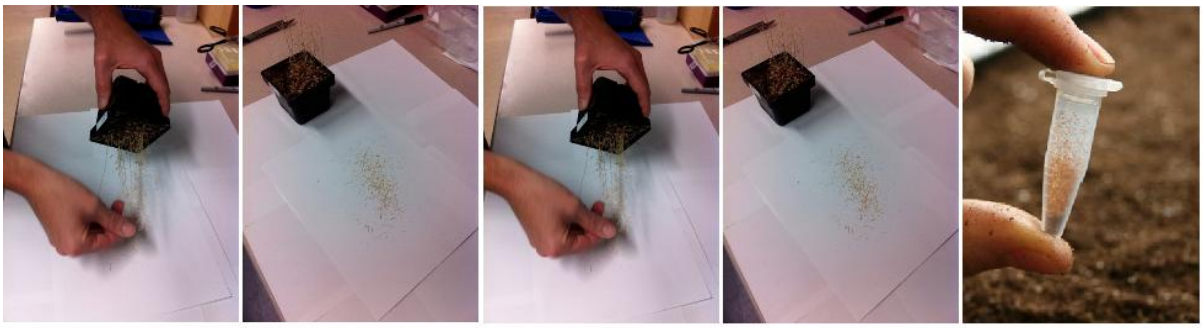


Figure 3.20 Harvesting of seeds

3.3.g.iv. Selection of Transformants (T_1, T_2 and T_3)

A transformation rate of approximately 15% is expected when using the Bent and Clough floral dipping method. In this experiment, by visual inspection, the transformation rate was slightly less at approximately 10% efficiency. As more than 100 seeds were spread on each dish in the first selection stage, it still resulted in at least 10 transformants per plate which were indicated by their bright green colour when compared to their non-transformed, brown, and non-germinating counterparts (Figure 3.21). After 10 days, and at the formation of secondary leaves, 2 transformants with secondary leaves from each plate were transferred to soil, where they were maintained until seeds produced.

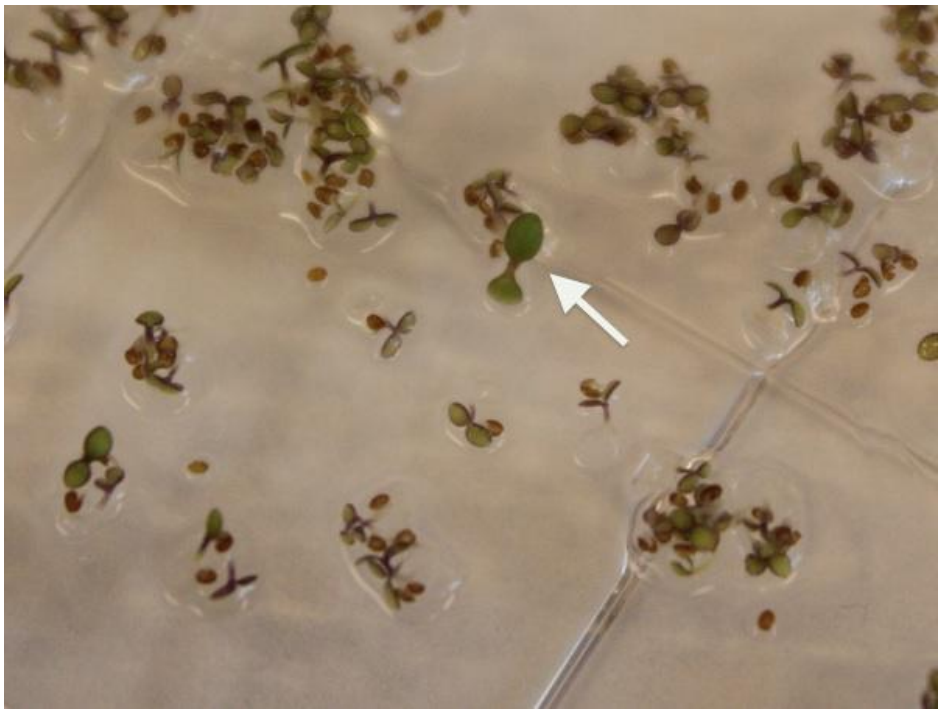


Figure 3.21 Selection of *Arabidopsis thaliana* transformants after *Agrobacterium*-mediated transformation using the floral dip method. Transformants indicated by green leaf colour and arrow.

3.3.h. DNA Extraction

PCR results of DNA extraction using the REDExtract-N-AMP™ Plant PCR kit indicated a band corresponding to *linA* at 475 bp in lanes 5-8 containing transformed samples designated LinA2(a), LinA2(b), LinA4(a) and LinA4(b). Lanes 3-4 (also containing suspected transformed samples and designated LinA1(a) and LinA1(b)) were negative for the *linA* gene. As expected, lane 2, which contained DNA from wild type *A. thaliana* (henceforth referred to as w/t), did not amplify any DNA (Figure 3.22).

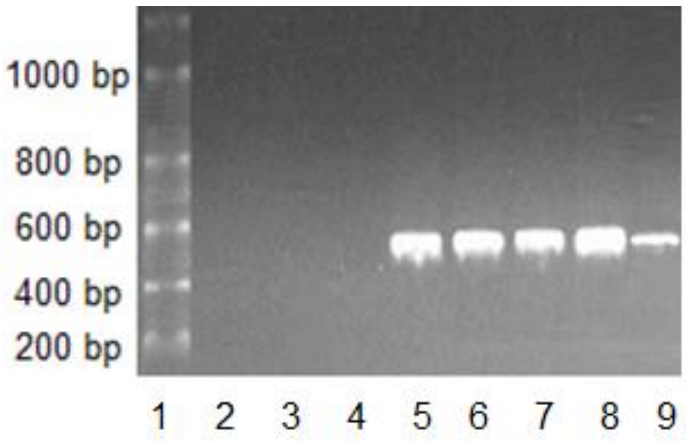


Figure 3.22 PCR results of DNA extraction using the REDExtract-N-AMP™ Plant PCR kit. Lanes 5-8, indicated a band corresponding to LinA at 475 bp. Lanes 3-4 were negative for the *linA* gene. Tthe wild type in lane 2 did not have a band at ~475 bp. Lane 9 represents the positive control, linA PCR product (from Figure 3.11).

Positive samples were confirmed as containing the *linA gene* by DNA sequencing. Negative samples were also sequenced to confirm the absence of the *linA* gene. Appendix Figures A4 and A5 show the results from samples LinA2(b) and LinA4(a) which were used for future experimentation and henceforth referred to as LinA2 and LinA4, respectively. LinA1(a) was used as a negative control, to show that the transformation process itself, had no bearing on the gene activity and is further referred to as LinA(-).

3.3.i. RNA Isolation

3.3.i.i. RNA Quantification

The upper aqueous phase containing RNA, from the TRIzol® Reagent (Ambion) reaction, was extracted and concentration was determined as shown in Table 3.2

Table 3.2 Biospectrophotometric determination of RNA concentration

	w/t	LinA2	LinA4	LinA(-)
RNA Concentration (µg/ml)	0.43	0.68	0.27	1.33
µl required to give 1 µg	2.33	1.47	3.70	0.75

3.3.i.ii. RT-PCR

The presence of mRNA and gene expression in the transgenic samples was indicated by a band at 475 bp in lanes 4 and 5, corresponding to LinA2 and LinA4 following RT-PCR (Figure 3.23).

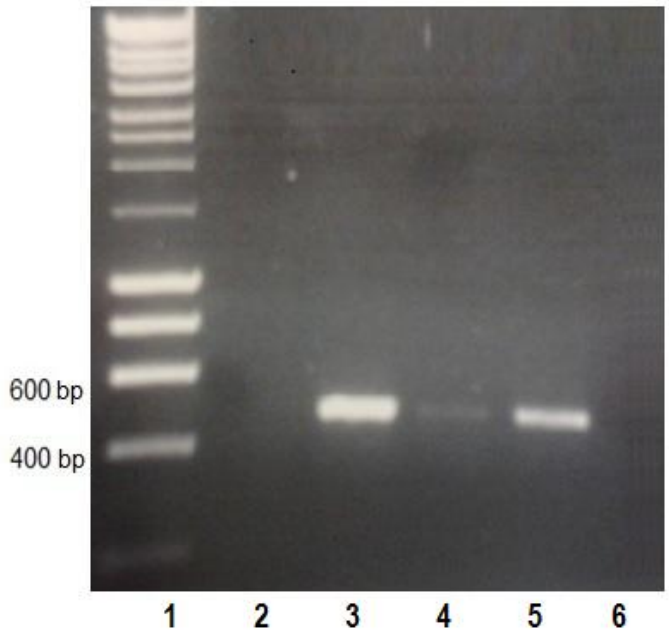


Figure 3.23 Gel electrophoresis of RT PCR. Lane 2 represents w/t *Arabidopsis thaliana*, lane 3 represents the *linA* PCR product used for cloning as a positive control, lanes 4 and 5 represent the transgenic lines LinA2 and LinA4, respectively and lane 6 is the negative line LinA(-)

3.3.j. Protein Analysis

Protein was extracted from each of the wild type, transgenic and negative control plant lines to determine the presence and activity of the LinA protein.

3.3.j.i. Protein Extraction

A standard curve for protein concentration using Bradford ULTRA Assay Kit (Westburg) was constructed using Bovine Serum Albumin (BSA). The measured absorbance for extracted proteins was compared against the standard curve to determine concentration values as follows in Figure 3.24 and Table 3.3.

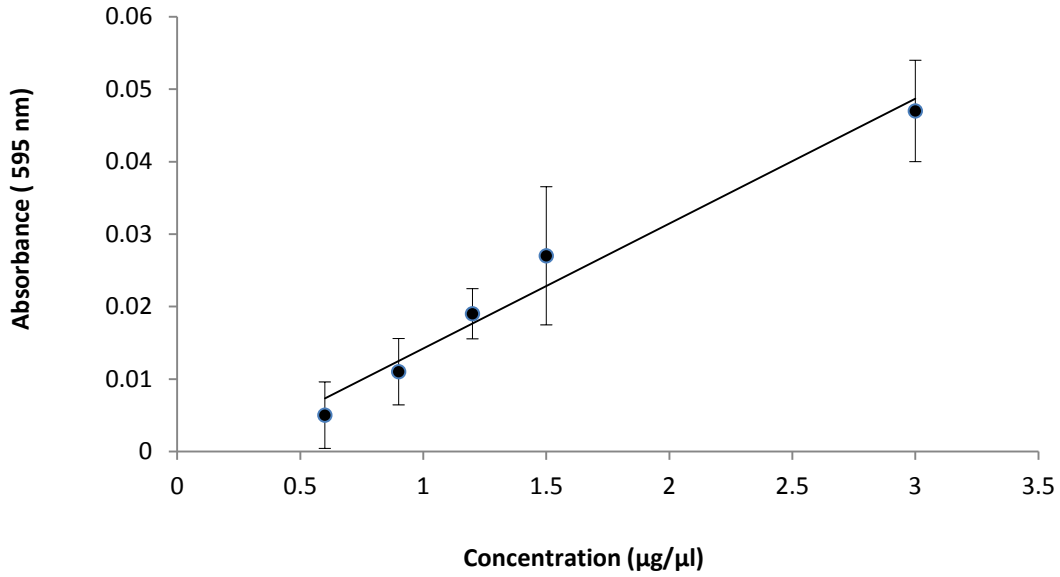


Figure 3.24 Standard curve for bovine serum albumin (BSA) using the Bradford ULTRA Assay Kit. Absorbance (595 nm) corrected for blank. Error bars represent standard deviation (n=3).

Table 3.3 Determination of extracted protein concentration using bovine serum albumin (BSA) standard curve (n=3).

	Plants grown in 0 µM lindane				**Plants grown in 30 µM lindane**			
	w/t	**LinA2**	**LinA4**	**linA(-)**	**w/t**	**LinA2**	**LinA4**	**linA(-)**
Absorbance (OD_{595})	0.005	0.009	0.008	0.020	0.013	0.022	0.009	0.030
Protein Concentration (µg/µl)	0.67	0.78	0.75	1.17	0.91	1.26	0.77	1.70
Standard Deviation	0.07	0.10	0.07	0.20	0.22	0.17	0.05	0.32

3.3.j.ii. Western Blotting

Proteins were separated using gel electrophoresis as seen in Figure 3.25.

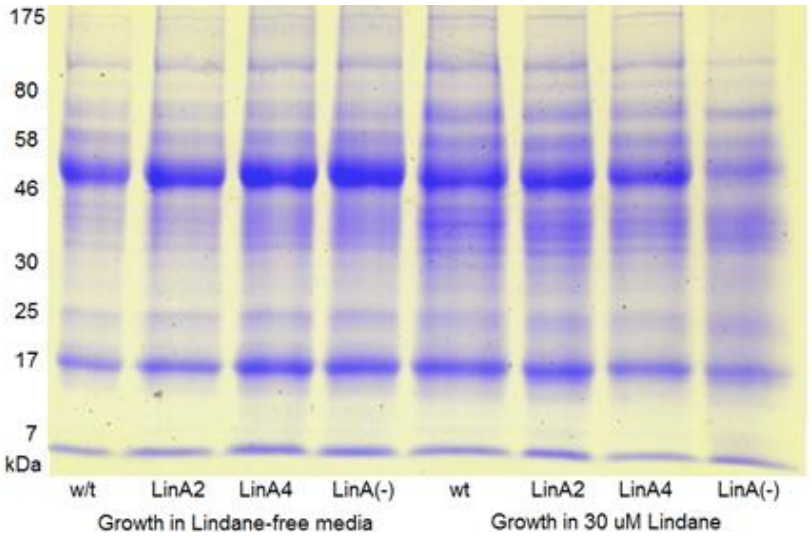


Figure 3.25 Protein separation using gel electrophoresis.

The antibody-probed proteins all gave unexpected banding at approximately 15 kDa and 55 kDa, likely due to non-specific binding with the small and large subunits of the highly abundant Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) protein. However, only the transgenic lines, LinA2 and LinA4, in lanes 3 and 4 respectively, have a band at 17 kDa, equal in size to the LinA protein (Figure 3.26).

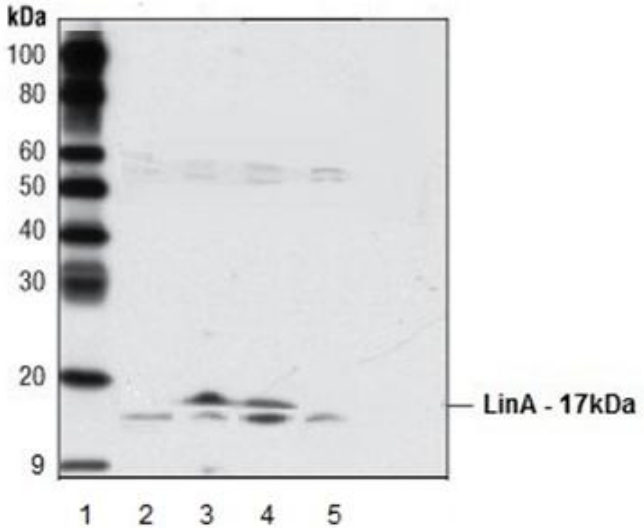


Figure 3.26 Western blot of extracted proteins using a LinA antibody. LinA2 and LinA4, in lanes 3 and 4 respectively, have a band at 17 kDa, equal in size to the LinA protein, whereas w/t and LinA(-), in lanes 2 and 5, respectively, do not.

Although not performed in this assay, the use of an *A. thaliana* specific loading control (or housekeeping gene) aside from RuBisCo, such as the phosphatase 2A coatomer subunits or the ubiquitin-conjugating enzyme, would have helped to normalize the levels of protein detected across the gel so as to aid in the interpretation of the Western blot result (Czechowski, *et al.,* 2005).

3.3.j.iii. Enzyme Activity Assay

Mercury thiocyanate, $Hg(SCN)_{2,}$ will form a complex with chloride ions (Cl^-) to absorb light at a 250-280 nm and accurately determine unknown Cl^- concentrations. A standard curve for Cl^- concentration using $Hg(SCN)_2$ was constructed using NaCl as a standard. The measured absorbance for extracted proteins incubated with 30 µM lindane was compared against the standard curve to determine the amount of Cl^- released and the specific activity of the LinA protein as follows in Figure 3.27 and Table 3.5. Sensitivity for the colorimetric method was tested from 0.2 ppm up to 14 ppm Cl^- based on the findings by Cirello-Egamino & Brindle (1995). The amount of Cl^- released prior to substrate incubation was also measured as a control to account for any spontaneous dissociation (Table 3.4). 1 unit of enzyme was defined as the activity required for the release of 1 µM Cl^- (0.03 ppm).

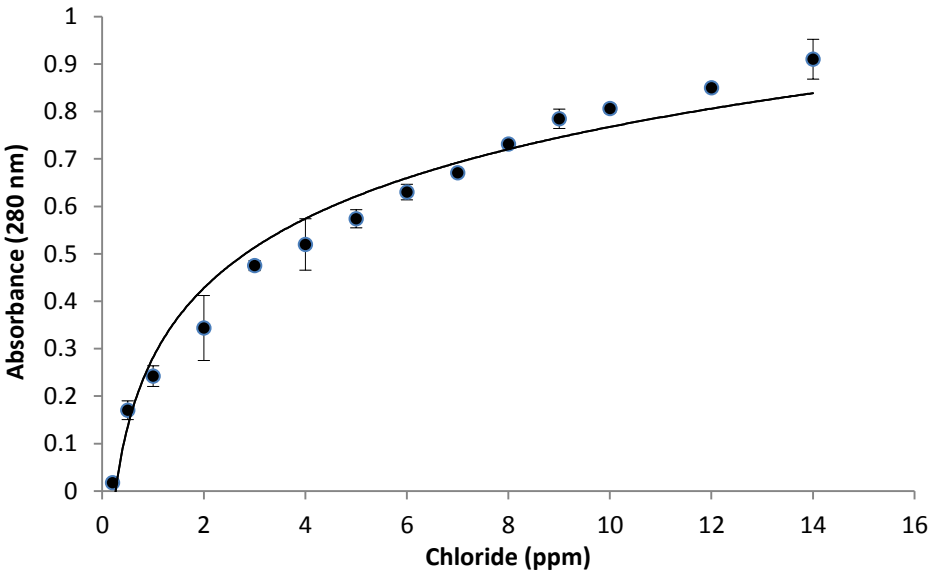


Figure 3.27 Colorimetric determination of chloride standard curve using mercuric thiocyanate. Absorbance (280 nm) corrected for blank. Error bars represent standard deviation (n=3).

Table 3.4 Specific activity of LinA protein prior to substrate incubation (n=3).

	Plants grown in 0 µM lindane				Plants grown in 30 µM lindane			
	w/t	**LinA2**	**LinA4**	**LinA(-)**	**w/t**	**LinA2**	**LinA4**	**LinA(-)**
Absorbance (OD_{260})	0.020	0.040	0.044	0.119	0.041	0.084	0.039	0.147
Cl^- released (ppm)	0.31	0.34	0.35	0.49	0.34	0.42	0.34	0.56
Total Activity (U)	8.76	9.60	9.76	13.78	9.67	11.78	9.58	15.69
Total Protein (µg)	33.33	38.77	37.26	58.60	45.52	62.88	38.60	85.11
Specific Activity (U/µg)	0.26	0.25	0.26	0.24	0.22	0.19	0.25	0.19
Standard Deviation	0.02	0.03	0.03	0.03	0.05	0.03	0.02	0.04

Table 3.5 Specific activity of LinA protein after 1 minute incubation with 30 µM lindane (n=3)

	Plants grown in 0 µM lindane				Plants grown in 30 µM lindane			
	w/t	**LinA2**	**LinA4**	**LinA(-)**	**w/t**	**LinA2**	**LinA4**	**LinA(-)**
Absorbance (OD_{260})	0.059	0.306	0.320	0.084	0.144	0.665	0.462	0.214
Cl^- released (ppm)	0.37	1.15	1.23	0.42	0.55	5.96	2.35	0.75
Total Activity (U)	10.46	32.46	34.69	11.78	15.48	168.20	66.44	21.29
Total Protein (µg)	33.33	38.77	37.26	58.59	45.52	62.88	38.60	85.11
Specific Activity (U/µg)	0.32	0.85	0.94	0.21	0.35	2.71	1.73	0.26
Standard Deviation	0.02	0.10	0.13	0.03	0.08	0.42	0.26	0.05

3.3.k. Growth of Arabidopsis thaliana Modified with linA

At t = 28 dap, growth was limited to the plates with 0 and 1 µM lindane for all non-transformed and transformed lines. However, initial growth could be seen for the plates at 34 µM, but did not continue after 14 dap (Figure 3.28).

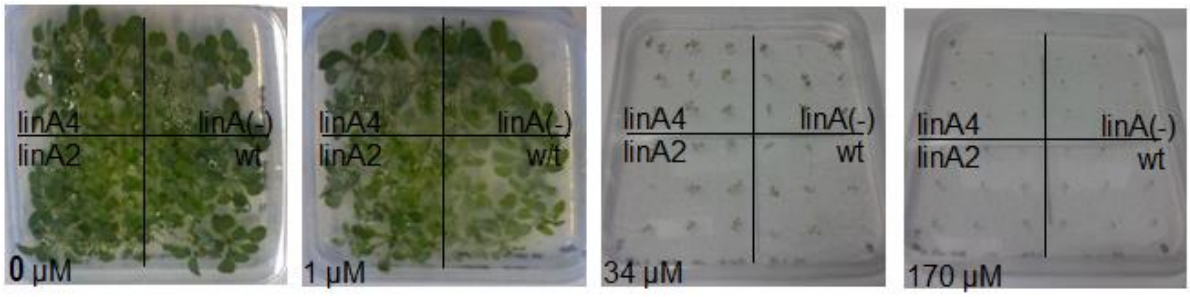


Figure 3.28 Growth comparison of wild type (w/t), transgenic (LinA2 and LinA4) and untransformed (linA(-)) lines on differing concentrations of lindane.

3.4. Discussion

Initial growth of w/t *A. thaliana* on lindane-contaminated media indicated the threshold toxicity to be at levels above 30 µM compared to 34 µM for a single species of bacteria and 174 µM for a bacterial consortium. The use of *A. thaliana* for the purpose of phytoremediation requires it to out-perform single species microbial growth and degradation as a minimum requirement. Although the bacterial lindane-degradation pathway in *S. japonicum* UT26 utilizes a number of different enzymes, one in particular, LinA, is completely unique in its structure and function showing no homology to any other known proteins. The first stable metabolic product resulting from the action of LinA is 1,2,4-TCB. Growth of wild type *A. thaliana* on 1,2,4-TCB-contaminated media revealed threshold toxicity to be at levels above 1.5 mM, considerably higher than wild type *A. thaliana* and *S. japonicum* UT26 in the presence of lindane. These findings support the hypothesis that single gene cloning of *linA* and its subsequent transformation into *A. thaliana* will result in a transgenic plant capable of enhanced lindane degradation based on the plant's natural xenobiotic degradation pathways.

Cloning of the *linA* gene using Gateway® Technology and *Agrobacterium*-mediated transformation into *A. thaliana* was successful in two lines, LinA2 and LinA4, as indicated after DNA extraction from the transformed plants, PCR of the extracted DNA using primers specific to the *linA* sequence and DNA sequence analysis when aligned against the known *linA* gene sequence. These lines were used for all subsequent experiments. The RT-PCR technique confirmed that the *linA* gene was active and being expressed in the genetically modified plants as indicated by its associated band in the agarose gel at the correct molecular length of 475 bp. Although RT-PCR can also be used to quantify exactly how active the gene is by comparing the unknown mRNA in the transgenic plants against standardized mRNA amounts, as seen in the study by De Lorenzo Prieto & Gonzalez Pastor (2007), enzyme activity was instead assessed directly by an enzymatic assay in this work.

Translated proteins, from the transgenic samples, were detected by Western Blotting using the LinA-antibody as a probe. As expected, the two confirmed transgenic lines

had bands at approximately 17 kDa, which correlates to that of the LinA protein. As the LinA enzyme is known to release Cl^- from the lindane molecule, enzymatic activity was colorimetrically determined after incubation with 30 µM lindane by measuring the amount of Cl^- released (Nagata, *et al*., 1993). The assay was performed on transgenic protein obtained from samples grown on both lindane-free and 30 µM lindane agar to determine if enzymatic activity was additive and increased in down-stream applications after growth on sub-threshold levels of lindane.

A one-way analysis of variance (ANOVA) was used to compare the specific enzyme activity means from each of the samples (significance level p = 0.05). When the means were found to differ from one another, a *post hoc* test was performed to determine which of group means were significantly different from one another. Although a number of *post hoc* tests exist, this work uses the Tukey *post hoc* test (significance level p = 0.05) as it was designed for use in situations with equal sample sizes per group and has been shown to accurately maintain alpha levels under model conditions assuming normality, homogeneity and independence (Stevens, 1999).

No statistically significant difference was found between the specific enzymatic activity of protein extracts not incubated with 30 µM lindane from Table 3.4, as determined by one-way ANOVA ($F(7,16) = 2.4$, $p = 0.07$). Their averaged means of 0.207 ± 0.083 U/µg, was therefore used to establish a baseline level of specific enzymatic activity. After incubation with 30 µM lindane, a statistically significant difference was found between the groups from Table 3.5 and the mean values from Table 3.4 ($F(8,23) = 98$, $p << 0.001$ No statistically significant difference was found between the non-transformed protein samples isolated from lindane-free and lindane-stress environments after incubation, and between the previously determined average baseline mean, suggesting that although there is an intrinsic ability of non-transformed *A. thaliana* to release Cl^-, it does not increase in the presence of lindane. The intrinsic ability of *A. thaliana* to release Cl^- was not further examined in this thesis.

The specific enzymatic activity was statistically significantly higher after incubation, as found in the transgenic lines LinA2 (0.846 ± 0.102 U/µg, $p << 0.001$) and LinA4 (0.939 ± 0.131 U/µg, $p << 0.001$), isolated from lindane-free environments, compared to all of the non-transformed lines and baseline mean (0.207 ± 0.083 U/µg). There were no statistically significant differences between the LinA2 and LinA4 means ($p = 0.38$). In the transgenic lines isolated from lindane stress environments, the tukey post-hoc test revealed that after incubation, the specific enzymatic activity was higher in LinA2 (2.713 ± 0.421 U/µg, $p << 0.001$) and LinA4 (1.732 ± 0.260 U/µg, $p << 0.001$) when compared to all of the non-transformed lines and baseline mean. Additionally, there were statistically significant differences between the LinA2 and LinA4 groups ($p = 0.026$), suggesting that the LinA2 line may have a better ability to metabolize organochlorines. The specific enzyme activity assay suggests that transformation with LinA does improve the ability of *A. thaliana* to release Cl^-, *in vitro.* However, larger sample sizes to determine the most effective transgenic line would warrant further investigation for future use in down-stream phytoremediation applications.

Regarding the additive effect of the LinA enzyme to release Cl^-, the specific enzymatic activity determined in protein samples harvested from plants grown on sub-threshold levels of lindane-stress was statistically significantly higher after incubation as found in the transgenic lines LinA2 (2.713 ± 0.421 U/µg, $p = 0.002$) and LinA4 (1.732 ± 0.260 U/µg, $p = 0.009$), when compared to their counterparts harvested from lindane-free environment, LinA2 (0.846 ± 0.102 U/µg) and LinA4 (0.939 ± 0.131 U/µg). These results suggest that initial growth in sub-threshold conditions of lindane may increase its subsequent metabolic capabilities.

Although not undertaken in this thesis, the literature states that crude protein extracts from *S. japonicum* UT26, subjected to the same colorimetric assay used in this thesis, were found to have a specific enzymatic activity of 37 U/mg (Nagata, *et al*., 1993). This is approximately 10^5-fold higher than that of *A. thaliana,* which may account for the lack of improved growth on lindane-contaminated media. As previously elucidated, determining the most effective transgenic line, and subsequently increasing the metabolic ability of the LinA protein through successive

generational growth on sub-threshold stress conditions, may help to increase the overall enzyme activity in transgenic plants for future use in down-stream phytoremediation applications *in situ.*

3.5. Conclusion

Despite the small sample size, initial findings from the DNA sequencing, RNA transcription, protein expression and the *in vitro* enzymatic assay supports the first aim of the hypothesis to develop a transgenic plant that should have superior growth when compared to the non-transformed lines in conditions of lindane-stress. However, this was not observed, *in vivo*. Phenotypically, *A. thaliana* transformed with the *linA* gene from *S. japonicum* UT26 appears to have no advantages with respect to growth when cultivated on lindane-contaminated agar and gives an identical growth pattern compared to the w/t and non-transformed lines, with threshold toxicity remaining below 34 µM. Regardless, the ability, even if limited, of the transgenic plant to express the LinA protein and exhibit enzymatic activity *in vitro* is evident from the preceding results.

The next chapter will further examine the ability of transgenic *A. thaliana* to remove lindane from its growth environment and will determine the extent of degradation, by measuring lindane and any known metabolic breakdown products accumulating in the media and / or plant using Gas Chromatography.

Chapter 4.

Gas Chromatography of Arabidopsis thaliana Modified with linA Grown under Conditions of Lindane-stress

4.1. Introduction

As the toxicity, distribution and persistence of lindane is well established, new bioremediation technologies, by which it can be safely and quickly removed from the environment, have been investigated. Bioremediation, which uses indigenous or foreign microorganisms, fungi, plants (phytoremediation) and their enzymes to biologically degrade organic waste to an innocuous state, or to levels below concentration limits is a low-cost, low-technology, and relatively low-disturbance alternative technique to traditional physical removal and chemical transformation methods (Vidali, 2001).

In studies where bacteria have been used for lindane decontamination in field situations, remediation capability was below that of laboratory studies and bioremediation occurred at relatively low concentrations, requiring both long-term inoculations and nutrient application (Raina, *et al*., 2008; Macek, *et al*., 2000; Eapen, *et al*., 2007). In phytoremediation, the literature states that the majority of plants lack the catabolic pathway for complete degradation and mineralization of these compounds when compared to microorganisms (Abilash, *et al*., 2009). Therefore, no such method for the clean-up of lindane on a global scale has been realized using natural microbial bioremediation and phytoremediation. As phytoremediation also offers other advantages including soil stabilization, carbon sequestration and biofuel production, the development of phytoremediation technologies is of significant interest. Improving plants for phytoremediation will likely result from transferring genes known to be involved in xenobiotic degradation from other plants, microbes and eukaryotes, specifically those that can be used to improve the uptake and degradation of lindane.

Chapter 3 saw the transformation and characterization of two transgenic *Arabidopsis thaliana* lines modified with *linA* from *Sphingobium japonicum* UT26. This work built upon preliminary results from a single proof of concept study whereby *A. thaliana* was modified with *linA* and demonstrated the ability to grow in the presence of normally toxic lindane concentrations and remove it from the medium in comparison to the wild type (w/t) model plant, which did not (De Lorenzo Prieto & Gonzalez

Pastor, 2007). Although the transgenic lines in this work did not display the same growth capabilities as those observed by De Lorenzo Prieto and Gonzalez Pastor (2007), specific enzymatic activity was observed in the presence of lindane (refer to Chapter 3.4. However, the ultimate fate and mass balance of lindane in the biological system has not yet been elucidated.

This chapter looks to investigate the removal, degradation and accumulation of lindane, 1,2,4-TCB and other potential intermediates from growth media and in *A. thaliana*, modified with *linA,* respectively. As this study is the first of its kind to date, model experiments were carried out *in vitro* using root tissue cultures grown in liquid culture and analysis was conducted by Gas Chromatography (GC). As lindane metabolized *in vitro* is subjected to the same enzymatic conditions as those in the parent plant, the principal purpose of using root tissue cultures in this study is to understand the enzymatic capacity of the LinA protein and any other associated reactions that operate in whole plants, and to minimize the initial expense and space requirements of greenhouse or field trials.

Although other methods of extraction and measurement, such as Solid Phase Microextraction (SPME) and Liquid Chromatography (LC), have been employed for lindane analysis, for the purpose of this research, which was not to develop or optimize these methodologies, the simplest, most common and most effective method, GC, was used. This also allowed a comparison and expansion of the study by De Lorenzo Prieto and Gonzalez Pastor (2007) to further develop a mechanistic understanding of the transgenic *A. thaliana* lindane-degradation pathway. Known concentrations of lindane were compared against experimental samples using GC analysis and the relative amounts remaining in the growth mediium, and within the plant tissues themselves, were quantified after 21 days, to reveal the extent of phytoremediation occurring in the wild type and transgenic plants.

4.2. Methods

4.2.a. Plant Tissue Culture

Approximately 2 x 100 µl *A. thaliana* w/t, LinA2, LinA4 and LinA(-) seeds (refer to Chapter 3.3.k) were placed in individual 1.5 ml Eppendorf tubes (i.e. 2 tubes per cell line). 1 ml of 70% Ethanol (Sigma) was added to each of the reaction tubes and gently inverted for 1 minute. 800 µl Reverse Osmosis (RO) purified water and 200 µl Economy Bleach (Ottimo Supplies) were added to the reaction tubes followed by 0.5 µl Tween-20 Ultrapure (Sigma) to give a final concentration of 0.05%. The mixture was gently inverted for 10 minutes. Seeds were then spun down in a bench-top centrifuge on short spin until it reached 5000 rpm. The liquid was removed from the tube and washed with 1µl RO water and repeated for a total of 5 washes. After the final wash, the liquid was removed and 300 µl of RO water was added to each of the tubes, which were covered in foil, placed in the fridge at 4 °C and left for 4 days to stratify or break seed dormancy.

4.2.a.i. BASTA Selection

After 4 days, the contents of each reaction tube were equally transferred to 2 - 15 ml falcon tube and made up to 4 ml with RO water (i.e. 4 tubes per cell line). 8 ml of hot 0.7% - ½ MS Plant Agar (Duchefa) was added to each tube. The agar and seeds were quickly mixed and poured onto 0.8% - ½ MS Plant Agar selective plates containing the antibiotic BASTA (20 µg/ml) (Sigma Aldrich). The seed mixture was evenly spread by gently swirling the plates. The selective plates were then sealed with micropore tape and placed in a growth cabinet at 22 °C with 16 hours of light and 8 hours of dark for 14 days (i.e. 4 plates per cell line).

4.2.a.ii. Root Tissue Culture

For each of the cell lines, 21 x 15 resistant transformants from the BASTA selective plates were transferred to 250 ml flasks containing 65 ml autoclave-sterilized Gamborg B5 Medium (Sigma) and 2% Glucose (w/v) (Sigma Aldrich). Root tissues

were washed prior to transfer in the PESTANAL® analytical standard grade fungicide, Benomyl (Fluka), to prevent fungal contamination. The Benomyl wash was prepared by dissolving it in 50 ml CG-MS grade acetonitrile (Fischer) to a final concentration of 0.02% (w/v) at 25 ºC. Flasks were maintained at room temperature on a reciprocating platform set at 60 strokes per minute. After 7 days, lindane was added to the flasks in predetermined concentrations of 0, 4, 34, 68, 102, 136 and 170 µM. All experimental conditions were performed in triplicate.

4.2.b. Liquid-Liquid Extraction of Lindane

4.2.b.i. Liquid-Liquid Extraction of Lindane from the Growth Medium

At T = 0 days after the addition of lindane, 5 mL of the Gamborg B5 liquid growth medium were subjected to liquid-liquid extraction to separate the lindane out. Prior to extraction, aldrin (Sigma Aldrich) was added to the 5 mL mixture as an internal standard, allowing a correction factor to account for the loss of any analyte during sample extraction.

The mixture was added to a separating funnel, along with 5 ml of the solvent Chloroform (Fischer). The mixture was then homogenized by inverting 5 times and the pressure was released from the funnel. The heavier organic fraction, containing the lindane, was collected from the funnel. The remaining liquid was washed a further 2 times with 5 ml of Chloroform, following the same procedure to extract any remaining lindane. The organic fraction was then left to evaporate in a fume cupboard and the remaining lindane was resuspended in 1 mL GC-MS grade acetonitrile (Fischer). Liquid-liquid extraction was also carried out on all samples at t= 7, 14 and 21 days, after the addition of lindane. Samples were stored at -20 °C until GC analysis.

4.2.b.ii. Liquid- Liquid Extraction of Lindane from Plant Tissue

At t = 21 days after the addition of lindane, the plant root tissue cultures were removed from the Gamborg B5 Medium, rinsed in RO water and dried to remove any

residual lindane. After drying, 5 ml of RO water was added to each sample which was then ground to a pulp using a glass micropestle and mortar before being subjected to liquid-liquid extraction to separate out the lindane. Prior to extraction, aldrin (Sigma Aldrich) was added to the 5 mL mixture as an internal standard, allowing a correction factor to account for the loss of any analyte during sample extraction. The mixture was added to a separating funnel, along with 5 ml of the solvent Chloroform (Fischer). The mixture was then mixed by inverting 5 times and the pressure was released from the funnel. The heavier organic fraction, containing the lindane, was collected from the funnel. The remaining liquid was washed a further 2 times with 5 ml of Chloroform, following the same procedure to extract any remaining lindane. The organic fraction was then left to evaporate in a fume cupboard and the remaining residue was resuspended in 1 mL GC-MS grade acetonitrile (Fischer). Samples were stored at -20 °C until GC analysis.

4.2.c. Gas Chromatography of Lindane Extracts

A TRACE GC Ultra Gas equipped with a splitless injector and a flame ionization detector (FID) (Thermo Fisher Scientific Inc.) was used in this study. Separation of samples was carried on a fused silica Rtx®-CLPesticides Column (proprietary Crossbond® phases) (30 m × 0.25 mm x 0.25 µm) (Restik) capable of achieving baseline resolution for more than 20 organo-chorinated analytes and providing reliable identification without the use of an MS detector (Restek, 2008). The injection volume was set at 10 µl and the carrier gas (Nitrogen) at a flow rate of 2 mL per minute. The oven temperature was programmed at 150 °C for 2 minutes with an equilibrium time of 2 minutes, followed by a rise of 5 °C per minute up to 220 °C (held for 0 minutes) which was then increased to a rise of 30 °C per minute to reach a final temperature of 300 °C (held for 0 minutes). The injector and detector port temperatures were maintained at 280 and 320 °C, respectively. Standard dilutions of lindane and 1,2,4-TCB were prepared to establish the robustness and sensitivity of GC as a method of quantification. The chromatograms from extracted medium and tissue samples were compared against the standard curves to determine the overall loss and accumulation of Lindane and its metabolite, 1,2,4-TCB after 21 days and to evaluate the effectiveness of the transgenic plant lines to remediate Lindane.

4.3. Results

4.3.a. Suitability of Gas Chromatography for the Separation, Detection and Quantification of Lindane and 1,2,4-TCB

4.3.a.i. Gas Chromatography of Acetonitrile

A triplicate of blank samples containing only the solvent GC-MS grade acetonitrile (Fischer) was used to determine the time for the solvent to peak and to establish a baseline chromatogram (Figure 4.1, Table 4.1).

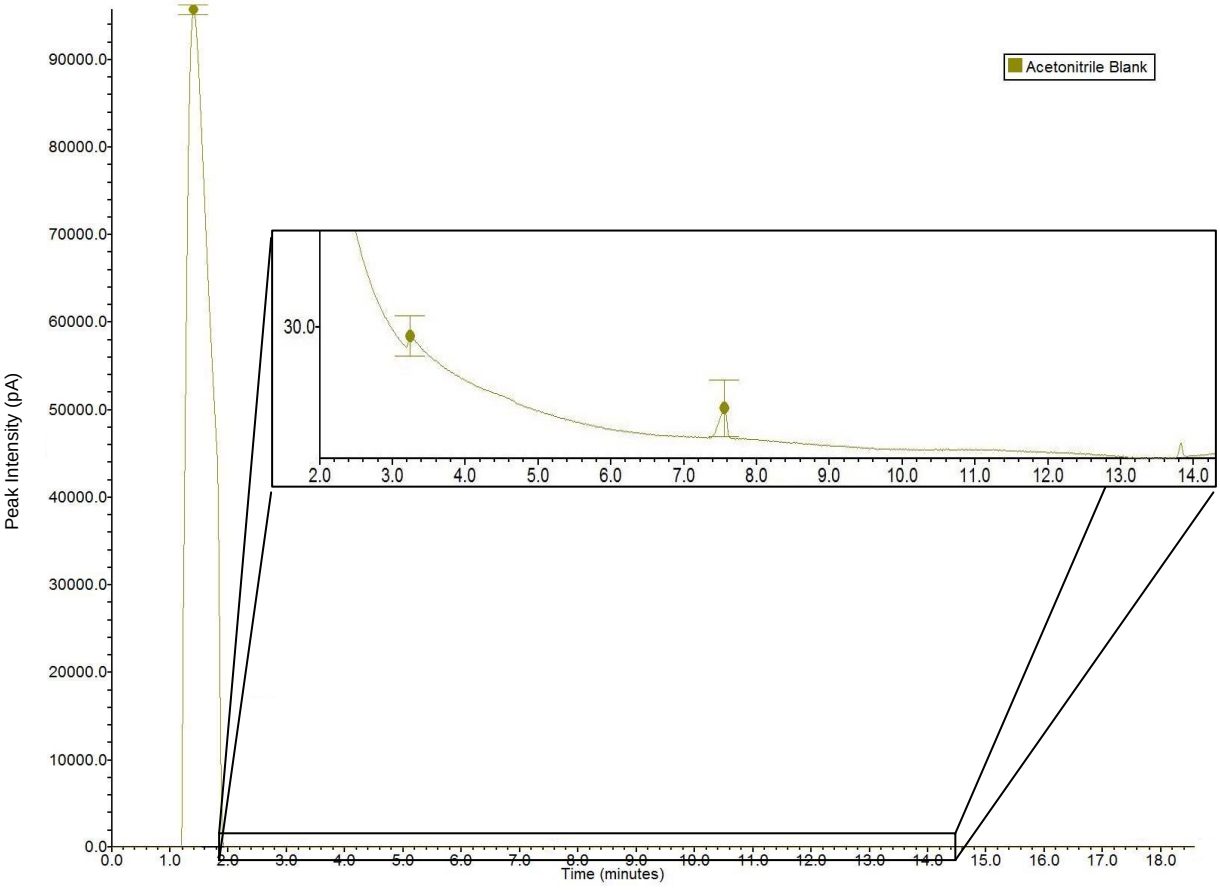


Figure 4.1 Chromatogram of the solvent, acetonitrile.
Error bars represent standard deviation (n=3).

Table 4.1 Chromatogram peak values and associated areas of the solvent, acetonitrile. Bracketed number represents standard deviation (n=3).

Peak Time (minutes)	Peak Mean (pA)	Area Mean (nonsensical units)
1.40	95686.52 (593.57)	2660949.25 (13256.88)
3.24	3.52 (0.59)	251.59 (63.08)
7.55	1.45 (0.81)	3.98 (2.30)

4.3.a.ii. Gas Chromatography of Lindane

A triplicate serial dilution series of lindane (dissolved in GC-MS grade acetonitrile) ranging from 10^4 mg/L down to 10^{-1} mg/L was used to determine the sensitivity of GC without the use of MS as a method for the detection of lindane (Figure 4.2). As the previous chromatogram of acetonitrile (Figure 4.1) established a base line with a large peak at T = 1.40 minutes, it is expected that the calculated area (nonsensical units) under this peak should remain relatively constant throughout the serial dilution series. On the contrary, with each serial dilution of lindane, a peak exhibiting a similar fold reduction in area would be indicative of the analyte.

The serial dilution series chromatogram of lindane gave three distinct peaks at T = 1.40 (not shown), 5.80 (not shown) and 11.75 minutes (Figure 4.2). As expected, the peak distinctive of acetonitrile at T = 1.40 minutes remained constant, while the other peaks had corresponding fold reductions with respect to the serial dilutions. As the lindane being analyzed was only 97% pure, it is not unusual to have more than one peak during compound separation. However, the peak at T = 5.80 minutes was only visible on the chromatograms of the 10^3 and 10^4 mg/L concentrations and the corresponding areas were only calculated to be 42.74 (SD 13.73) and 428.75 (SD 38.72), respectively (not shown). These findings, along with an observable peak and corresponding areas for the 1 to 10^4 mg/L concentrations at T = 11.75 minutes, suggest that this peak is a more reliable indicator of lindane, especially at lower concentrations (Table 4.2).

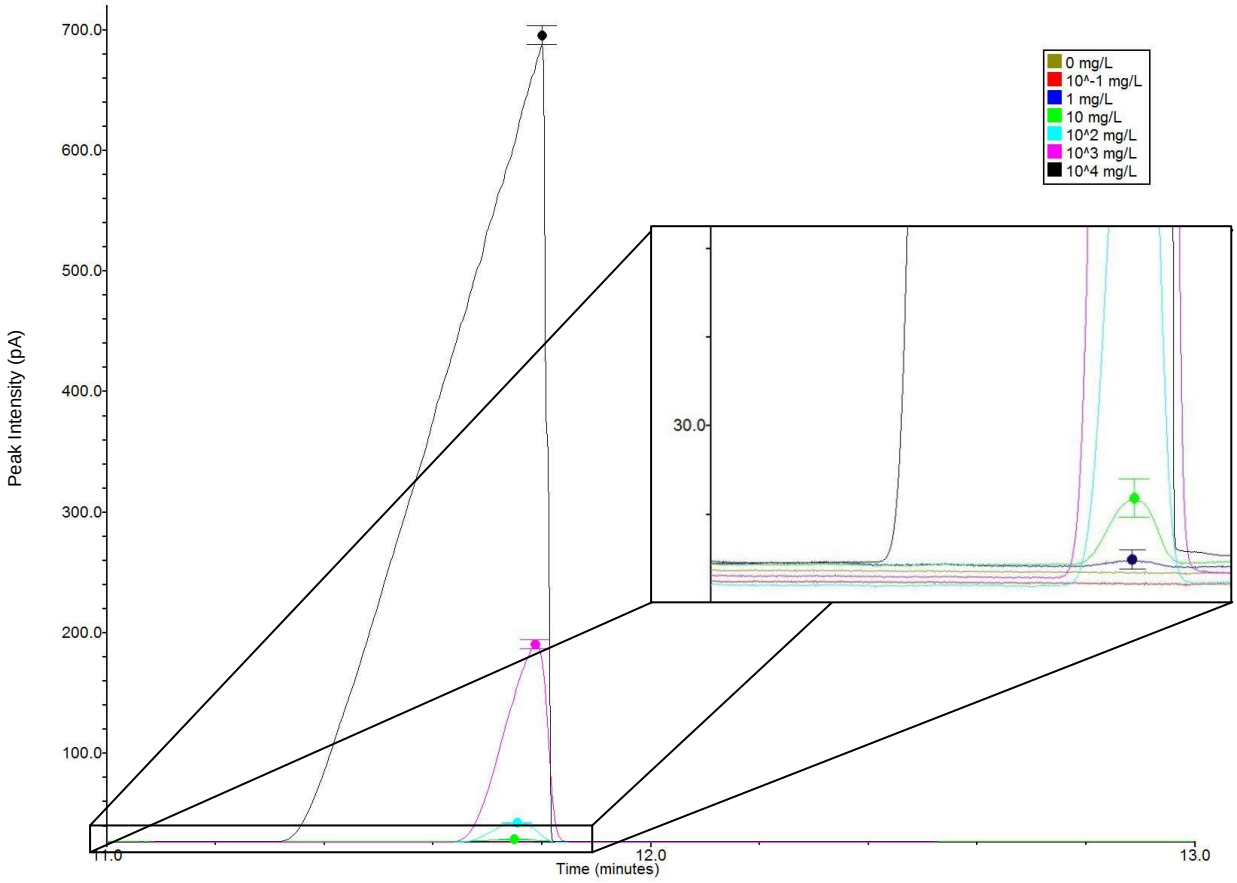


Figure 4.2 Serial dilution series chromatogram of lindane.
Error bars represent standard deviation (n=3).

Table 4.2 Area comparison between T = 1.40 minutes (acetonitrile) and 11.75 minutes (lindane). Bracketed number represents standard deviation (n=3).

Lindane Concentration (mg/L)	**Area under Peak at T = 1.40 minutes**	**Area under Peak at T = 11.75 minutes**
0	2660921.50 (13256.88)	0.00 (0.00)
10^{-1}	2660949.25 (13256.88)	0.00 (0.00)
1	2701387.25 (55218.52)	0.82 (0.21)
10	2672017.50 (14783.31)	7.03 (1.81)
10^{2}	2661492.25 (26163.14)	87.15 (3.60)
10^{3}	2708379.00 (44505.87)	875.49 (21.74)
10^{4}	2664334.50 (58974.45)	8944.22 (171.77)

4.3.a.iii. Gas Chromatography of 1,2,4-TCB

A triplicate serial dilution series of the metabolite; 1,2,4-TCB (dissolved in GC-MS grade acetonitrile), ranging from 10^4 mg/L down to 10^{-1} mg/L; was used to determine the sensitivity of GC without the use of MS as a method for the detection of 1,2,4-TCB (Figure 4.3). As the previous chromatogram of acetonitrile (Figure 4.1) established a base line with a large peak at T = 1.40 minutes, it is expected that the calculated area under this peak should remain relatively constant throughout the serial dilution series. On the contrary, with each serial dilution of 1,2,4-TCB, a peak exhibiting a similar fold reduction in area would be indicative of the analyte.

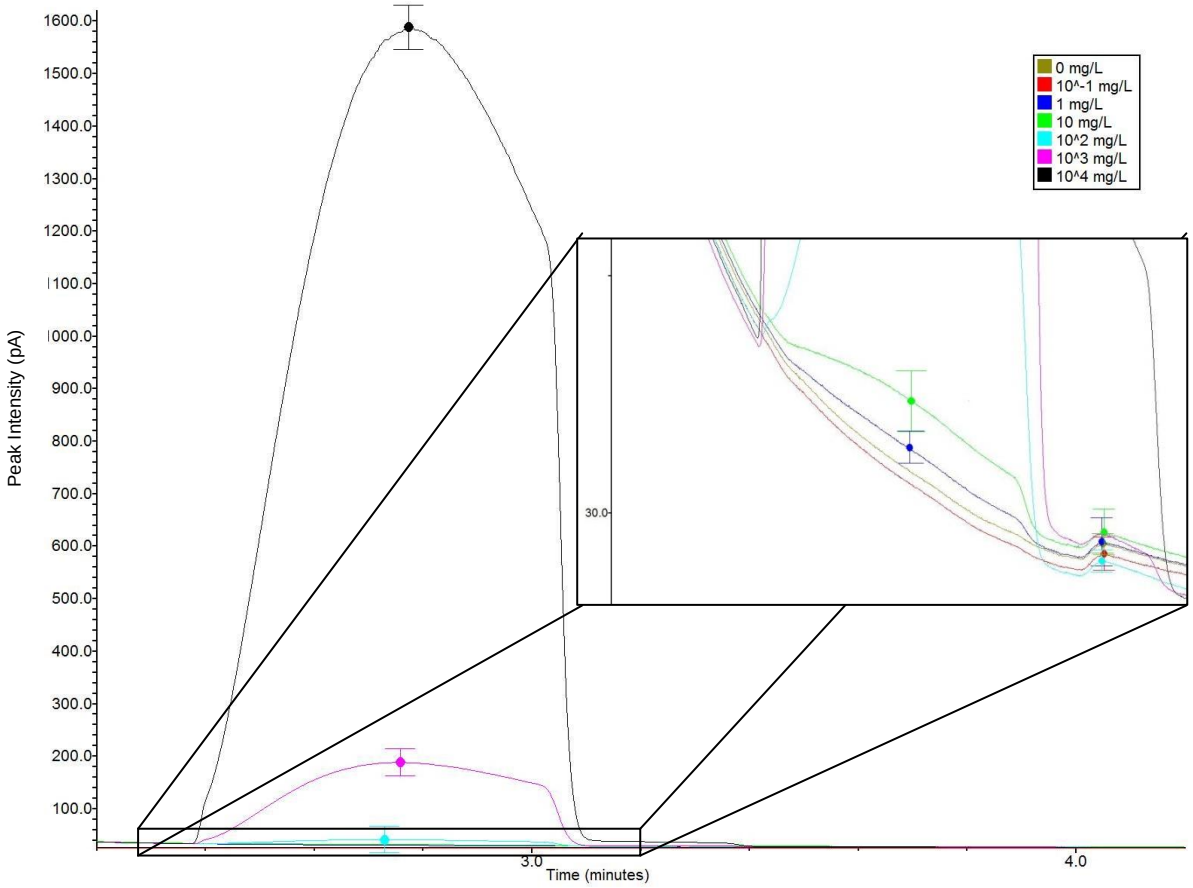


Figure 4.3 Serial dilution series chromatogram of 1,2,4-trichlorobenzene (TCB). Error bars represent standard deviation (n=3).

The serial dilution series chromatogram of 1,2,4-TCB gave two distinct peaks at T = 1.40 and 2.75 minutes. As expected, the peak at T = 1.40 minutes remained constant, while the other peak was visible and had corresponding fold reductions from 10^4 down to 1 mg/L concentrations indicative of the analyte 1,2,4-TCB (Table 4.3). A smaller, third peak at T = 3.08 minutes was also seen. As 1,2,4-TCB is only ≥ 99% pure, this is not unexpected. However, the peak intensity and corresponding areas remained relatively constant and were deemed unsuitable for as an indicator for 1,2,4-TCB.

Table 4.3 Area comparison between T = 1.40 minutes (acetonitrile) and 2.75 minutes (1,2,4-trichlorobenzene (TCB)). Bracketed number represents standard deviation (n=3).

1,2,4-TCB Concentration (mg/L)	Area under Peak at T = 1.40 minutes	Area under Peak at T = 2.75 minutes
0	2661682.25 (33315.60)	0.00 (0.00)
10^{-1}	2678164.25 (29748.24)	0.00 (0.00)
1	2678164.25 (30318.54)	5.01 (2.05)
10	2661107.25 (39745.84)	48.62 (3.63)
10^2	2668749.25 (29217.35)	540.11 (19.63)
10^3	2669726.25 (23513.35)	5026.18 (32.72)
10^4	2672467.75 (48137.70)	45960.99 (945.34)

4.3.a.iv. Separation of Lindane, 1,2,4-TCB and Aldrin

To account for any loss of analyte during the liquid-liquid extraction process, the organochlorine, aldrin, was added to each sample at a concentration of 10^2 mg/L prior to extraction as an internal standard. Aldrin is well established as an effective internal standard for many organo-chlorinated pesticides, including lindane, in GC analysis (Lehotay, *et al.*, 1995). Additionally, resolution of aldrin is possible with the Rtx®-CLPesticides Column used in this work (Restik, 2008). The chromatograms and data in Appendix Figure A6 and Table A1 show that at 10^2 mg/L, aldrin has a peak at T = 14.37 minutes and a calculated area of 301.60 under the peak. Figure 4.4 shows the separation capability of the Rtx®-CLPesticides Column for lindane, its metabolite 1,2,4-TCB, and the internal standard aldrin, to give 3 distinct peaks. Following extraction, the area of aldrin as calculated from under the peak at T =

14.37 minutes, is compared against the known area of 301.60 and a correction factor can then be applied to all peak data across the chromatogram.

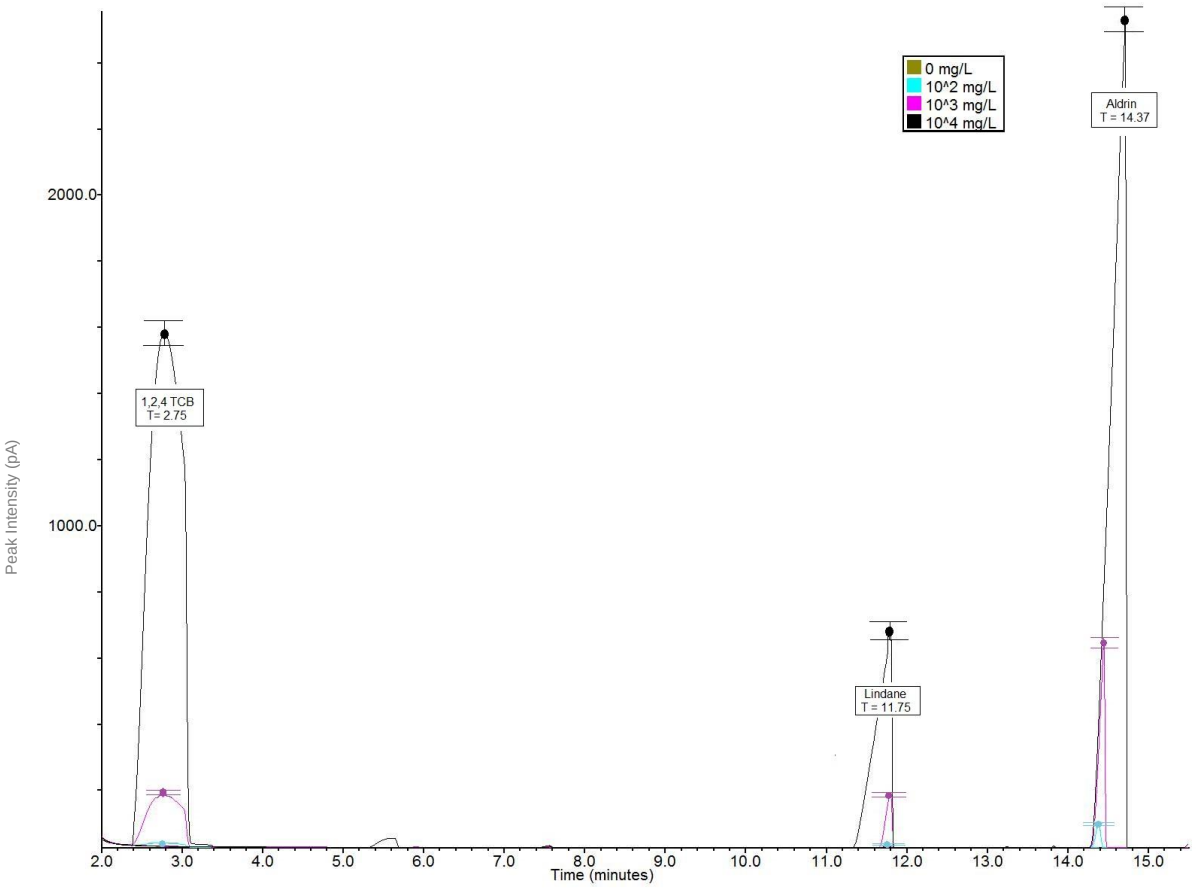


Figure 4.4 Separation of lindane, 1,2,4-trichlorobenzene (TCB) and the internal standard, aldrin. Error bars represent standard deviation (n=3).

4.3.b. Gas Chromatography of Liquid-Liquid Extracted Residues

The calculated area for extracted residues as determined by their chromatograms was compared against the standard curves to assess the effectiveness of the transgenic plant lines to remediate lindane by accounting for its overall loss or accumulation of its metabolite, 1,2,4-TCB after 21 days. Plant tissue was not analysed at T=0 as the experimental set-up was limited to a finite amount of starting material in order to reduce the possibility of contamination and to allow optimal growth. Lindane concentration in plant tissue at T=0 was therefore assumed to be 0 µM.

4.3.b.i. Standard Curves of Lindane and 1,2,4-TCB

Standard curves for both lindane and 1,2,4-TCB were constructed (Figure 4.5 and Figure 4.6) using experimental design concentrations (Appendix Figure A7, Figure A8 and Table A2). The limit of detection (LOD) was calculated using the formula,

$$LOD = 3.3 * SD_{(standard\ curve)} / Slope_{(standard\ curve)}$$

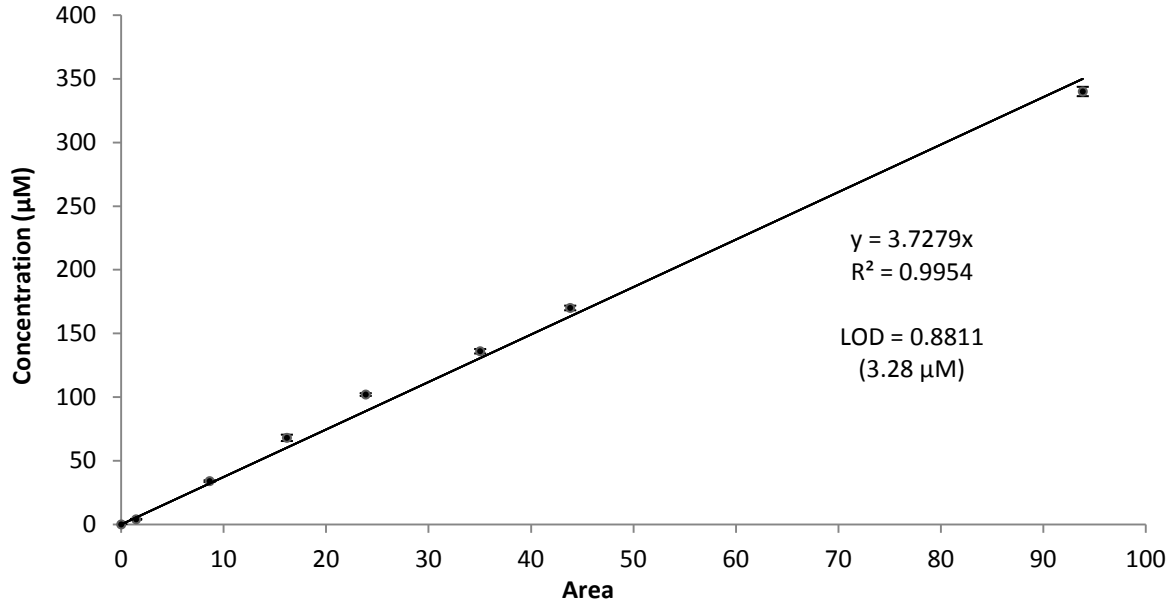


Figure 4.5 Standard curve of lindane using experimental concentrations (prior to extraction). Error bars represent standard deviation (n=3).

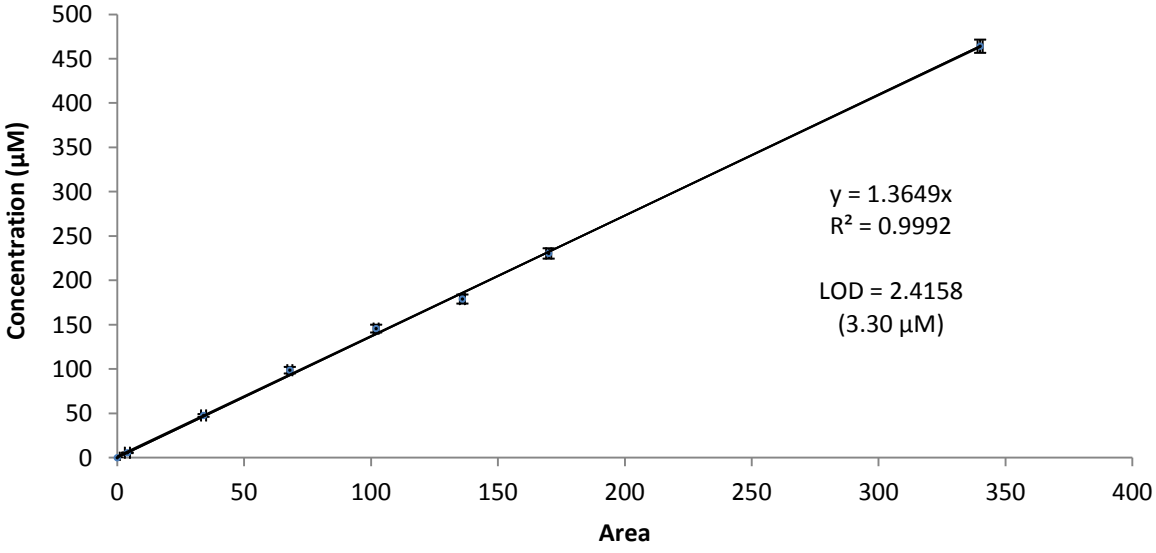


Figure 4.6 Standard curve of 1,2,4-trichlorobenzene (TCB) using experimental concentrations (prior to extraction). Error bars represent standard deviation (n=3).

4.3.b.ii. GC of *A. thaliana* w/t Medium and Root Tissue

The extracted organic fractions from *A. thaliana* w/t at T = 0 (medium) (Figure 4.7) and 21 days (medium and ground root tissues) (Figure 4.8 and Figure 4.9) were analyzed by GC and compared against the standard curve (Table 4.4).

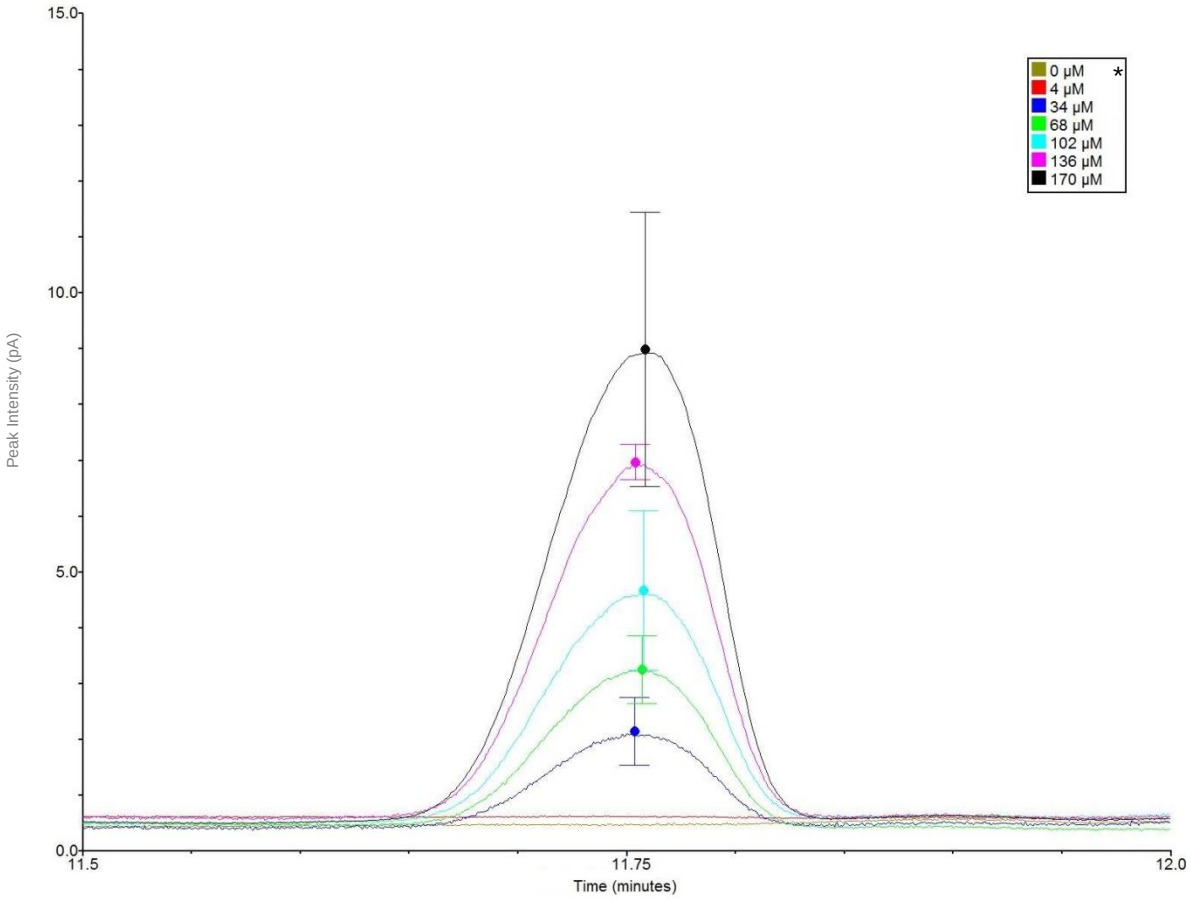


Figure 4.7 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* w/t medium at T= 0 days after the addition of lindane. Error bars represent standard deviation (n=3).
* Concentration represents starting concentration at T=0 days (prior to extraction)

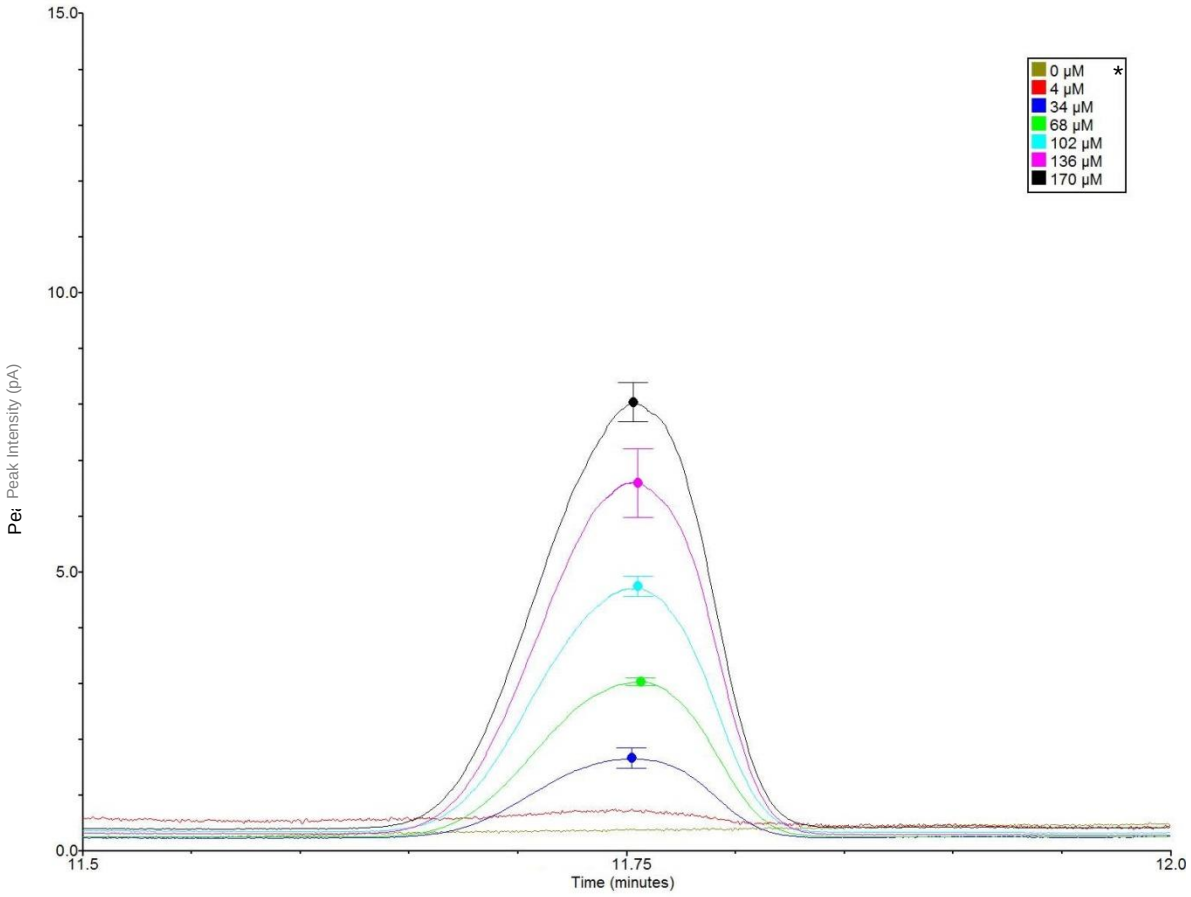


Figure 4.8 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* w/t medium at T= 21 days after the addition of lindane. Error bars represent standard deviation (n=3).
* Concentration represents starting concentration at T=0 days (prior to extraction)

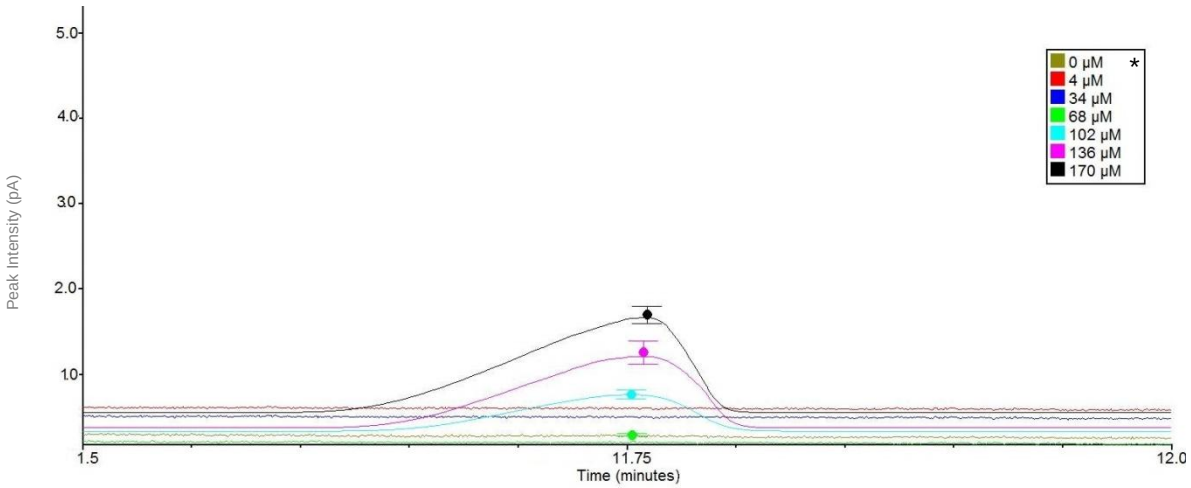


Figure 4.9 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* w/t root tissue at T= 21 days after the addition of lindane. Error bars represent standard deviation (n=3).
* Concentration represents starting concentration at T=0 days (prior to extraction)

Table 4.4 Concentration of *Arabidopsis thaliana* w/t samples (after extraction) as calculated using the standard curves. Bracketed number represents standard deviation (n=3).

Molarity prior to extraction (µM)	Area Under the Curve			Molarity After Extraction (µM)		
	Media		Plant	Media		Plant
	T= 0	T= 21	T=21	T= 0	T= 21	T=21
0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
4	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
34	7.10 (1.58)	7.70 (0.35)	0.00 (0.00)	26.38 (5.87)	28.61 (1.30)	0.00 (0.00)
68	15.87 (1.24)	14.81 (0.16)	0.00 (0.02)	58.94 (4.61)	55.02 (0.59)	0.00 (0.07)
102	25.40 (4.10)	23.50 (0.32)	2.33 (0.15)	94.38 (15.23)	87.30 (1.19)	8.66 (0.56)
136	36.51 (0.66)	36.00 (1.89)	4.61 (0.33)	135.65 (2.45)	133.74 (7.02)	17.13 (1.23)
170	44.22 (5.51)	43.32 (0.85)	6.70 (0.28)	164.28 (20.47)	160.94 (3.16)	24.89 (1.04)

4.3.b.iii. Mass Balance of Lindane in the *A. thaliana* w/t System

To determine if there was any loss of lindane in the system after 21 days, the total number of moles of solute was calculated from the 10 µl injection volumes for each sample using the following equation,

$$\text{Number moles of solute} = \text{Molarity} \times \text{Volume}$$

The total number of moles at 21 days was compared against the initial number of moles in the system at T = 0 days (Table 4.5).

Table 4.5 Mass balance of lindane after T = 21 days in the *Arabidopsis thaliana* w/t system. Bracketed number represents standard deviation (n=3).

Molarity Prior to Extraction (µM)	Number of moles of solute (pmol)			Total number of moles of solute at T = 21 (pmols)	Gain / Loss (-) (%)
	Media		Plant		
	T= 0	T= 21	T=21		
0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	n/a
4	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	n/a
34	0.26 (0.06)	0.29 (0.01)	0.00 (0.00)	0.29 (0.01)	8.42
68	0.59 (0.05)	0.55 (0.01)	0.00 (0.00)	0.55 (0.01)	-6.65
102	0.94 (0.15)	0.87 (0.12)	0.09 (0.01)	0.96 (0.12)	1.67
136	1.35 (0.02)	1.34 (0.07)	0.17 (0.01)	1.51 (0.07)	11.22
170	1.64 (0.20)	1.61 (0.03)	0.25 (0.01)	1.86 (0.03)	13.11

4.3.b.iv. GC of Transgenic *A. thaliana* LinA2 Medium and Root Tissue

The extracted organic fractions from transgenic *A. thaliana* LinA2 at T = 0 (medium) (Figure 4.10) and 21 days (medium and ground root tissues) (Figure 4.11 and Figure 4.12) were analyzed by GC and compared against the standard curve (Table 4.6).

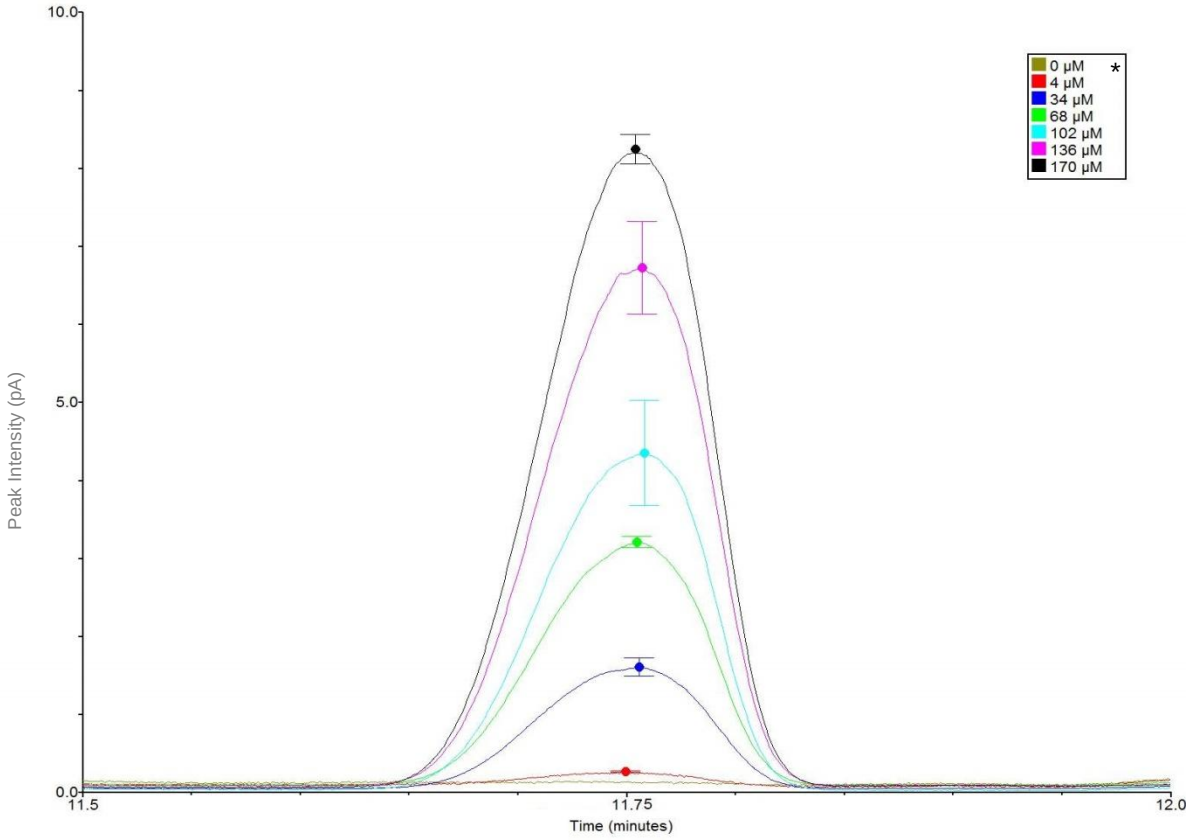


Figure 4.10 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* LinA2 medium at T= 0 days after the addition of lindane. Error bars represent standard deviation (n=3). * Concentration represents starting concentration at T=0 days (prior to extraction)

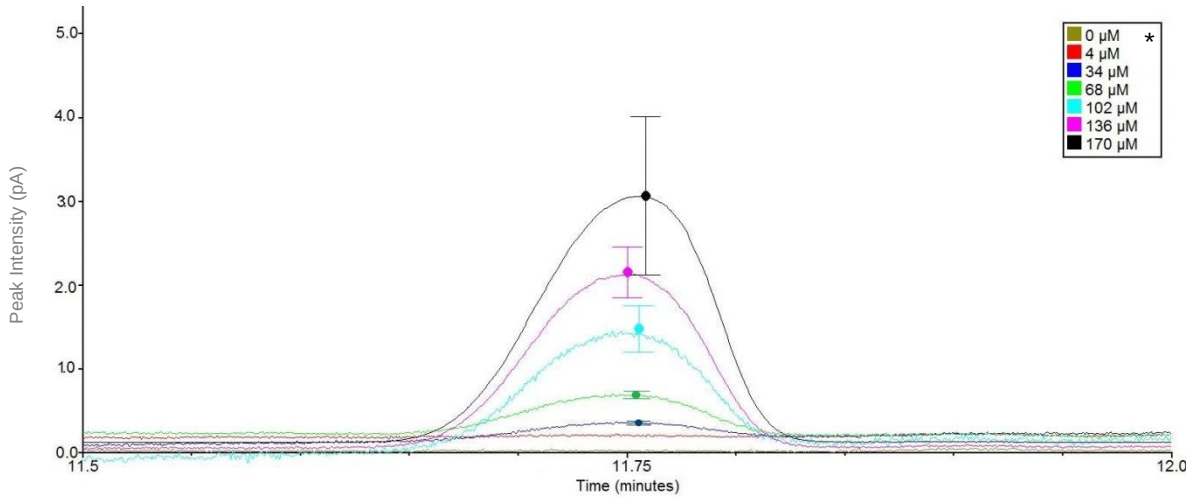


Figure 4.11 Chromatogram of lquid-liquid extracted residues from *Arabidopsis thaliana* LinaA(2) medium at T = 21 days after the addition of lindane. Error bars represent standard deviation (n=3). * Concentration represents starting concentration at T=0 days (prior to extraction)

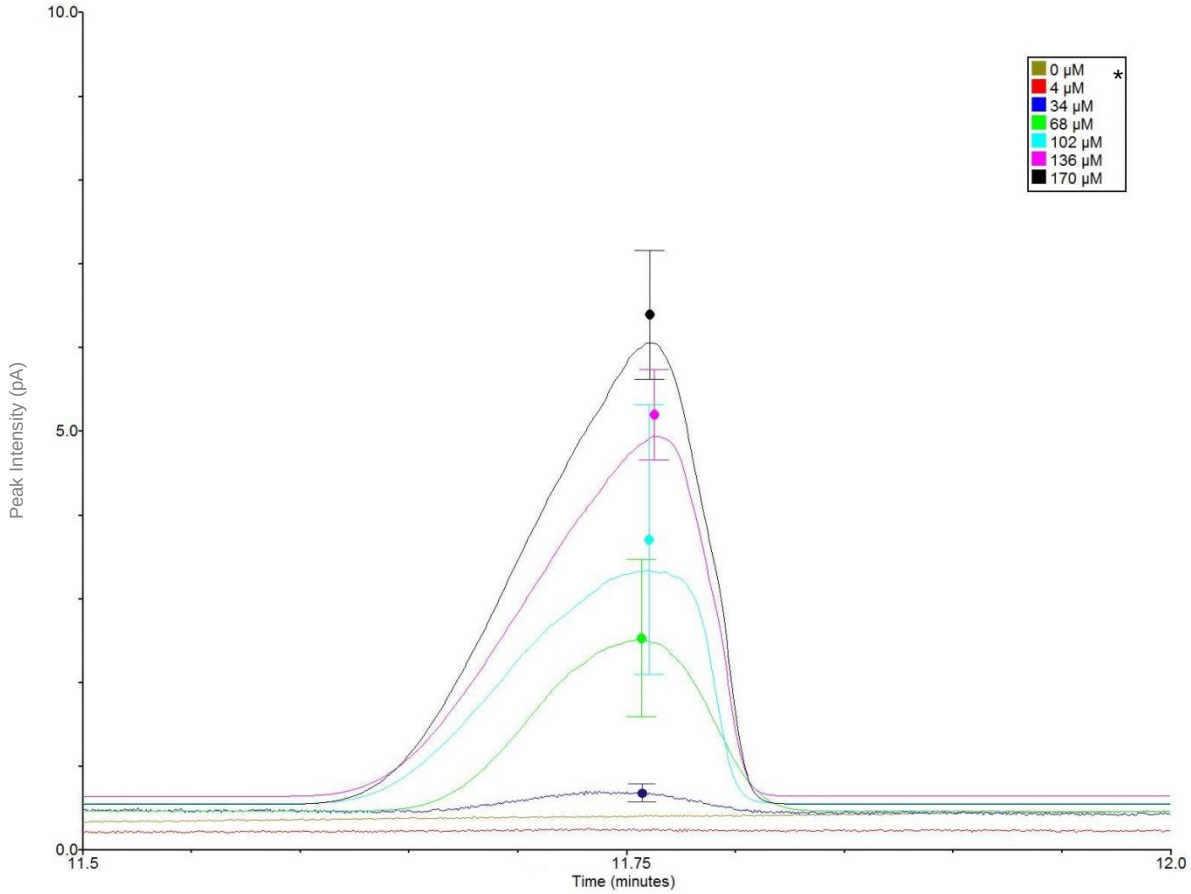


Figure 4.12 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* LinA2 root tissue at T= 21 days after the addition of lindane. Error bars represent standard deviation (n=3). * Concentration represents starting concentration at T=0 days (prior to extraction)

Table 4.6 Concentration of *Arabidopsis thaliana* LinA2 Samples (after extraction) as calculated using the standard curves. Bracketed number represents standard deviation (n=3).

Molarity Prior to Extraction (µM)	Area Under the Curve			Molarity After Extraction (µM)		
	Media		Plant	Media		Plant
	T= 0	T= 21	T=21	T= 0	T= 21	T=21
0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
4	1.52 (0.01)	0.00 (0.00)	0.00 (0.00)	5.66 (0.04)	0.00 (0.00)	0.00 (0,00)
34	8.42 (0.25)	1.50 (0.01)	1.56 (0.25)	31.30 (0.93)	5.57 (0.04)	5.83 (0.93)
68	16.78 (0.15)	4.06 (0.15)	12.34 (2.15)	62.34 (0.56)	15.08 (0.56)	45.84 (7.99)
102	25.31 (1.94)	8.64 (0.80)	17.07 (4.23)	94.02 (7.21)	32.10 (2.97)	63.42 (15.71)
136	35.70 (1.68)	12.78 (0.82)	24.43 (1.75)	132.64 (6.24)	47.48 (3.05)	90.76 (6.50)
170	44.10 (0.49)	15.72 (2.01)	31.10 (1.98)	163.84 (1.82)	58.40 (7.47)	115.54 (7.36)

4.3.b.v. Mass Balance of Lindane in the *A. thaliana* LinA2 System

To determine if there was any loss of lindane in the system after 21 days, the total number of moles of solute was calculated from the 10 µl injection volumes for each sample using the following equation,

$$\text{Number moles of solute} = \text{Molarity} \times \text{Volume}$$

The total number of moles at 21 days was compared against the initial number of moles in the system at T = 0 days (Table 4.7).

Table 4.7 Mass balance of lindane after T = 21 days in the transgenic *Arabidopsis thaliana* LinA2 system. Bracketed number represents standard deviation (n=3).

Molarity Prior to Extraction (µM)	Number of moles of solute (pmol)			Total number of moles of solute at T = 21 (pmols)	Gain / Loss (-) (%)
	Media		Plant		
	T= 0	T= 21	T=21		
0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	n/a
4	0.06 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	-100.00
34	0.31 (0.01)	0.06 (0.00)	0.06 (0.00)	0.12 (0.00)	-63.68
68	0.62 (0.01)	0.15 (0.01)	0.46 (0.02)	0.61 (0.02)	-2.26
102	0.94 (0.07)	0.32 (0.03)	0.63 (0.11)	0.95 (0.11)	1.59
136	1.33 (0.06)	0.48 (0.03)	0.91 (0.11)	1.39 (0.12)	4.22
170	1.64 (0.02)	0.58 (0.07)	1.16 (0.28)	1.74 (0.29)	6.17

4.3.b.vi. GC of Transgenic *A. thaliana* LinA4 Media and Root Tissue

The extracted organic fractions from transgenic *A. thaliana* LinA4 at T = 0 (media) (Figure 4.13) and 21 days (medium and ground root tissues) (Figure 4.14 and Figure 4.15) were analyzed by GC and compared against the standard curve (Table 4.8).

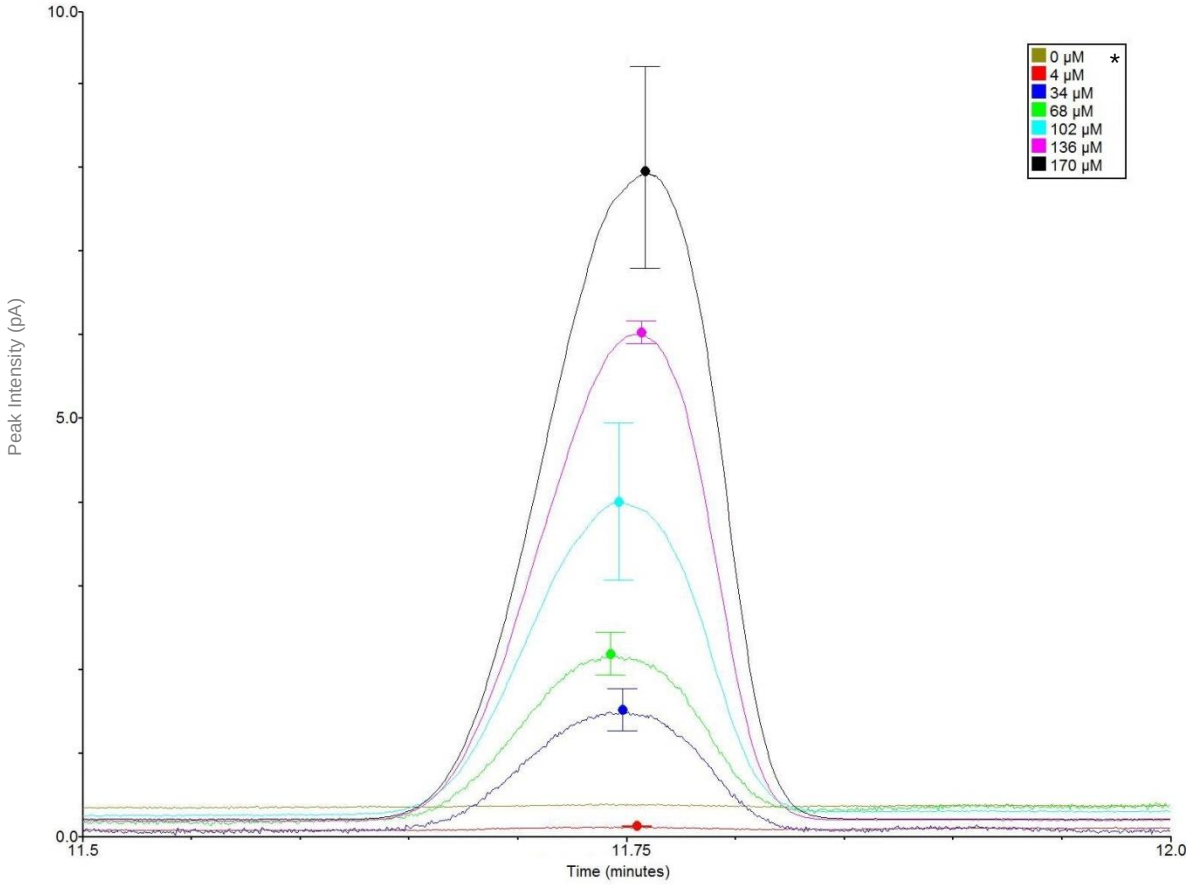


Figure 4.13 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* LinA4 medium at T= 0 days after the addition of lindane. Error bars represent standard deviation (n=3). * Concentration represents starting concentration at T=0 days (prior to extraction)

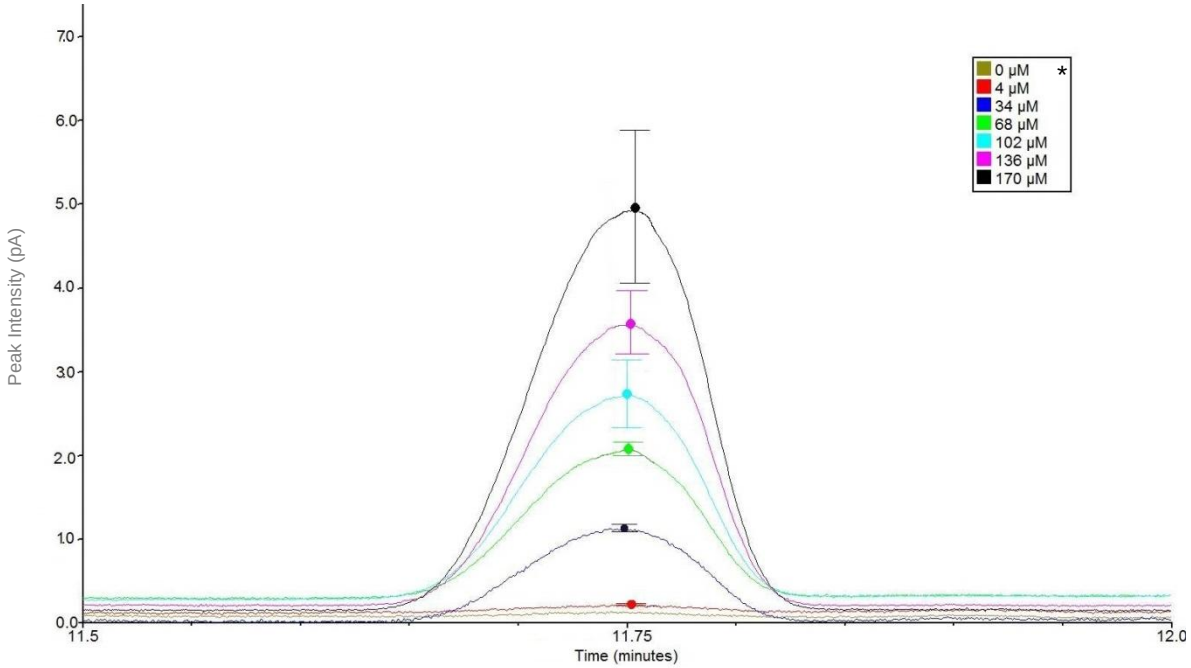


Figure 4.14 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* LinA4 medium at T= 21 days after the addition of lindane. Error bars represent standard deviation (n=3). * Concentration represents starting concentration at T=0 days (prior to extraction)

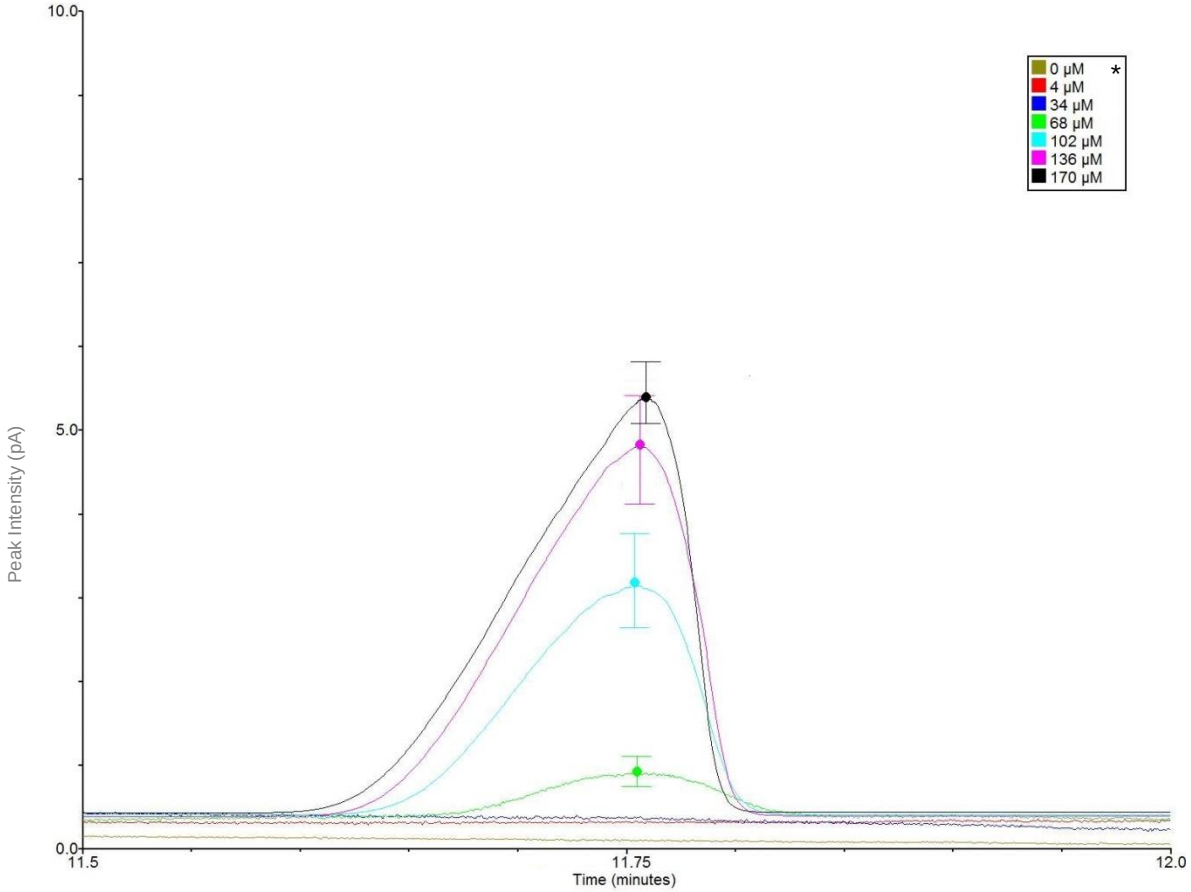


Figure 4.15 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* LinA4 root tissue at T = 21 days after the addition of lindane. Error bars represent standard deviation (n=3). * Concentration represents starting concentration at T=0 days (prior to extraction)

Table 4.8 Concentration of *Arabidopsis thaliana* LinA4 samples (after extraction) as calculated using the standard curves. Bracketed number represents standard deviation (n=3).

Molarity Prior to Extraction (µM)	Area Under the Curve			Molarity After Extraction (µM)		
	Media		Plant	Media		Plant
	T= 0	T= 21	T=21	T= 0	T= 21	T=21
0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
4	0.00 (0.01)	0.00 (0.01)	0.00 (0.00)	0.00 (0.04)	0.00 (0.04)	0.00 (0.00)
34	8.47 (0.83)	3.38 (0.15)	0.00 (0.00)	31.47 (3.08)	14.27 (0.56)	0.00 (0.00)
68	15.41 (0.85)	8.62 (0.20)	11.32 (0.51)	57.56 (3.16)	32.02 (0.74)	42.05 (1.89)
102	25.28 (2.58)	12.43 (1.12)	15.19 (1.43)	93.93 (9.58)	46.18 (4.16)	56.43 (5.31)
136	34.33 (0.39)	16.27 (0.98)	23.99 (1.62)	127.54 (1.45)	60.44 (3.64)	89.13 (6.02)
170	43.83 (2.69)	23.34 (1.98)	28.84 (1.18)	162.84 (9.99)	86.71 (7.36)	107.14 (4.38)

4.3.b.vii. Mass Balance of Lindane in the *A. thaliana* LinA4 system

To determine if there was any loss of lindane in the system after 21 days, the total number of moles of solute was calculated from the 10 µl injection volumes for each sample using the following equation,

$$\text{Number moles of solute} = \text{Molarity} \times \text{Volume}$$

The total number of moles at 21 days was compared against the initial number of moles in the system at T = 0 days (Table 4.9).

Table 4.9 Mass balance of lindane after T = 21 days in the transgenic *Arabidopsis thaliana* LinA4 system. Bracketed number represents standard deviation (n=3).

Molarity Prior to Extraction (µM)	Number of moles of solute (pmol)			Total number of moles of solute at T = 21 (pmols)	Gain / Loss (-) (%)
	Media		Plant		
	T= 0	T= 21	T=21		
0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	n/a
4	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	n/a
34	0.32 (0.00)	0.14 (0.01)	0.00 (0.00)	0.14 (0.01)	-54.66
68	0.57 (0.03)	0.32 (0.01)	0.42 (0.02)	0.74 (0.02)	29.40
102	0.94 (0.10)	0.46 (0.04)	0.56 (0.05)	1.03 (0.07)	9.24
136	1.28 (0.01)	0.60 (0.03)	0.89 (0.06)	1.50 (0.07)	17.27
170	1.63 (0.10)	0.87 (0.07)	1.07 (0.04)	1.94 (0.09)	19.04

4.3.b.viii. GC of *A. thaliana* LinA(-) Media and Root Tissue

The extracted organic fractions from *A. thaliana* LinA(-) at T = 0 (medium) (Figure 4.16) and 21 days (medium and ground root tissues) (Figure 4.17 and Figure 4.18) were analyzed by GC and compared against the standard curve (Table 4.10).

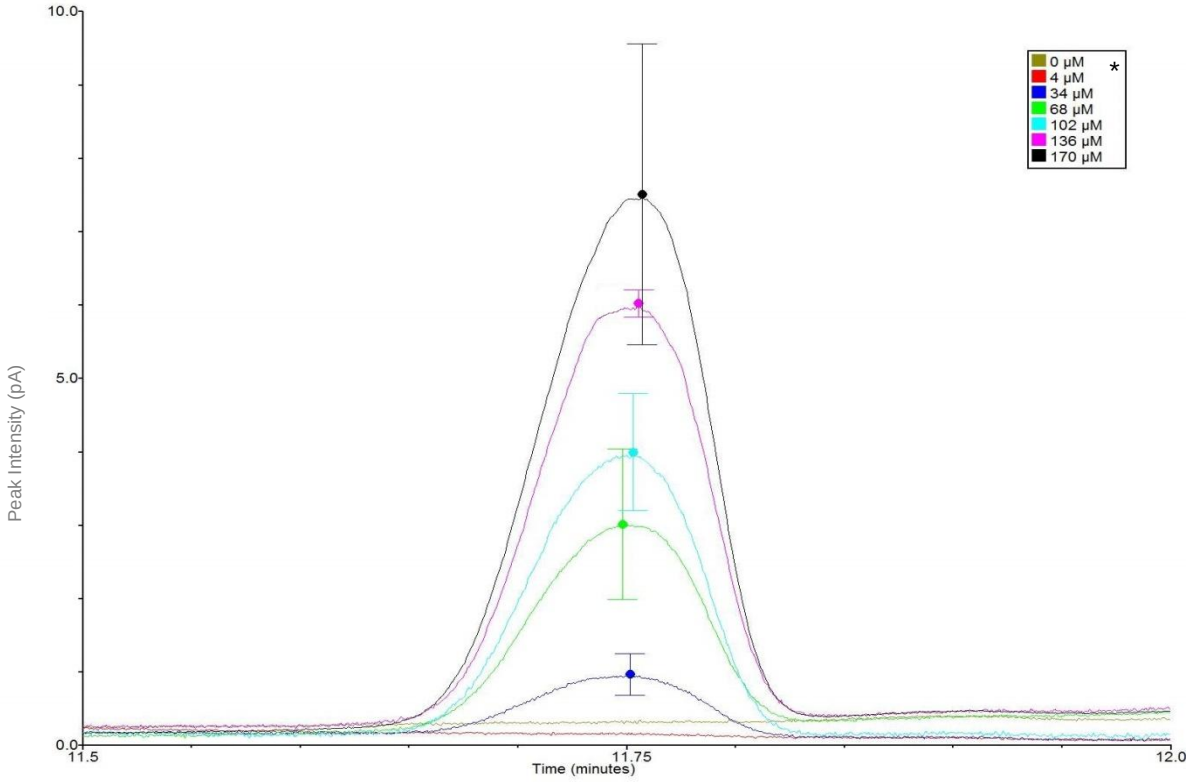


Figure 4.16 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* LinA(-) medium at T= 0 days after the addition of lindane. Error bars represent standard deviation (n=3). * Concentration represents starting concentration at T=0 days (prior to extraction)

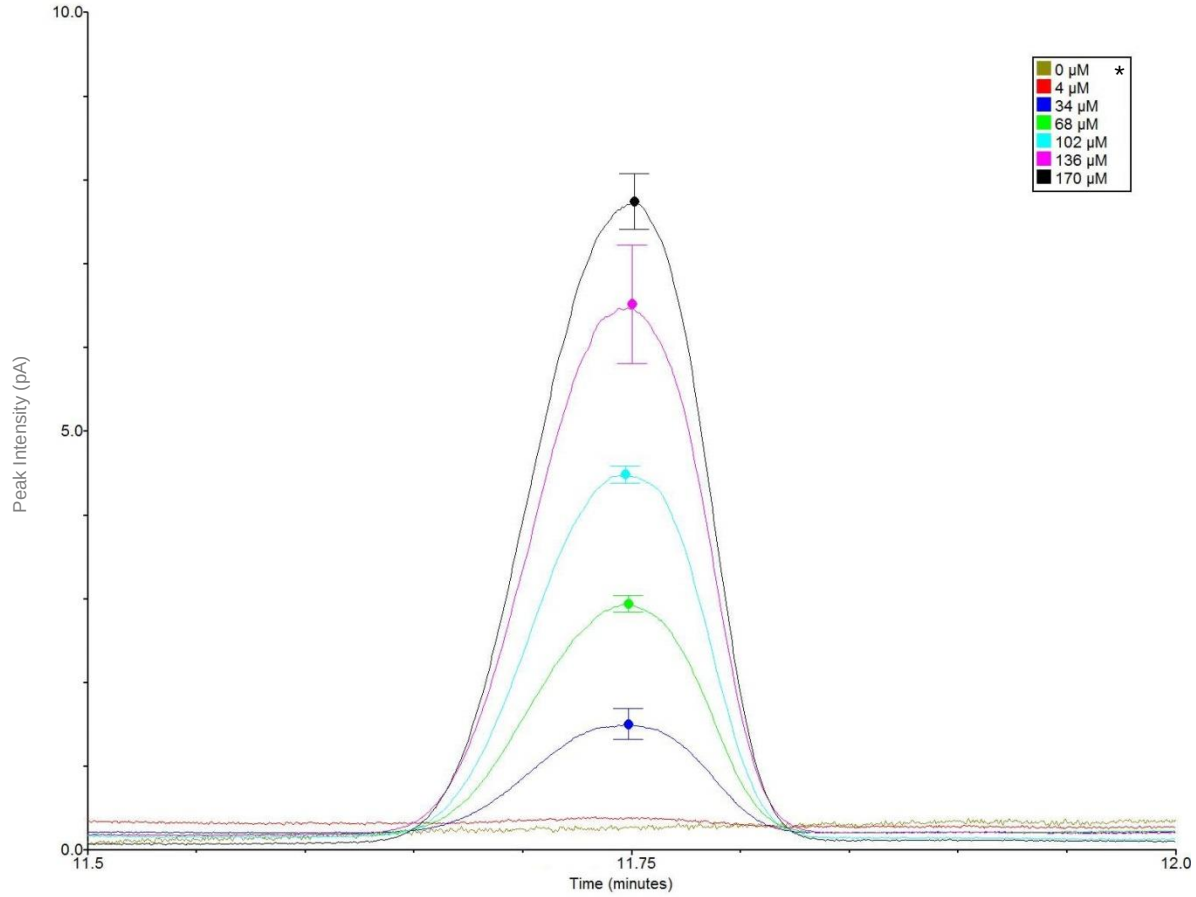


Figure 4.17 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* LinA(-) medium at T= 21 days after the addition of lindane. Error bars represent standard deviation (n=3).
* Concentration represents starting concentration at T=0 days (prior to extraction)

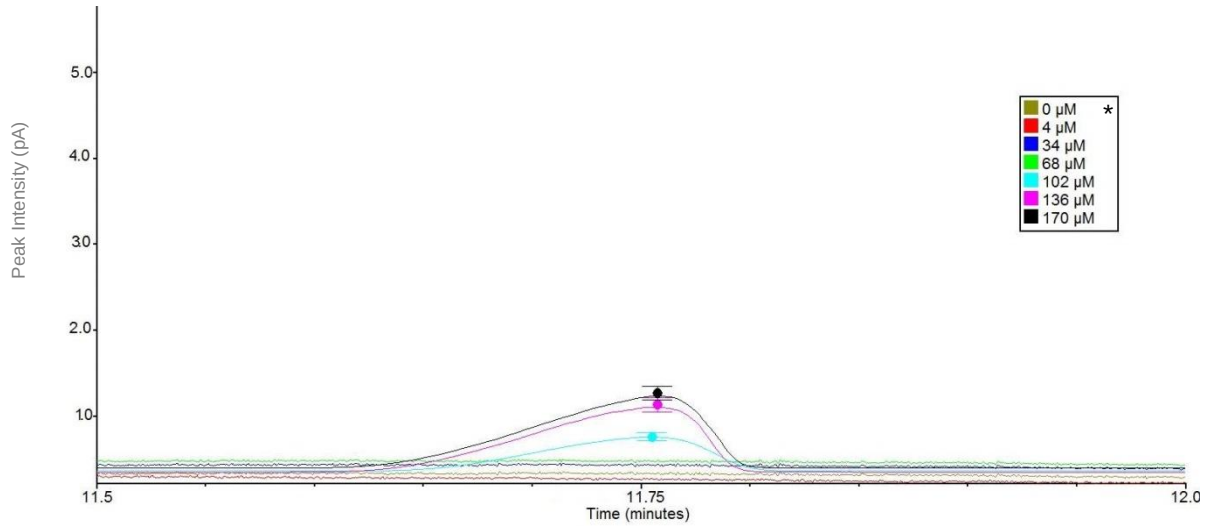


Figure 4.18 Chromatogram of liquid-liquid extracted residues from *Arabidopsis thaliana* LinA(-) root tissue at T= 21 days after the addition of lindane. Error bars represent standard deviation (n=3).
* Concentration represents starting concentration at T=0 days (prior to extraction)

Table 4.10 Concentration of *Arabidopsis thaliana* LinA(-) samples (after extraction) as calculated using the standard curves. Bracketed number represents standard deviation (n=3).

Molarity Prior to Extraction (µM))	Area Under the Curve			Molarity After Extraction (µM)		
	Media		Plant	Media		Plant
	T= 0	T= 21	T=21	T= 0	T= 21	T=21
0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
4	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
34	8.38 (0.75)	8.19 (0.46)	0.00 (0.00)	31.13 (2.79)	30.43 (1.71)	0.00 (0.00)
68	15.94 (2.01)	15.66 (0.20)	0.00 (0.00)	59.22 (7.47)	58.18 (0.74)	0.00 (0.00)
102	24.56 (1.78)	23.22 (0.23)	2.16 (0.15)	91.24 (6.61)	86.26 (0.85)	8.02 (0.56)
136	35.11 (0.38)	33.64 (1.82)	4.12 (0.21)	130.44 (1.41)	124.98 (6.76)	15.31 (0.78)
170	43.33 (4.56)	42.81 (0.98)	5.04 (0.19)	160.98 (16.94)	159.03 (3.64)	18.72 (0.71)

4.3.b.ix. Mass Balance of Lindane in the *A. thaliana* LinA(-) system

To determine if there was any loss of lindane in the system after 21 days, the total number of moles of solute was calculated from the 10 µl injection volumes for each sample using the following equation,

$$\text{Number moles of solute} = \text{Molarity} \times \text{Volume}$$

The total number of moles at 21 days was compared against the initial number of moles in the system at T = 0 days (Table 4.11).

Table 4.11 Mass balance of lindane after T = 21 days in the *Arabidopsis thaliana* LinA(-) system. Bracketed number represents standard deviation (n=3).

Molarity Prior to Extraction (µM)	Number of moles of solute (pmol)			Total number of moles of solute at T = 21 (pmols)	Gain / Loss (-) (%)
	Media		Plant		
	T= 0	T= 21	T=21		
0	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	n/a
4	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	n/a
34	0.31 (0.03)	0.30 (0.02)	0.00 (0.00)	0.30 (0.02)	-2.27
68	0.59 (0.07)	0.58 (0.01)	0.00 (0.00)	0.58 (0.01)	-1.75
102	0.91 (0.07)	0.86 (0.01)	0.08 (0.01)	0.94 (0.01)	3.34
136	1.30 (0.01)	1.25 (0.07)	0.15 (0.01)	1.40 (0.07)	7.55
170	1.61 (0.17)	1.59 (0.04)	0.19 (0.01)	1.78 (0.04)	10.42

4.4. Discussion

Although concentrations of 10^{-1} mg/L were below the limits of detection in this assay, the calibration graphs revealed a linear correlation (fold reduction) between both 1,2,4-TCB and lindane concentration (mg/L) and the calculated area under the peak at T = 2.4 and 11.75 minutes, respectively, in the concentration range of 1 to 10^4 mg/L. As the lowest lindane concentration to be used in the experimental protocol (at 4 µM) is greater than the calculated LOD of 3.28 µM and separation was visible between lindane, 1,2,4-TCB along with the internal standard aldrin, it was concluded that GC carried out using a fused silica Rtx®-CLPesticides Column without the subsequent use of MS is a suitable and sensitive enough method for the determination of both lindane and its metabolite, 1,2,4-TCB.

While lindane concentrations of 4 µM are within the LOD, the GC did not detect it from the w/t, LinA4 and LinA(-) extracts with a starting concentration of 4 µM at T = 0 days after the addition of lindane (Table 4.4, Table 4.8 and Table 4.10). Although the use of an internal standard was in place to correct for any analyte loss during extraction, it cannot correct for an amount that is below the LOD. This loss was ultimately attributed to the extraction process and not from natural attenuation or degradation of lindane, as the extraction took place immediately after the addition of lindane. In fact, all of the experimental samples analyzed at T = 0 days after the addition of lindane also gave starting concentrations at less than the initial amount added to the system (Table 4.4, Table 4.6, Table 4.8 and Table 4.10). Although the internal standard aldrin should correct for any inefficiencies in the extraction process, it may be that some of the solute was immediately sequestered to the root tissue and was not freely available in the media to be assayed. However, this was not measured as the experimental design did not allow for root tissue to be analyzed at T = 0 days after the addition of lindane. As this study is the first of its kind to develop a feasible model of transgenic phytoremediation of lindane and its ultimate fate within the biological system, the method of GC carried out using a fused silica Rtx®-CLPesticides Column detection is still a viable and acceptable option. However, future studies involving whole plants and actual field trials (outside of the laboratory) would benefit from the investigation into the use of separation methods such as SPME (Abdel-Latif, 2003).

The only observable and distinct peaks were detected at T = 11.75 minutes, indicative of lindane (Figure 4.2). The calculated areas for each peak at T = 11.75 minutes, in each condition, for each cell line of *A. thaliana,* were compared against the standard curve of lindane (Figure 4.5) to give the final concentrations remaining in the medium and plant tissues at T = 21 days after the addition of lindane. None of the samples assayed at T = 0, 7, 14 or 21 days after the addition of lindane, from either the medium and plant tissues, exhibited a peak at T = 2.40 minutes, which is where any 1,2,4-TCB residues would appear on the chromatogram (Figure 4.3).

The presence of 1,2,4-TCB would be indicative of lindane metabolism or degradation by LinA, abiotic transformants or native plant enzymes. In samples where a loss of lindane from the system has been observed, the absence of 1,2,4-TCB suggests that it and any other potential metabolite(s) may be undergoing further degradation, supporting the hypothesis that native plant enzymes, such as Cytochrome P450s would continue to degrade any organic metabolites. To determine if there was any loss of lindane in the system after 21 days, the total number of moles of solute were calculated from the 10 µl injection volumes for each sample and the total number of moles at T = 21 days were compared against the initial number of moles in the system at T = 0 days (Table 4.12).

Table 4.12 Mass Balance of lindane after T = 21 days from all of the *Arabidopsis thaliana* systems.

Sample	**w/t**		**LinA2**		**LinA4**		**LinA(-)**	
Molarity Prior to Extraction (µM)	**Gain / Loss (%)**	**Half-life (Days)**	**Gain / Loss (%)**	**Half-life (Days)**	**Gain / Loss (%)**	**Half-life (Days)**	**Gain / Loss %**	**Half-life (Days)**
0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
4	n/a	n/a	-100.00	* / **	n/a	n/a	n/a	n/a
34	8.42	n/a	-63.68	14.41	-54.66	18.43	-2.27	639.40
68	-6.65	210.39	-2.26	640.44	29.40	n/a	-1.75	854.42
102	1.67	n/a	1.59	n/a	9.24	n/a	3.34	n/a
136	11.22	n/a	4.22	n/a	17.27	n/a	7.55	n/a
170	13.11	n/a	6.17	n/a	19.04	n/a	10.42	n/a

* this value was not considered as the 100% loss was most likely due to experimental error and a low limit of detection as observed in the other systems at 4 µM.

** sample concentrations measured at 7 days and 14 days (not shown) were also undetectable, making the half-life calculation mathematically impossible to determine

Although there appears to be accumulation of lindane in some of the plant tissues of the non-transgenic *A. thaliana* w/t and LinA(-) varieties, the overall amount of lindane (pmols) at T = 21 days exceeds the initial amount of solute in the system, which is theoretically impossible. These results suggest that the single wash, prior to liquid-liquid extraction, did not sufficiently remove solute that was adhering to the outsides of the root tissue, thereby resulting in lindane detection.

A. thaliana w/t and LinA(-) both demonstrated an overall loss of lindane in the system, for the 68 µM starting concentration samples, of 6.65 and 1.75%, respectively (Table 4.12). Using the formula,

$$\text{Half-life} = (\text{elapsed time} \times \log 2) / \log (\#\ \text{moles}_{t=0} / \#\ \text{moles}_{t=21})$$

the half-life rates of lindane in these plant cell lines, were calculated as 210.39 and 854.42 days, respectively (Table 4.12). The half-life rate of 210.39 days from the w/t (Col-0) is comparable to the half-life rates for aerobic microbial biodegradation, ranging from 31 to 413 days, as reported by Howard *et al.* (1991). However, the lack of lindane uptake and degradation in the higher concentration samples suggests that the loss of lindane is likely due to inconsistencies in extraction, instrument measurement and small sample size. Regardless of the reason for the loss, intrinsic or experimental error; when compared to single species microbial degraders, such as *S. japonicum* UT26 (which have a threshold of 34 µM), the non-transgenic line does not exhibit an increased ability to degrade lindane with respect to half-life rates, and is not suitable for phytoremediation or warrant further investigation, despite the degradation at a higher concentration.

Conversely, in the transgenic *A. thaliana* LinA2 and LinA4 plant cell lines, there is a considerable loss of lindane in the system for samples with a starting concentration of 34 µM. The lines were found to have a decrease of 63.68 and 54.66%, respectively, at T = 21 days, equating to a half-life rate of 14.41 and 18.43 days; a substantial rate increase of approximately 50% when compared to those for aerobic microbial biodegradation. The samples with a starting concentration of 68 µM present conflicting data with the LinA2 line showing a reduction of 2.26%, or a half-

life rate of 640.44 days, whereas the LinA4 line has a net gain of 29.39%; most likely related to residues left over from the wash (Table 4.12).

Together, these findings suggest that the transgenic lines have a superior degradation capability when compared to microbes at the same concentration, warranting further investigation to assess the feasibility of transgenic plants for the phytoremediation of lindane.

4.5. Conclusion

A. thaliana transformed with the *linA* gene from *S. japonicum* UT26 confers the ability to remove lindane from the surrounding medium as observed in the *A. thaliana* LinA2 and LinA4 root tissue culture experiments at concentrations of 34 µM with half-life rates increases of approximately 50% when compared to microbial lindane-degraders. These findings are contrary to those of *A. thaliana* w/t and the negative control LinA(-), which were not able to remove it at improved rates.

However, after 21 days, the combined amount of lindane in the medium and plant tissue remained relatively constant when compared to the initial concentration, suggesting that although the transgenic plants were capable of removing it from the medium, aside from the samples with a starting concentration of 34 µM, they were unable to degrade it, and instead, accumulated or sequestered the lindane in their root tissues.

When compared to whole plant agar growth observations (refer to Chapter 3.3.l), whole plants grown under the same conditions are not able to survive, despite the increased protein activity (refer to Chapter 3.3.k.iii) and increased rate of lindane degradation in plant tissues. These findings suggest that other factors controlling lindane uptake or those involved in associated metabolic pathways may be inhibiting the degradation *in vivo*.

The next chapter will investigate the ability of the transgenic plants to remove and degrade lindane at the level of protein expression. Proteomic analysis using isobaric tags for relative and absolute quantification (iTRAQ) will provide an in-depth understanding into the functional processing at a metabolic level and will work to establish links between the environmental stress, organism survival and remediation capability.

Chapter 5.

Protein Expression in Wild Type and Transgenic Arabidopsis thaliana Grown in Conditions of Lindane-stress

5.1. Introduction

The previous chapters have revealed several differences between wild type (w/t) and transgenic *Arabidopsis thaliana* when grown under conditions of lindane-stress. Specifically, changes in LinA enzyme activity, lindane uptake and lindane accumulation, can be seen when the plants are in the presence of the organochlorinated pollutant. The most probable explanations for these variations are modifications occurring at the level of protein expression within the transgenic plant cells. Investigation of such proteomic and related metabolic-pathway alterations are beneficial to establish links between the environmental stress, organism survival and remediation capability when exploring the use of genetically-modified plants for phytoremediation.

Although genetic, enzymatic and phenotypic analyzes can provide information regarding gene expression, enzymatic activity and the potential of organisms to adapt under conditions of stress; proteomic analysis provides an in-depth understanding into the functional processes occurring at a metabolic level. In this chapter, the proteomes of w/t and transgenic *A. thaliana* (modified with *linA*) when grown in the presence and absence of 30 µM lindane (sub toxic threshold) have been compared using isobaric tags for relative and absolute quantification (iTRAQ); a quantitative, high throughput strategy intended for proteome-wide investigation of protein expression levels.

5.2. Methods

5.2.a. Protein Concentration and Quantification

Wild type and transgenic *A. thaliana* plant lines, w/t and LinA2 respectively, were grown in the presence and absence of lindane, their tissues harvested and protein extracted as previously mentioned (refer to Chapter 3.3.k). Two replicates from each of the two phenotypes (w/t and transgenic) were grown under the two distinct growth conditions (absence and presence of lindane) and were assayed using the 8 available isobaric tags. This allowed comparisons to be made under a distinct set of experimental conditions, thereby removing any possible chemical, biological or mechanical variation that might be caused by using two different iTRAQ kits.

Extracted proteins were concentrated using the Amicon® Ultra-0.5 Centrifugal Filter Device, with a buffer exchange using 0.5 M Triethylammonium bicarbonate (TEAB) (Sigma Aldrich), according to the manufacturer's protocol. Protein concentration was quantified using the Bradford ULTRA Assay Kit (Westburg) on a Nanodrop 2000 Spectrophotometer (Thermo Scientific). As comparatively equal amounts of total proteins from each phenotype are integral in maintaining the accuracy of the iTRAQ assay when quantifying the relative abundance of peptides, the final amount of protein to be labelled for further analysis was determined from the protein sample with the lowest concentration. Total proteins from each sample equating to 30 µg were collected, dried and re-suspended in a final volume of 20 µl - 0.5 M TEAB.

5.2.b. iTRAQ Labelling

2 µl of reducing reagent, 50 mM tris(2-carboxyl)phosphine (TCEP), was added to each assay tube. The reaction mixture was vortexed and incubated for 1 hour at 60 °C. 1 µl of 200 mM methyl methanethiosulfonate (MMTS) cysteine blocking reagent was added to each tube, which was vortexed and incubated for 10 minutes at RT. The proteins were then digested using trypsin (ThermoScientific) reconstituted in 50 µl - 500 mM TEAB. 3.75 µl of trypsin and 3.25 µl acetonitrile (Fischer) were added to each tube to give a final volume of 30 µl. The reaction mixture was vortexed and incubated overnight (O/N) at 37 °C. 100 µg Cytochrome C (Sigma Aldrich) was also

resuspended in 50 µl - 500 mM TEAB and digested with trypsin to be used as a control in the experimental analysis.

Room temperature iTRAQ Reagents 8-plex labels (Ab Sciex) were spun for 2 minutes at 1000 x g and 70 µl of 100% isopropanol (Fischer) was added to each label. The tube was then vortexed to mix and quickly centrifuged to bring the mixture back to the bottom of the tube. The contents of one iTRAQ Reagents – 8 plex tube were then mixed with one tube containing the digested protein sample (Table 5.1). The mixtures were incubated at room temperature for 3 hours after which time, the contents of each iTRAQ Reagents – 8 plex-labelled peptide tube were pooled together split into 2 aliquots, dried in a vacuum centrifuge at room temperature and stored at -20 °C.

Table 5.1 List of phenotypes used and the corresponding iTRAQ labels

Phenotype	Replicate 1 iTRAQ label	Replicate 2 iTRAQ label
wild type	113	114
LinA2	115	116
wild type + lindane	117	118
LinA2 + lindane	119	121

5.2.c. HPLC Separation of Dried and Labelled Peptides

The dried peptides were re-suspended in aqueous buffer A (25% acetonitrile in water, 0.1% formic acid). Separation was performed on a BioLC HPLC unit (Dionex, UK) using a POLYSULFOETHYL A column (PolyLC, USA) 21 cm length, 2.1 mm internal diameter and 5 µm pore size. The separation programme consisted of 100% buffer A for 5 minutes, 0 – 5% buffer B (25% acetonitrile in water, 0.1% formic acid, 500 mM potassium chloride) for 1 minute, 5 - 30% buffer B for 30 minutes, 30 - 35% buffer B in 5 minutes, 35 – 100% buffer B in 5 minutes, 100% buffer B for 5 minutes, and finally 100% buffer A for 10 minutes. A flow rate of 0.2 ml/min was maintained with an injection volume of 70 µl. A UV detector (UVD170U) and Chromeleon software v6.50 (Dionex/LC packings, The Netherlands) were used to monitor the

chromatograms as fractions were collected every 30 seconds using a Foxy Jr. fraction collector (Dionex, UK). The fractions were then dried in a vacuum centrifuge (Eppendorf) and samples were stored at -20 °C.

5.2.d. C18 Clean-up

To prevent contaminant interference (by common biological compounds and buffers) from affecting the sensitivity and quality of downstream LC-MS applications, the HILIC fractions were cleaned using UltraMicroSpin Columns (Nest) according to the manufacturer's guidelines prior to vacuum centrifugation (Eppendorf).

5.2.e. RPLC-MS analysis

RPLC-MS was conducted using an Ultimate 3000 HPLC (Dionex) coupled to a QStar XL Hybrid ESI Quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems (now ABSciex)). Samples were re-suspended in 20 µL buffer A (3% acetonitrile, 0.1% formic acid) before loading 9 µL onto a Acclaim PepMap 100 C18 column, 3 µm particle size, 15 cm length, 75 µm diameter, 100 Å pore size (Dionex). With a flow of 300 µL min-1, buffer A was exchanged with buffer B (97% acetonitrile, 0.1% formic acid) to form a linear gradient as follows: 3% B (0 - 5 min), 3 – 35% B (5 - 95 min), 35 – 90% B (95 - 97 min), 90% B (97 - 102 min), 3% B (102 - 130 min). The mass detector range was set to 350 - 1800 m/z and operated in positive ion mode. Peptides with +2, +3, and +4 were selected for fragmentation.

5.2.f. Data Analysis

Peptide fragments were submitted to an in-house Mascot proteomics search engine (Matrix Science) for protein identification based on the SwissProt *A. thaliana* protein sequence database. The raw data output from Mascot was subsequently run through IsobariQ to filter the Mascot data for false hits and produce relative tag intensities for each individual peptide spectral match (PSM) (Arntzhen 2013).

A cluster analysis using principal components analysis (PCA) was carried out on the relative tag intensities for each indentified PSM. As the relative abundance of each peptide relates to a specific protein across the different phenotypes and growth conditions, the PCA is therefore indicative of variations in the global proteome.

Quantification was obtained at an 80% confidence level from proteins identified by two or more peptides using an R-script algorithm (Evans, *et al*. 2013). An increased abundance of protein was indicated by positive fold changes and a decrease in the abundance of proteins by a negative fold change. Indentified proteins were mapped onto metabolic pathways using KEGG Mapper – Search & Color Pathway, searching against the *A. thaliana* specific pathways (Kanehisa Laboratories 2014).

5.3. Results

Extracted and concentrated proteins were quantified to ensure an equal amount of total protein from each sample was labelled and assayed for further use (Figure 5.1 and Table 5.2).

5.3.a. Protein Concentration

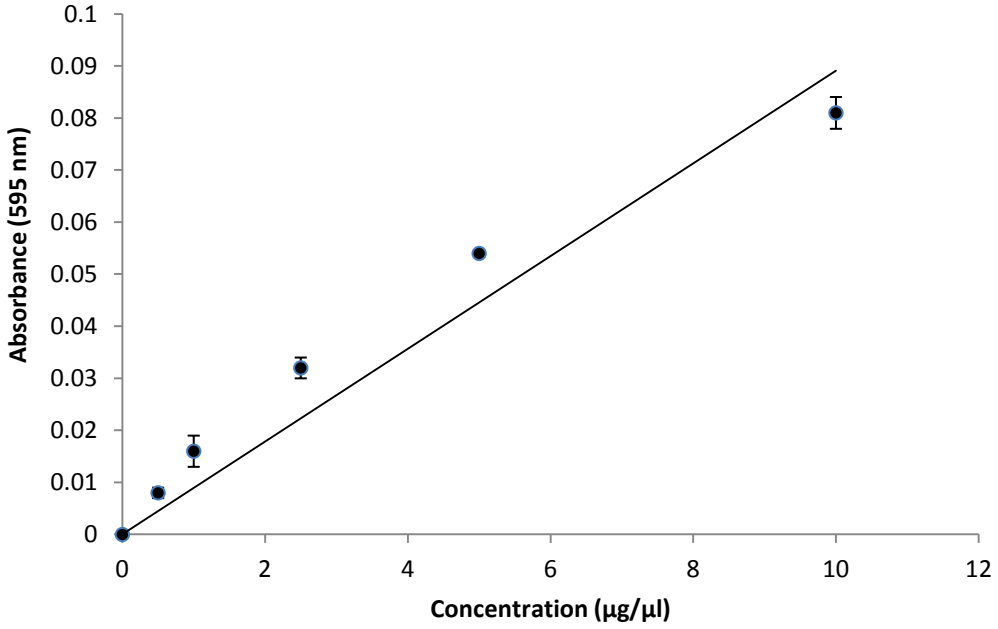


Figure 5.1 Standard curve for Bovine Serum Albumin (BSA) using the Bradford ULTRA Assay Kit.
Absorbance (595 nm) corrected for blank
Error bars represent standard deviation

Table 5.2 Determination of extracted protein concentration (after Amicon® concentration) using Bovine Serum Albumin (BSA) standard curve

	Plants grown in 0 µM lindane				**Plants grown in 30 µM lindane**			
	w/t(1)	**w/t(2)**	**LinA2(1)**	**LinA2(2)**	**w/t(3)**	**w/t(4)**	**LinA2(3)**	**LinA2(4)**
Absorbance (OD_{595})	0.025	0.015	0.010	0.016	0.014	0.041	0.024	0.033
Concentration (µg/µl)	1.61	0.92	0.57	0.97	0.83	3.02	1.48	2.17
Standard Deviation	0.20	0.14	0.03	0.08	0.06	0.12	0.10	0.08

The protein sample with the lowest concentration, LinA2(1), was used to determine final amount of protein needed from each sample. As the final volume available for

collection was 50 µl, a maximum amount of 30 µg of total proteins from each sample were collected, dried and resuspended in a final volume of 20 µl - 0.5 M TEAB.

5.3.b. HPLC Separation

The number of unique peptides obtained in each fraction collected during separation was plotted as a function of fraction number to obtain the resolution of the chromatographic separation (Figure 5.2).

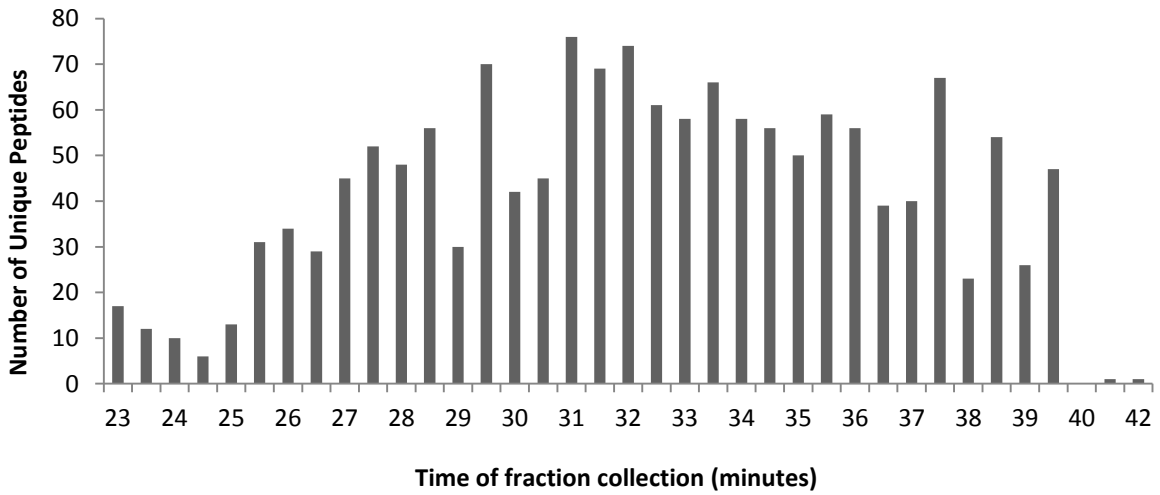


Figure 5.2 Number of unique peptides obtained in collected fractions.
(Fractions were not collected for 40.5 and 41.5 minutes)

The relatively even distribution of unique peptides obtained across the different collected fractions during the time period of 23.5 to 39.5 minutes indicates an efficient separation of peptides during HPLC.

5.3.c. Principal Components Analysis

A cluster analysis using Principal Components Analysis (PCA) was carried out on the relative reporter ion abundance for each of the peptides identified by tandem mass spectrometry. The result of the PCA analysis is presented in Figure 5.3.

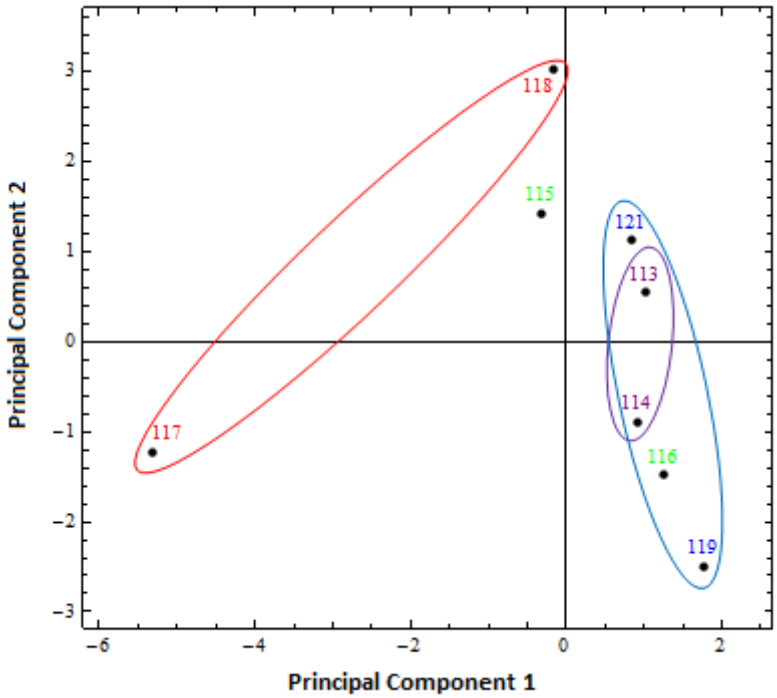


Figure 5.3 Principal components analysis (PCA) of labelled tags from each of the different *Arabidopsis thaliana* phenotypes grown in the presence and absence of 30 µM lindane. Purple = wild type in the absence of lindane, green = transgenic in the absence of lindane, red = wild type in presence of 30 µM lindane and blue = transgenic in presence of 30 µM lindane

The PCA shows separation along Principal Component 1 with the biological replicates from each phenotype and growth condition clustering together, indicating similar protein expression patterns. The presence of three distinct protein expression patterns across the four phenotypes can also be observed. Specifically, the protein expression profile in the w/t plants exposed to lindane is distinctly different from its transgenic counterpart (in the same growth condition), suggesting that lindane has different effects on plants depending on the presence or absence of the *linA* gene. No difference was detected in the absence of lindane, between the two different plant types, w/t and transgenic. Likewise, no difference was detected in the proteome from the same plant type under differing growth conditions, absence and presence of lindane.

5.3.d. Protein Identification and Quantification

Analysis of isobariQ data identified 304 proteins with two or more unique peptides. The abundance of proteins present in the transgenic plants grown in the presence of lindane, relative to those from the w/t plants were compared (Figure 5.4).

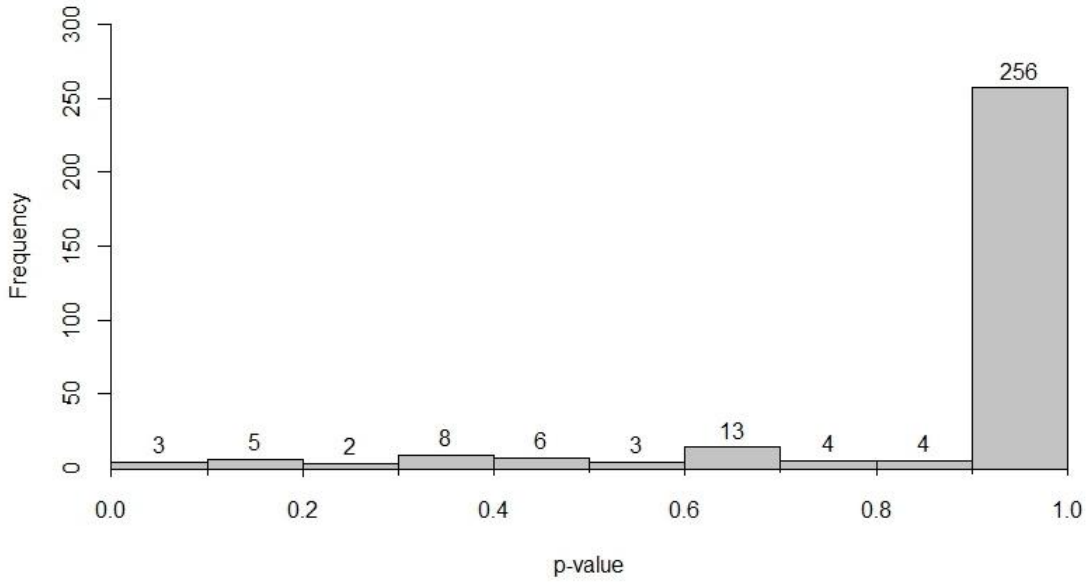


Figure 5.4 Relative abundance of proteins.

The median fold change and functions of the proteins with differential abundance in transgenic plants relative to w/t plants, both grown in the presence of lindane, and identified at 80% confidence with multiple test correction, as identified from the R-script algorithm is presented in Figure 5.5 and Table 5.3, respectively.

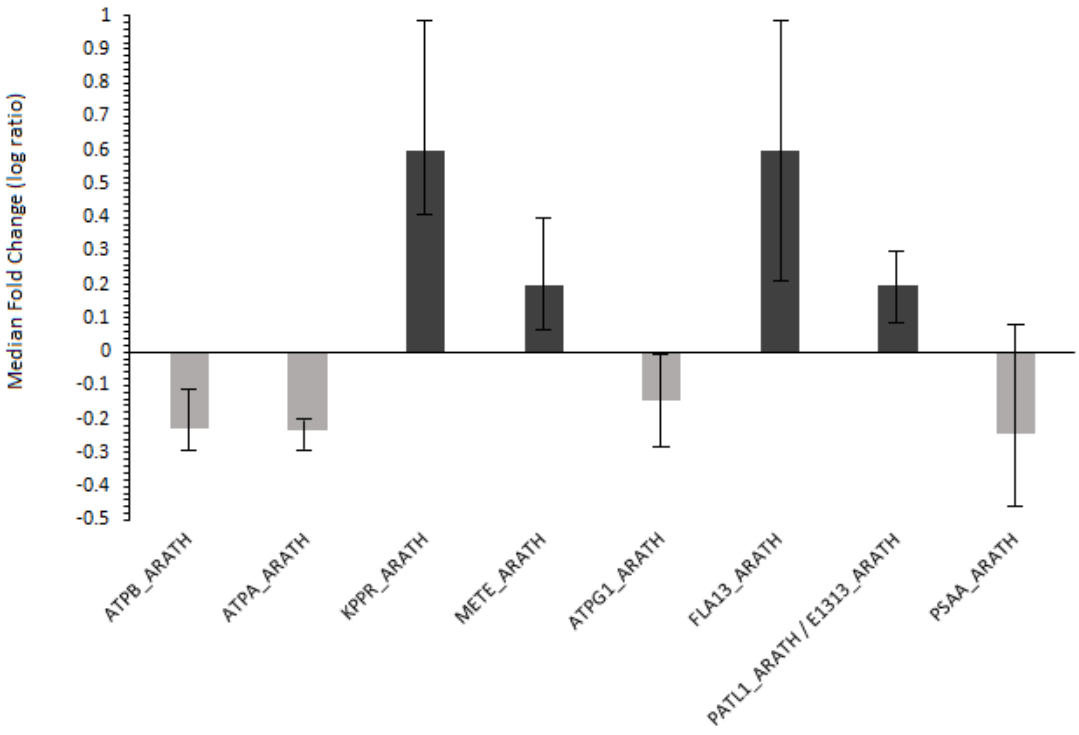


Figure 5.5 Median fold change of proteins with differential abundance in transgenic plants relative to wild type plants, both grown in the presence of lindane. (Identified at 80% confidence with multiple test correction).

Table 5.3 List of proteins and their functions with differential abundance in transgenic plants relative to wild type plants, both grown in the presence of lindane. (Identified at 80% confidence with multiple test correction).

Locus	Regulation	Full Protein Name	Function
ATPA_ARATH	Down	ATP synthase subunit alpha, chloroplastic	Produces ATP from ADP in the presence of a proton gradient across the membrane.
ATPB_ARATH	Down	ATP synthase subunit beta, chloroplastic	Produces ATP from ADP in the presence of a proton gradient across the membrane. The catalytic sites are hosted primarily by the beta subunits
KPPR_ARATH	Up	Phosphoribulokinase, chloroplastic	Carbohydrate biosynthesis; Calvin cycle
METE1_ARATH	Up	5-methyltetra-hydropteroyltrigluta-mate homocystein methyltransferase 1	Catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine resulting in methionine formation.
ATPG1_ARATH	Down	ATP synthase gamma chain 1, chloroplastic	Produces ATP from ADP in the presence of a proton gradient cross the membrane. The gamma chain is believed to be important in regulating ATPase activity and the flow of protons through the membrane proton channel complex
FLA13_ARATH	Up	Fascilin-like arabinogalactan protein 13	May be a cell surface adhesion protein.
PATL1_ARATH*	Up	Pattelin-1	Carrier protein that may be involved in membrane-trafficking events associated with cell plate formation during cytokinesis. Binds to some hydrophobic molecules and promotes their transfer between the different cellular sites. Binds to phosphoinositides.
E1313_ARATH*	Up	Glucan endo-1,3-beta-glucosidase 13	Hydrolysis of (1->3)-beta-D-glucosidic linkages in (1->3)-beta-D-glucans
PSAA_ARATH	Down	Photosystem I P700 chlorphyll a apoprotein A1	Primary electron donor of photosystem I (PSI), which transfers its electrons to the spectroscopically characterized acceptors A0, A1, FX, FA and FB in turn.

* The likelihood of either PATL1_ARATH or E1313_ARATH (based on the peptide sequences) being present and regulated within the genome is equally plausible and indistinguishable given the available peptide data and algorithm processing power

Information compiled from The UniProt Consortium (2014)

The protein response of the transgenic plants in the presence of lindane showed a lower abundance of proteins involved in energy production and an increase in the expression of the membrane transfer proteins.

5.3.e. Metabolic Pathways

All identified proteins were mapped onto metabolic pathways and analysis indicated that down-regulated proteins were linked to the photosynthetic pathways (Figure 5.6). By including insignificant proteins, a broader scope of all interactions that may be occurring is possible. Up-regulated proteins did not map onto any pathways.

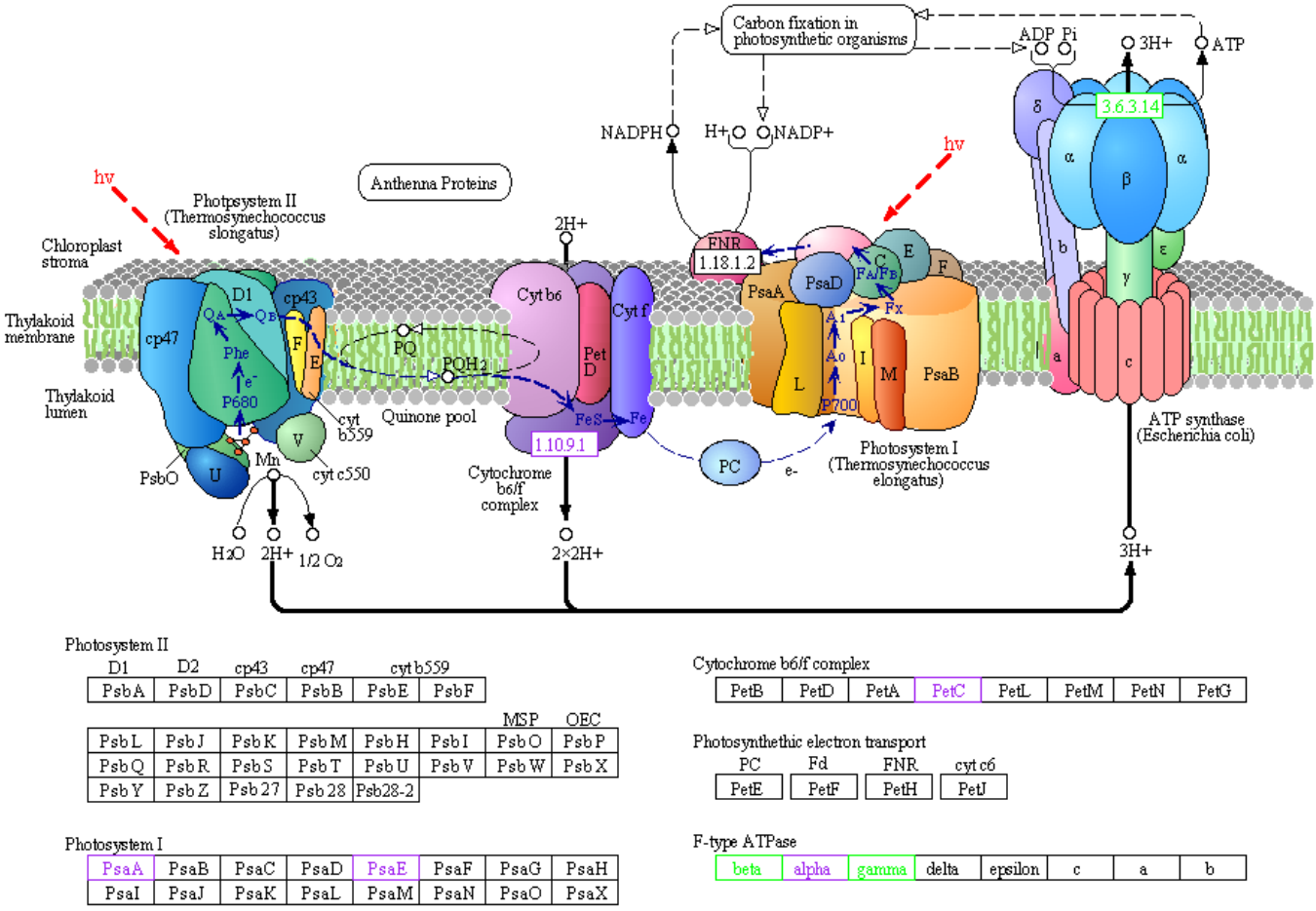


Figure 5.6 Mapping of regulated proteins onto the photosynthetic pathways. Significantly down-regulated proteins are green and insignificant are purple

5.4. Discussion

This chapter sought to understand the differences in the global proteome of w/t and transgenic *A. thaliana* when grown in the absence and presence of lindane. The results of the PCA reveal a significant difference in the relative abundance of proteins between w/t and transgenic *A. thaliana* in the presence of lindane (Figure 5.3). Only 8 proteins with differential abundance in transgenic plants, relative to w/t plants and grown in the presence of lindane, were identified at 80% confidence with multiple test correction, from the R-script algorithm (Figure 5.4). Their regulation and functions (Figure 5.5 and Table 5.3, respectively) revealed that under conditions of stress, energy generation pathways are found to be down-regulated in the transgenic cells. Specifically, proteins involved in Photosystem (PS) I and ATP-ase pathways are highlighted (Figure 5.6).

In a study examining the phytotoxicity mechanisms of *A. thaliana*, Zhang, *et al*., (2013) reported that lindane increases the detoxification enzymatic activities of catalase (CAT) and peroxidase (POD), while inhibiting the activity of superoxide dismutase (SOD). Further, in systems relating to photosynthesis, the efficiency of PS I and II were also revealed to be down-regulated. Ott, *et al*., (1999) have also observed a decrease in the electron transport rate between PSI and PSII under conditions of stress and have suggested that this regulatory function could inhibit superoxide formation and aid plants in avoiding reactive oxygen species (ROS) toxicity. An over-accumulation of superoxide, outpacing the activity of antioxidant superoxide enzymes can trigger programmed cell death (Foyer & Noctor, 2009).

Considering the above studies, it is not surprising that the proteins related to energy metabolism are in relatively lower abundance, as the transgenic plant has already been shown to have specific enzymatic activity in metabolizing lindane (refer to Chapter 3.3.k.iii). When compared to the w/t plant under conditions of lindane-stress, less energy is required for survival by the transgenic plant as other metabolic and detoxification enzymes are able to control the production and accumulation of ROS to ultimately reduce oxidative stress.

The up-regulated proteins did not strongly map onto any of the metabolic pathways. However, two of the proteins are implicated as having membrane or cell surface functions such as adhesion and trafficking. As the transgenic plant is capable of breaking down lindane, it is possible that other catalytic processes are occurring requiring the binding and movement of proteins and molecules into different cellular spaces and pathways. This hypothesis would support the up-regulation of the two remaining proteins, KPPR_ARATH (P25697) and METE1_ARATH (O50008), which are both reported to be involved in various pathways of biosynthesis (The UniProt Consortium 2014).

However, the relatively small number of differential proteins identified makes it difficult to accurately elucidate which pathways are being up- or down-regulated to a significant extent. A number of explanations exist to explain the low number of identified peptides:

- The presence of the over-abundant RuBisCO protein in the protein extract limited the number of peptides that could be reliably identified. Although the possibility exists to reduce the concentration of RuBisCO from the protein extract, the likelihood of further compromising the overall protein content is probable.

- Although within the detectable limits of 20 to 100 µg, as defined in the protocol, the total amount of labelled protein for each sample was still relatively low at 30 µg. Increasing the total amount of starting protein may increase the number of detectable proteins potentially lost during the tryptic digestion; repeated rounds of drying and rehydration; and C18 clean-up processes prior to LC-MS.

- Theoretically speaking, given unlimited MS time and sample, it is possible to use a strategy capable of attaining superior coverage when compared to that achieved with limited time and / or sample, providing good coverage to begin with. However, these limits are highly dependent, with diminishing returns over time.

5.5. Conclusion

Despite careful optimisation of each step in the iTRAQ workflow, an optimal coverage of the proteome was not achieved. Further improvements, with respect to protein extraction; RuBisCo reduction; total protein content; sample size; and MS time coverage, warrant further study to realize the full potential of iTRAQ for analysis of prospective phytoremediative species. Implementing a second quantitative proteomics approach in tandem with iTRAQ, such as traditional two- dimensional gel electrophoresis or protein microarrays, may be helpful in identifying additional differentially expressed proteins.

Nonetheless, the results from this chapter support the idea that the global proteome of wild type *A. thaliana* is distinctly different from its transgenic counterpart when grown in the presence of lindane. Therefore, the differential protein expression may be attributed to the expression of the LinA protein. It is likely that the effect of lindane on the transgenic plants does not induce stress conditions but instead triggers a catabolic response which may consequently lead to the biosynthesis and activation of other molecules and enzymatic pathways.

Chapter 6.

Conclusions and Future Work

6.1. Introduction

The aim of this thesis was to develop a single gene (*linA*)-modified plant for the phytoremediation of lindane. As is evident from the literature review and research results in this thesis, the development of an effective remediation strategy to safely and quickly remove the highly toxic, ubiquitous and persistent organic pollutant from the environment is paramount. Phytoremediation is an aesthetically, environmentally and economically-friendly biotechnology using plants that are able to sequester and transform organic waste to a non-toxic state, or to sub-threshold levels below concentration limits. However, as plants are often deficient in the catabolic enzymes for complete degradation and mineralization of these pollutants, there is potential for accumulated toxins to be released back into the environment or food chain. Improving plants for the phytoremediation of lindane may be achieved by transferring genes known to be involved in xenobiotic degradation from other organisms such as microbes.

The catabolic genes and enzymes involved in lindane degradation have been extensively studied in *Sphingobium japonicum* UT26 (Pal, *et al*., 2005). The first of these enzymes, HCH dehydrochlorinase (LinA), mediates the initial transformation of lindane in a specialized pathway by catalyzing the first two dehydrochlorination steps of lindane to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN), followed by its spontaneous conversion to 1,2,4-trichlorobenzene (1,2,4-TCB) (Nagata, *et al*., 1999). Preliminary results from a single proof of concept study have shown that the model plant *Arabidopsis thaliana* modified with *linA* from *S. japonicum* UT26 is able to grow in the presence of normally toxic lindane concentrations, and remove it from the media whereas the wild type (w/t) plant is not (De Lorenzo Prieto & Gonzalez Pastor, 2007).

This work proposed to build on the above feasibility study and successfully developed a mechanistic understanding of w/t and transgenic *A. thaliana* in response to lindane. Phenotypic growth, enzymatic activity, degradation rates, metabolite production and changes to the global proteome under increasing concentrations

were studied using a variety of synthetic biological, chemical separation and analytical techniques. The differences seen are summarised below.

6.2. Growth and Characteristics of A. thaliana Modified with linA

The first and second aims of this thesis were to transform *A. thaliana* with the *linA* gene from *S. japonicum* UT26 and to observe the growth and characteristics of transgenic *A. thaliana* on control, sub-threshold and toxic concentrations of lindane and 1,2,4-TCB. Despite the small sample size, initial findings from the DNA sequencing, RNA transcription, protein expression and the *in vitro* enzymatic assay confirm the presence of the *linA* gene, its subsequent transcription and translation, and have demonstrated the catabolic activity of the protein to release Cl^- from lindane. Together, these results suggest that the transgenic lines should have superior growth when compared to the non-transformed lines, when grown in conditions of lindane-stress. However, this was not observed *in vivo*. Phenotypically, *A. thaliana* transformed with the *linA* gene from *S. japonicum* UT26 appears to have no advantages, with respect to growth, when cultivated on lindane-contaminated agar and gives an identical growth pattern to the w/t and non-transformed lines, with threshold toxicity remaining below 34 µM. Regardless, the ability, even if limited, of the transgenic plant to express the LinA protein and exhibit enzymatic activity, *in vitro*, supports the theory that *A. thaliana,* and possibly other plants amenable to *Agrobacterium*-mediated transformation, are capable of integrating the *linA* gene into their genome, to eventually yield a functioning form of the LinA protein, for potential use in the phytoremediation of lindane.

6.3. Uptake and Degradation of Lindane

Chapter 4 aimed to examine the ability of transgenic *A. thaliana* to remove lindane from its growth environment and determined the extent of degradation, by measuring lindane and any known metabolic breakdown products accumulating in the medium and / or plant, using Gas Chromatography. *A. thaliana* transformed with the *linA* gene from *S. japonicum* UT26 had the ability to remove lindane from the surrounding media as observed in the transgenic *A. thaliana* LinA2 and LinA4 root tissue culture

experiments at concentrations of 34 µM. Half-lives decreased approximately 50% when compared the half-life degradation rates of microbial lindane degraders. These findings are contrary to those of *A. thaliana* w/t and the negative control LinA(-), at the same concentrations, which were not able to remove it at improved rates. 1,2,4-TCB was not detected in any of the samples after 21 days suggesting that it is being further metabolized by naturally occurring plant enzymes into a compound undetectable by GC, or at least by the column used for GC in this work. Although this finding supports the hypothesis that naturally occurring enzymes are capable of breaking down 1,2,4-TCB, further investigation into other compounds would need to be undertaken to confirm this.

At higher concentrations, the combined final amount of lindane in the medium and transgenic plant tissue remained relatively constant (when compared to the initial concentration), suggesting that although the transgenic plants were capable of removing it from the medium, they were unable to degrade it, and instead, accumulated or sequestered the lindane in their root tissues. When compared to the whole plant agar growth observations, plant tissue cultures displayed an improved ability to grow in a higher concentration of lindane (34 µM). These findings suggest that other factors controlling lindane uptake or those involved in associated metabolic pathways may be inhibiting the degradation *in vivo*.

6.4. Changes to the Global Proteome

Chapter 5 reports quantitative changes in the global proteome of *A. thaliana*, modified with *linA*, ascertaining whether protein regulation has been affected at the individual level, within the plant xenobiotic degradation pathway or within any other metabolic pathway(s) by employing proteomic analysis using isobaric tags for relative and absolute quantification (iTRAQ). Despite careful optimization of each step in the iTRAQ workflow, an optimal coverage of the proteome was not achieved. Nonetheless, the results from this chapter supported the idea that the global proteome of w/t *A. thaliana* is distinctly different from its transgenic counterpart when grown in the presence of lindane, primarily affecting the enzymes involved in Photosystems I and II. Although undetected in the iTRAQ results, the differential

protein expression may be attributed to the expression of the LinA protein itself. It is likely that the effect of lindane on the transgenic plants does not induce stress conditions but instead triggers a catabolic response which may consequently lead to the biosynthesis and activation of other molecules and enzymatic pathways.

6.5. General Conclusions

Although other bioremediation tactics have previously been applied to clean up lindane from the soil, strategies using w/t organisms have been ineffective and may result in the toxic compound being released back into the environment (Bhatt, *et al*., 2009). The development of transgenic plants that actively take up, degrade and detoxify lindane may be one way to reduce its ecological impact. The main purpose of this study was to determine whether transgenic plants expressing a bacterial *linA* gene were able to dechlorinate lindane to a less toxic intermediate or proceed to full mineralization. To test this, *Agrobacterium*-mediated transformation was used to introduce a w/t bacterial *linA* gene into *A. thaliana*.

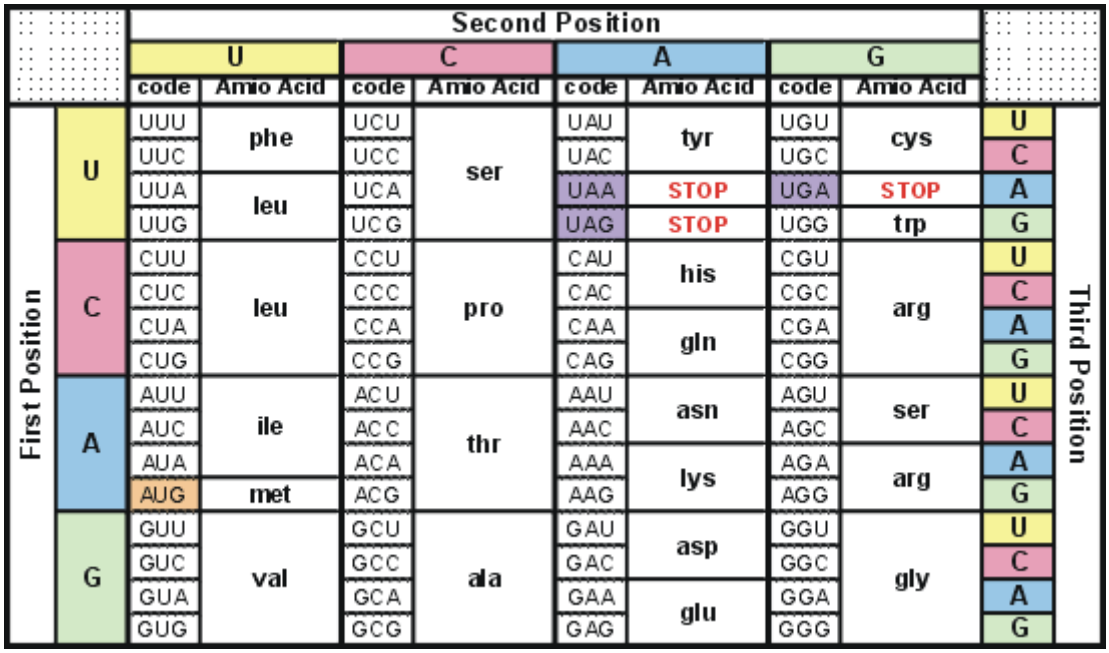
Previous efforts to produce a transgenic lindane-degrading plant using the *linA* gene have resulted in plants that are able to remove lindane from its growth media and grow at normally toxic concentrations of 34 µM. However, the resulting metabolites were not described. In this thesis, lindane has been shown to accumulate in the transgenic plant root tissues at concentrations of 68 µM and higher while at 34 µM, over 50% of the compound is removed from the nutrient medium and is undetected in the plant tissues. The metabolite, 1,2,4-TCB, was also not detected *in planta*, implying additional metabolism or full mineralization utilizing other innate enzymes. Metabolism of 1,2,4-TCB is suspected to occur due to the presence of Cytochrome P450s (refer to Chapter 3.3) (Marco-Urrea, *et al*., 2009; Eilers, *et al*., 1999). However, the methods empolyed in this work did not look to confirm the actual presence of Cytrochome P450s. Further investigation into Cytochrome P450s, other plausible plant xenobiotic metabolic enzymes and their metabolic products utilizing is necessary to fully understand the degradative pathway of lindane in transgenic *A. thaliana*.

Results from RT-PCR, western blotting, enzymatic activity and proteomic analyses indicated that the *linA* gene was being expressed in the transgenic plant's roots and shoots, *in vitro*. However, *in vivo*, they failed to display improved growth characteristics on lindane-contaminated medium when compared to the w/t. This is likely caused by a lack of adequate protein expression or harmful interactions with other molecules. The conflicting results suggest that the transgenic plant expressing the LinA enzyme may be useful in the phytoremediation of lindane-contaminated environments but its efficiency and the ultimate fate of the xenobiotic *in planta* must considered for future study.

6.6. Future Work

The work in this thesis has provided valuable information with respect to the feasibility of phytoremediation as a strategy to remove lindane from the environment and the specific response of *A. thaliana* (modified with a bacterial *linA* gene) in its presence. However, it has also raised a number of new concepts worth considering.

Firstly, the immediate investigation of codon preference would provide insight with respect to the complex regulation of proteins. Codons are used by organisms to translate mRNA into proteins. As seen in the table of codons shown in Figure 6.1, the genetic code is redundant with more than 61 codons encoding for 20 different amino acids. Codon preferences exist as a part of the gene expression process in organisms and may provide another level to control the expression of proteins. Studies in the genetic engineering of plants using bacterial genes have shown codon preference to be an important factor in successful protein expression (USDA National Institute of Food and Agriculture, 2014; Wang, *et al*., 2005). Plants with preferential codon usage may lack the tRNA to complement the bacterial codons or may make the tRNA at such low levels resulting in few copies within the cell to accommodate translation of the desired mRNA. Successful engineering of transgenic plants, using bacterial DNA for the phytoremediation of POPs, may therefore be enhanced by synthetic coding regions substituting codons preferred by plants to those preferred by bacteria.



First Position	Second Position: U				Second Position: C				Second Position: A				Second Position: G				Third Position
	code	Amio Acid	code	Amio Acid	code	Amio Acid	code	Amio Acid	code	Amio Acid	code	Amio Acid	code	Amio Acid	code	Amio Acid	
U	UUU	phe	UCU	ser	UAU	tyr	UGU	cys	U								
	UUC		UCC		UAC		UGC		C								
	UUA	leu	UCA		UAA	STOP	UGA	STOP	A								
	UUG		UCG		UAG	STOP	UGG	trp	G								
C	CUU	leu	CCU	pro	CAU	his	CGU	arg	U								
	CUC		CCC		CAC		CGC		C								
	CUA		CCA		CAA	gln	CGA		A								
	CUG		CCG		CAG		CGG		G								
A	AUU	ile	ACU	thr	AAU	asn	AGU	ser	U								
	AUC		ACC		AAC		AGC		C								
	AUA		ACA		AAA	lys	AGA	arg	A								
	AUG	met	ACG		AAG		AGG		G								
G	GUU	val	GCU	ala	GAU	asp	GGU	gly	U								
	GUC		GCC		GAC		GGC		C								
	GUA		GCA		GAA	glu	GGA		A								
	GUG		GCG		GAG		GGG		G								

Figure 6.1 Table of codons

Reproduced from USDA National Institute of Food and Agriculture (2014)

Secondly, future work in this area should focus on determining the most suitable plant(s) for the transgenic phytoremediation of lindane. *Withania somnifera,* an important medicinal plant also known as Indian Ginseng, is likely to be better suited for phytoremediation owing to a larger biomass, rapid growth, easier culturing and harvesting and an innate ability to amass substantial amounts of lindane in its root, shoot and stem matrix (Abhilash & Singh, 2009a). Recent studies have shown that *Agrobacterium*-mediated transformation of *W. somnifera* is possible implying that the same methodology employed to modify model plant *A. thaliana* for phytoremediation studies may subsequently be applied to improve upon the natural capabilities of *W. somnifera* (Pandey, *et al*., 2010).

The results presented in this thesis imply that at concentration levels of 34 µM, the transgenic plant is successfully able to remove lindane from the medium, without its subsequent accumulation. Excluding the enzymes involved in the plant xenobiotic degradation pathway, and considering only the actions of LinA, 1,2,4-TCB is the compound most likely to be detected after incubation with lindane. The absence of 1,2,4-TCB suggests that naturally occurring plant enzymes may be involved in its degradation. The study of molecular interaction networks, referred to as

Interactomics, has successfully been adapted for use in the isolation of protein heterocomplexes in plants (Rohila, *et al.*, 2004). Investigating other protein-protein interactions involved in plant xenobiotic metabolism, and resulting metabolites, is key in establishing the overall efficiency of phytoremediation. This knowledge will provide insight into the potential accumulation of any toxic intermediates which may release back into the environment and / or food chain; and ultimately, any plants accumulating these toxic intermediates will likely require the use of clean-up technologies, post-phytoremediation, off-setting any environmental or economic benefits.

Finally, this work is specifically applicable to densely populated and developing countries such as India, the largest end-user and manufacturer of lindane in the world. Over 6000 tons of lindane were produced by India for domestic usage and export from 1997 to 2006, with restricted use still being permitted due to its cost and availability (Abhilash & Singh, 2009a). In North India, where land is predominantly used for crop production, lindane-contaminated soils are a serious concern due to its large-scale industrial production along with its continued agricultural use as a pesticide (Abhilash, *et al*., 2008). As several plant species within North India, including *W. somnifera*, have already demonstrated naturally abundant growth on lindane-contaminated soils, phytoremediation appears to be well-suited for the clean-up of these sites to ensure their continued and future use for crop cultivation.

However, due to its high hydrophobicity, the ability of Lindane to be taken up by plants is generally considered to be inefficient and difficult. This accumulation and resultant toxicity renders phytoremediation less useful without supplementation and optimization by means of rhizoremediation or phytostimulation. *Cytisus striatus* is another plant that has been proposed as a candidate phytoremedatiative species (Kidd, *et al*., 2008). In a previous study, two culturable endophytic and rhizosphere bacterial species, *Rhodococcus erythropolis* ET54b and *Sphingomonas* sp. D4, were found to be associated with *C. striatus* growing on an HCH-contaminated site (Becerra-Castro, *et al,.* 2011). In 2013, Becerra-Castro, *et al.* found that when these plants were inoculated with the two bacterial species and grown in the presence of HCH, a 120–160% increase in root and shoot biomass was observed followed by a

decrease in the activity of the anti-oxidative defence enzymes ascorbate peroxidise (APOD) and superoxide dismutase (SOD), essentially protecting the plants against any toxic effects from the contaminant. Inoculating *C. striatus* with this combination of bacterial strains showed higher dissipation of HCH isomers and could therefore be another promising approach for the remediation of Lindane.

The main findings from this thesis highlight the complex relationship between cross-kingdom genetic engineering, xenobiotic uptake, protein expression, and *in vivo* versus *in vitro* assays, to the study of phytoremediation. The multifaceted developmental process in the formation of transgenic plant species has driven researchers to employ a laboratory and model-based approach to studying phytoremediation. Although beneficial, the feasibility approaches employed to gain a mechanistic understanding of biological systems are often not conclusive enough to close in on the differences between experimental lab results and actual application in- field studies. The nature and properties of a laboratory test cannot be considered independently of the environment the new technology will eventually be used in. While almost impossible to include in the experimental set-up, bench assays tend to neglect the impact of other factors such as species competition, chemical exudates, external nutrients, pharmacological agents and inclement environmental conditions – all of which may seriously alter the experimental outcome. Therefore, a major shift of current research strategies towards actual in-field studies, under containment, using whole plants more suited to the phytoremediation of lindane is necessary to fully realize a bioremediation method by which it can be safely and quickly removed from the environment.

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