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Identification and characterization of an adenosine triphosphate binding cassette (ABC) transporter EcABCB1 involved in the transport of alkaloids in Eschscholzia californica

Uzuegbu, Perpetua
Identification and characterization of an adenosine triphosphate binding cassette (ABC) transporter EcABCB1 involved in the transport of alkaloids in *Eschscholzia californica* by

Perpetua Nwanyichukwu Uzuegbu

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Abstract

California poppy (*Eschscholzia californica*) roots and suspension cultures accumulate an assortment of structurally and functionally diverse benzylisoquinoline alkaloids, notably the antimicrobial sanguinarine in the cell and the media. In *E. californica* cell suspension cultures, alkaloid biosynthesis is induced by treatment with certain biotic and abiotic elicitors, which activates plant defense responses. Sanguinarine accumulated in the media is taken up into the cell through a potential active transport process. A large and widely distributed superfamily of proteins found in bacteria, fungi, plants and animals called adenosine triphosphate binding cassette (ABC) transporters have been implicated in the influx of alkaloids in plants. We report the isolation and characterization of a full-length cDNA encoding a homologous ABC transporter EcABCB1 from California poppy. The gene encoding EcABCB1 transcripts were detected in all California poppy plant organs. Evidence has shown that EcABCB1 is an inducible, plasma membrane localized, multi-specific ABC transporter sensitive to vanadate.
Acknowledgement

I would like to thank God Almighty for his faithfulness and continuous sustenance throughout the period of my studies. I am immensely and eternally grateful to my supervisor Dr. David A. Bird, for giving me the opportunity to engage in this interesting research, for his guidance and support all through the years and for taking the time out of his busy schedule to proofread my work and offer useful advice and comments. Also, I wish to express my sincere thanks and gratitude to my co-supervisor Dr. Peter J. Facchini, for providing a platform that allowed for the start of this interesting research work; for availing some of the materials, lab space and instruments and most importantly for his contributions which helped to steer the research forward. My sincere appreciation goes to my committee members Dr. Samuel Marcus and Dr. Ed Yeung, for all the useful advice they gave me with regards to my research and career path. Many thanks to my external/internal examiner Dr. Sarah Childs for all the useful comments.

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My sincere gratitude goes to Sean McDonald, who helped me the last batch of alkaloids from the media of cell suspension cultures. I thank Rebecca Joy Dalgarno for helping me collect the qPCR data of EcABC1B1 in whole plants. I will not fail to recognize the support I enjoyed from all the staffs at the Mt Royal University lab support centre.

Words alone cannot express how grateful I am to my family, my dad, mum, my ever caring brothers and sisters and my sisters’ in-law for their moral and financial support. Finally, my special thanks goes to my husband Anthony Azubuike, for his unwavering support throughout the period of my studies. For his willingness to listen to my daily ordeals and give me a piece of his wisdom. Anthony I am sincerely grateful and God bless you abundantly.
Dedication

This work is dedicated to my parents Mr & Mrs James Uzuegbu, my brothers Innocent, Augustine, and Leonard Uzuegbu; my sisters Lucy and MaryRose and my sister in-law Ogechukwu Juliet.
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<th>Definition</th>
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<tr>
<td>4’OMT</td>
<td>3’-hydroxy-N-methyl-(S)-coclaurine 4’-O-methyltransferase</td>
</tr>
<tr>
<td>6’OMT</td>
<td>Norcoclaurine 6-O-methyltransferase</td>
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<tr>
<td>ABC</td>
<td>Adenosine triphosphate binding cassette</td>
</tr>
<tr>
<td>ABCG</td>
<td>Adenosine triphosphate binding cassette transporter G</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine 5’-triphosphatase</td>
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<tr>
<td>BBE</td>
<td>Berberine Bridge Enzyme</td>
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<tr>
<td>BCIP (X-gal)</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>BIA</td>
<td>Benzylisoquinoline alkaloid</td>
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<tr>
<td>BLAST</td>
<td>Basic local alignment search</td>
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<tr>
<td>BtuC</td>
<td>Vitamin B12 ABC transporter</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>CFS (CYP719A5)</td>
<td>(S)-cheilanthifoline synthase</td>
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<td>CID</td>
<td>Collision-induced dissociation</td>
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<td>CjABCB1</td>
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<td>Coclaurine N-methyltransferase</td>
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<td>DNA</td>
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<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>DW</td>
<td>Dry weight</td>
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<tr>
<td>EcABCB1</td>
<td><em>Eschscholzia californica</em> adenosine triphosphate binding cassette transporter B1</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
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</tr>
<tr>
<td>EMM</td>
<td>Edinburgh minimal media</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
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<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>GW</td>
<td>Gateway</td>
</tr>
<tr>
<td>H⁺</td>
<td>Hydrogen ion</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>I-KI</td>
<td>Iodine-potassium-iodide</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>lacZ</td>
<td>Beta-galactosidase gene</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass divided by charge</td>
</tr>
<tr>
<td>MalF</td>
<td>Maltose transporter F</td>
</tr>
<tr>
<td>MalG</td>
<td>Maltose transporter G</td>
</tr>
<tr>
<td>MetI</td>
<td>Methionine transporter</td>
</tr>
<tr>
<td>Mg⁺</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>MIRA</td>
<td>Mimicking intelligent read assembly</td>
</tr>
<tr>
<td>ModB</td>
<td>Molybdate ABC transporter</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MRP</td>
<td>Multidrug resistance-associated protein</td>
</tr>
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<td>MSH</td>
<td>Methylstylopine hydroxylase</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
</tr>
<tr>
<td>P6H</td>
<td>Protopine 6-hydroxylase</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-schiff</td>
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<td>PBS</td>
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<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<tr>
<td>qPCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>RT</td>
<td>Retention time</td>
</tr>
<tr>
<td>S.O.C</td>
<td>Super optimal broth supplemented with glucose</td>
</tr>
<tr>
<td>SBP</td>
<td>Streptavidin binding peptide</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH</td>
<td>Sulphydryl</td>
</tr>
<tr>
<td>SR</td>
<td>Sanguinarine reductase</td>
</tr>
<tr>
<td>TBO</td>
<td>Toluidine blue O</td>
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<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlyenediamine</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
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<tr>
<td>TNMT</td>
<td><em>(S)</em>-tetrahydroprotoberberine cis-N-methyltransferase</td>
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<tr>
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<td>Tyrosine decarboxylase</td>
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<tr>
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<tr>
<td>UTP</td>
<td>Uridine-5'-triphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WBC</td>
<td>WHITE-BROWN COMPLEX</td>
</tr>
</tbody>
</table>
Chapter One: Introduction

1.1 Preamble

*Eschscholzia californica* (California poppy) is an annual/perennial herbaceous plant belonging to the family Paparavaceae, genus Eschscholtzia and order Ranunculales. It is indigenous to western North America where it is commonly used as an ornamental [1, 2]. Its root accumulates a wide variety of benzophenanthridine alkaloids, such as sanguinarine, protopine, marcapine, scoulerine and berberine. Berberine for instance is used to aid digestion and as an antidiarrhetic [3]. Sanguinarine has antimicrobial properties, in addition to its ability to cause apoptosis and disrupt the microtubule network [4-6]. The biosynthetic pathway of benzophenanthridines has been widely studied using California poppy cell cultures, mainly because of the inducibility of the enzymes involved in the biosynthetic pathway with microbial or fungal elicitor [7] or methyl jasmonate [8]. California poppy cell suspension cultures have the remarkable ability to secrete alkaloids into the media, making them a viable tool for investigating the transport and biosynthetic pathway of alkaloids. According to [9], California poppy cell suspension cultures treated with sodium orthovanadate responded by accumulating more alkaloid within the cells, while those treated with yeast extract elicitor responded by accumulating about the same amount of alkaloid but most of them gets secreted into the media. Based on this report, induction of alkaloid production occurs in both vanadate and yeast extract elicitor treated cells. Therefore, inhibition of transport is probably the reason why the induced alkaloids accumulate in the cells of vanadate-treated cell cultures instead of them being secreted into the media. In [9], they argued that if the inhibition of transport is due to the sodium orthovanadate inhibiting the H⁺
ATPase in cells, then the overall effect on alkaloid production would have been the inhibition of the total amount of alkaloids produced however, this is not the case as induction still occurs. Based on this observation, they suggested that Adenosine triphosphate binding cassette (ABC) transporters could be involved in the transport of alkaloids in the cell suspension cultures of California poppy, but no transporter has been associated with this activity [9]. In another alkaloid producing plant *Coptis japonica*, ABC transporters (CjABCB1 and CjABCB2) have been implicated in the import of alkaloid berberine [10, 11].

This study aims to identify a transporter involved in the transport of alkaloids in *E. californica*. The scope of secondary metabolism, a short review on ABC transporters and research objectives are all highlighted in chapter one. All the methods used to achieve the research objectives are presented in chapter two. In chapter three, EcABCB1 cloning with data in support of the involvement of EcABCB1 in the transport of alkaloids in California poppy are presented in chapter four, results from pharmaceutical studies and expression analysis done in California poppy cell suspension cultures are presented while the anatomy of the California poppy root is presented in chapter five.

1.2 Secondary metabolites

Secondary metabolites are natural products not directly involved in the growth and development of the organism producing it, but play indirect roles in the organism’s ability to survive [12, 13]. The term secondary metabolites was first defined by Albrecht Kossel in 1891 [14]. Other names like phytochemicals, anti-nutritional factors, natural products and plant xenobiotics have also been used to describe secondary metabolites [12, 15]. They are structurally and functionally diverse, low molecular weight compounds [12, 15]. Some of the established
sources of secondary metabolites include bacteria, corals, fungi, plants, snails, sponges and tunicates [16].

Information available from the sequences of genes involved in the primary and secondary metabolism, has given more insight on how new genes involved in secondary metabolism arise [17]. Gene duplication is thought to be the origin of new genes required for primary metabolism in plants [17]. Similarly, genes involved in secondary metabolism, are believed to have evolved from the genes involved in primary metabolism. For examples, some members of the cytochrome-p450 gene family are known to be involved in the primary metabolism of steroids while others are known to be involved in the secondary metabolism [17].

The divergence in function shared among genes of the same family such as in the p450 gene family suggests that they may have been redundant in function at least until mutation occurred [17]. This is in support of the hypothesis that secondary metabolites may be products of mutations which have persisted for a very long time [12, 18, 19]. Evidence supporting this hypothesis has been demonstrated in the study performed on resveratrol biosynthesis [19]. The enzyme stilbene synthase involved in the biosynthesis of resveratrol has been shown to have evolved by gene duplication and can be found in several other phylogenetically different plants [19]. It was then concluded that natural products that arose through this process are the ones favored for selection if they are advantageous to the plant [19].

Secondary metabolites are structurally diverse and are found across different plant species [13, 15, 20]. They include, polyketides, terpenes, alkaloids, and phenols [20]. Secondary metabolites like alkaloids, cyanogenic glycosides, toxic amino acids and saponins are toxic to mammals but advantageous to plants where they play a role in protecting plants from herbivory
and pathogens [15, 21]. Gibberellins, a plant hormone, belonging to a group of secondary metabolites known as diterpenes play a role in controlling seed germination [22], while others produce allelopathic responses by secreting chemicals harmful to other invading plants species [23]. All these together aid plants adapt to their environments [15, 21]. For the purpose of this thesis report, focus will be on alkaloids as plant secondary metabolites.

1.3 Brief description of alkaloids

The term alkaloid meaning alkali-like [24] was first used by W. Meiβner in 1819 [25]. Alkaloids are mostly derived from amino acids and so contain nitrogen in their chemical structure. Hence, they are often referred to as nitrogenous compounds [26, 27]. Amino acids like anthranilic acid, arginine, histidine, lysine, nicotinic acid, ornithine, phenylalanine, tryptophan, tryptamine, and tyrosine, are reportedly the precursor of most alkaloids [28]. Alkaloids are bitter in taste and majority of them have basic properties. They are widely varied in structure and function, with thousands of them found in plants [26, 27]. Various functions have been ascribed to plant alkaloids: (i) they are a source of nutrition for plants’ metabolic activities, (ii) they play defensive roles in plants, (iii) they are harmless end product of metabolism [29]. However, most established report favor their role in plants’ defence [23].

1.4 Uses of alkaloids

For centuries, alkaloids have been used traditionally as medicine largely due to their biological activity, and some have continued to be used in modern medicine. For instance, morphine which was first isolated from *Papaver somniferum* (opium poppy) in 1806 by Friedrich Wilhelm Sertürner. Its chemical structure was not unraveled until 1923 [14]. Before the
isolation of morphine, the opium poppy was traditionally used by Romans for murder and as a sleep portion and the active ingredient was morphine [28].

The use of two closely related compounds vincristine and vinblastine alkaloids, commonly known as the vinca alkaloids derived from the plant Catharanthus roseus (periwinkle) dates back as far as 1910 [30]. Traditionally, a leaf infusion was used in Brazil for the treatments of hemorrhage and scurvy, as a mouthwash, and in treating wounds [30]. In British West Indies, periwinkle was used to treat ulceration in diabetic patients while in the Philippines and South Africa, it was used in the treatment of hypoglycemia [30]. The traditional use of the vinca alkaloids as hypoglycemic agent inspired further research, which led to their current use in the treatment of leukemia and other forms of cancer [30, 31]. Another alkaloid berberine has long been used in the treatment of gastrointestinal diseases like diarrhoea in Asia [32]. Recent reports have shown that it has antibacterial and antimalarial activities [33].

About 25% of present day drug components originate from plants [34]. For a example: salicylic acid originally derived from the bark of the white willow tree is a major component of the drug aspirin [34] morphine and its derivatives from opium poppy are used as an essential component of analgesics [14, 31, 35]; while quinine from cinchona tree is used as an anti-malaria drug [25, 29].

Some other examples of plants’ alkaloids and their uses are as follows: caffeine is used as stimulant and anti-diuretic; nicotine as an insecticide, cocaine is used in local anaesthesia. Taxol, vinblastine and vincristine are used in treating cancer [25, 29]. Sanguinarine, berberine and chelerythrine have antimicrobial properties [36, 37]. Sanguinarine and chelerythrine also have anti-inflammatory properties [36, 38].
1.5 Drawbacks to the use of plants as a source of valuable secondary metabolites

The use of biological and medicinally active secondary metabolites like alkaloids has been limited by their availability in plants. Most of them are accumulate in such a small quantity making less available for commercial use in the industries [3]. Until recently, the functions and biosynthesis of many secondary metabolites has remained unsettled. With the recent technological advancements in biochemical, and pharmaceutical companies coupled with the available molecular biology tools, scientists have gained deeper understanding into the function and biosynthesis of many previously uncharacterized secondary metabolites such as the berberine alkaloid [10, 11, 31, 39, 40]. However, a few selected plant cells such as that of Coptis japonica can produce large quantities of secondary metabolite such as berberine alkaloid for commercial uses [6]. Cell cultures have also been a very useful tool in studying the biosynthetic pathways and transport mechanisms of these plant secondary products.

1.6 Localization of alkaloids

Most alkaloids are reportedly found at sites prone to attack by either herbivores or pathogens, such as in the inflourescence, shoot tips, and outer layers of stems and roots and they accumulate in such organs as the vacuoles where they are secluded from interfering with plant’s biological processes [28].

1.7 Classification of alkaloids

Classification of alkaloids have not been very easy probably due to their complex chemical and ambiguous nature. This notwithstanding, three different types of classification have been recognized, and they could be based on i) their physiological roles; ii) chemical structures; and iii) biosynthetic pathway [25]. However, the most commonly used method of classification
is based on the biosynthetic pathway of the alkaloids and this will be method of choice in this thesis report.

1.7.1 Classification based on biosynthetic pathway

The majority of alkaloids are classified based on their biosynthetic pathway because, not all the alkaloids are derived from amino acids [27-29]. Based on the biosynthetic pathway of alkaloids, they can be grouped as proto alkaloids, pseudo alkaloids and true alkaloids. Proto alkaloids lack nitrogen in their heterocyclic ring but have amino acid precursors. They are mostly derived from tyrosine and tryptophan. Examples are: phenethyamine alkaloids (Hordenine), indole alkaloids (Yohimbine) and pyrrolizidine alkaloids (stachydrine) [25], (Figure 1).
Figure 1: structures of representative members of proto, pseudo and true alkaloids. The class to which each structure belongs to is enclosed in parenthesis.
The carbon skeleton of pseudo alkaloids have nitrogen in their heterocyclic ring which is not derived from the core of an amino acid. Instead, pseudo alkaloids are linked to the pathways for the amino acid synthesis. The nitrogen found in the heterocyclic ring of pseudo alkaloids can be contributed by an amino acid during the transamination reaction in the presence of a suitable aldehyde or ketone [25]. Some pseudo alkaloids are derived from the amino acid precursors, while others are a resultant product of transamination and amination reactions of pathways linked with the precursors or postcursors of amino acids [25]. They are found mostly in the plants belonging to the following families: Apocynaceae, Buxaceae, Escalloniaceae, Liliaceae, Ranunculaceae, Garyaceae, and Solanaceae. Examples are the purine alkaloids (caffeine and theobromine), steroid alkaloids (cyclopamine and solanidine), terpenoid alkaloids (aconitine and gentianine), and aromatic alkaloids (capsaicin) [25] (Figure 1).

True alkaloids have nitrogen in the heterocyclic ring and are derived from amino acids such as L-histidine, L-lysine, L-ornithine, L-phenylalanine, L-tryptophan and L-tyrosine (Fig 1) [25, 27]. For instance, isoquinoline alkaloids which are true alkaloids are synthesized from the amino acids phenylalanine and tyrosine [25]. True alkaloids are bioactive and usually soluble in water [25]. Examples include; morphine, sanguinarine, berberine and cocaine. All the alkaloids considered in this thesis are true alkaloids.

True alkaloids are grouped further according to the structure of their backbone. Examples include alkaloids of the major group quinoline, indole, ergot, simple tetrahydroisoquinlines, phenethylamino, piperidine, indolizidine, quinolizidine, pyrrolizidine, imidazole, acridone and pyridine. Most of the alkaloids used in this study are true alkaloids and fall under the major
group simple tetrahydroisoquinoline alkaloids. For this reason, this major major class will be discussed further.

1.8 Simple tetrahydroisoquinoline alkaloids

These groups of alkaloids are structurally simple just as the name implies. They are usually derived from the core of one aromatic amino acid residue such as the phenylalanine or tyrosine [41]. So they have just one aromatic core and a methylenedioxy substituent [41]. Simple tetrahydroisoquinoline alkaloids are formed from a condensation reaction between phenylethylamines and the aldehydes [42]. They are the major component of the structural backbone of the basic alkaloid 1-benzylisoquinolines (BIAs) [42].

1.8.1 1-Benzyliisoquinoline alkaloids

1-benzyliisoquinoline forms the structural backbone of different kinds of alkaloids with diverse chemical structures [43]. Examples include the benzophenanthridines (sanguinarine), morphinans (morphine), phthalideisoquinolines (noscapine); pavines (eschscholtzidine); bisbenzyliisoquinolines (tubocurarine); benzylisoquinoline (papaverine, aporphine) and proberberine (berberine) [43, 44].

Members of the BIA class share in common the tyrosine precursor in the early stages of their biosynthetic pathway [28]. They are found in most plants belonging to such families as Papavaraceae, Berberidaceae, Ranunculaceae, Menispermaceae, Fumariaceae, Fabaceae, Annonaceae, Eupomatiaceae, Aristolochiaceae, Magnoliaceae, Lauraceae, Monimiaceae and Nelumbonaceae.
1.8.2 Benzophenanthridines

Benzophenanthridines are a subclass of benzylisoquinoline alkaloids. Many of the members of this subclass are thought to possess ecophysiological roles and antimicrobial properties [45, 46]. They are mostly found in plants belonging to the families, Papavaraceae and Fumeriaceae [7, 46-55]. They are generally toxic and their toxicity is based on their structure which has a mix of aromatic and heterocyclic ring (Figure 1) and their ability to react with SH-containing compounds. They toxify cells through the intercalation of double stranded DNA, inhibition of transcription and replication of DNA as well as binding to the negatively charged membrane surfaces [55]. They also inhibit cytosolic and membrane bound enzymes such as Na⁺/K⁺ ATPases, lipoxygenases, etc. [55]. However, plants have a mechanism to keep their toxicity in check, this they do by keeping the toxic compounds in vesicles and vacuoles [55].

1.8.2.1 Biosynthesis of benzophenanthridines

Radioactively labeled carbon experiments, gene discovery and enzymology have been very useful in elucidating the different biosynthetic pathways of benzylisoquinoline alkaloids (BIAs) [28, 53]. For instance, radioactive labeled experiment with ¹⁴C showed that (S)-nocorclaurine is involved in the BIA biosynthesis. (S)-nocorclaurine is formed through the condensation of dopamine and 4-hydroxyphenylacetaldehyde, both of which were derived from tyrosine [28, 53]. (S)-N-methylcoclaurine is derived from (S)-nocorclaurine through the enzyme specific action of O-methyltransferase and N-methytransferase. 3’ hydroxylation of (S)-N-methylcoclaurine is catalyzed by (S)-N-methylcoclaurine-3’-hydroxylase (CYP80B1) followed by 4’methylation catalyzed by 4’-O-methyltransferase (4’OMT) to yield (S)-reticuline [53]. Two enzymatic pathways branch out from (S)-reticuline, both leading to the biosynthesis of different
groups of alkaloids such as the benzopehenanthridines, protoberberines and morphinans. Each of these groups has different properties and roles for the plants.

The conversion of (S)-reticuline to (S)-scoulerine, a reaction catalyzed by berberine bridge enzyme (BBE) is the first step devoted towards the biosynthesis of benzophenanthridines is [28, 48, 53] (Figure 2).
Figure 2: Schematic representation of benzylisoquinoline alkaloid biosynthetic pathway. Enclosed in brackets are the subgroups to which the alkaloids belong. The enzymes shown are the ones studied and reported in chapter four. Each arrow represents an enzymatic step.
Elicitation with biotic elicitor such as yeast extracts can activate two signal events depending on the dosage of the elicitor. At low concentration, the elicitor activates a G-protein which is coupled to phospholipase A2 at the plasma membrane. The activated A2 goes on to trigger lysophosphatidyl choline (LPC) peak. This peak then spreads out in the cytoplasm to stimulate the Na⁺/H⁺ antiporters at the tonoplast and simultaneously triggers the efflux of protons from the vacuole. This results in a change in the pH of the cytoplasm. This pH change in turn signals the expression of alkaloid biosynthetic gene [56]. However, at high elicitor concentration, a peak of jasmonate and hypersensitive cell death is stimulated, all in a different pathway without acidifying the cytoplasm [47, 56, 57].

1.9 Indications of transport in California poppy

Some plants such as the California poppy respond to pathogenic attack by producing phytoalexins [9, 47, 51]. Cell suspension culture of *E. californica*, has been shown to secrete benzylisoquinoline alkaloids into the media [7, 46-55]. They also respond to fungal elicitor preparation by accumulating more benzophenanthridines and secreting them into the media [9, 47, 58]. Induction of the cells can be done with abiotic (methyl jasmonate) and biotic elicitors (fungal extract), making them a good model system for the study of plant chemical compounds and their pathways as in the case of alkaloids [9, 47, 51].

Accumulation of alkaloids in California poppy cells incubated for 6 hours with fungal elicitor preparation has been reported [47]. Two BIA biosynthetic enzymes namely berberine bridge enzyme (BBE) and protopine-6-hydroxylase (P6H), found downstream the pathway of benzophenanthridine alkaloid biosynthesis have been reported to be induced when *E.californica* cells are incubated with fungal elicitor preparation [51]. Proteome and transcriptome analysis
have shown some proteins increase after treatment with elicitor and biosynthetic enzymes constitute only a small portion of the upregulated proteome [47].

1.9.1 Organ specific localization of members of B1As

Benzophenanthridines have been reported to accumulate in different cell types, such as in the idioblasts of *Sanguinaria canadensis* and laticifers of *Chelidonium majus* [59]. Evidence has also shown that some alkaloids accumulate in plant tissues different from the tissues expressing the biosynthetic genes. For instance, nicotine alkaloids from *Nicotiana* spp. accumulate in the leaf vacuoles whereas its synthesis occurs in the roots. In *Coptis japonica*, its main alkaloid berberine accumulates in the rhizome while the berberine biosynthetic genes are expressed in the root [3, 10]. Some intermediates have been reported to move from cell to cell within a plant organ. For instance, in *Catharanthus roseus*, the intermediates for vinblastine alkaloid move from the parenchymatous phloem to the epidermis and finally to the laticifers and idioblasts [3]. All these evidence suggest that some kind of transport mechanism is involved in the movement of these compounds and or their intermediates from one cell, organ and or tissue to the other.

ABC transporters have been implicated in the transport of secondary metabolites in many species of metabolites producing plants. For example, ABC transporters have been implicated in the transport of auxin [60-64], and cuticular lipids [64-66], diterpenes [67], phenolics [68-70], auxins [68], 4-Nitroquinoline N-oxide, berberine alkaloid [10, 11] and catharanthine [71]. Among the ABC transporters implicated in the transport of the above mentioned metabolites, CjABCB1 [10] and CjABCB2 [11] from *Coptis japonica* are the only known ‘B’ subfamily members involved in the transport of the alkaloid notably berberine [10, 11]. More recently,
another ABC transporter CrTPT2 belonging to the ABCG subfamily member from *Catharantus roseus* has been implicated in the transport of alkaloid catharanthine [71].

**1.10 Adenosine triphosphate Binding Cassette (ABC) transporters**

ABC transporters are broad superfamily of proteins found in living organisms including bacteria, fungi, plants and animals where they employ the energy of ATP hydrolysis to transport a wide array of molecules. They are one of the largest transporter groups [72]. Their involvement in a variety of processes such as in the biosynthesis of chlorophyll, assembly of iron/sulphur clusters, movements of the stomata, and maybe the flux of ion. Many have been identified and their abundance varies from one species to another. For instance, there are about 129 ABC transporters in *Arabidopsis*, 69 in *E.coli*, 51 in *Drosophila*, 58 in *Caenorhabdittis* and 28 in *Saccharomyces cerevisiae* [73], 121 in *Oryza sativa* and 50-70 in humans [74].

A typical ABC transporter consist of two nucleotide binding domain (NBD) facing the cytosolic side of the lipid bilayer where it couples ATP binding and hydrolysis to transport of substrates and 2 transmembrane domains (TMD) mainly composed of six α-helices which span across the membrane to form the translocation pore [75, 76]. However three distinct folds have been recognized in the TM α-helices of ABC transporters suggesting a high degree of heterogeneity among different ABC transporters [72]. Based on these different folds, ABC transporters have been grouped into three classes, namely: Type I ABC importers, Type II ABC importers and ABC exporters [72].

Some examples of Type I ABC importer class is the ModB, a molybdate transporter, two maltose transporter MalF and MalG and methionine transporter (MetI). A single subunit of the TMD of each of ModB, MalF, MalG and MetI is made up of six, eight, six and five α-helices
respectively. So a full transporter for each of these would compose of twelve, fourteen twelve and ten TM helices respectively[72].

Type II ABC importer class has been found in BtuC; vitamin B12 importer and its homologue HI1471 from *Haemophilus influenza*. In this group of ABC transporters, the full transporter has a total of twenty TM helices. Which means a subunit of the TM helix is composed of ten α-helices [72].

The TMD subunit of the ABC exporter class contains six α-helices, accordingly, a full transporter contains twelve TM α-helices [72]. Substrates bind to the TMDs of ABC importers thus enabling them to be specific for the substrate they transport although many are not substrate specific [73]. The formation of hydrogen bonds between substrates and the ABC protein is thought to be responsible for the substrate specificity of the ABC transporter like the p-glycoprotein [77].

For proteins in the ABC superfamily, the TMDs are not conserved among families, but the NBDs are. The NBDs have about 200 amino acids residues consisting of seven distinct and highly conserved sequence motifs including the Aromatics (Y-residue), Walker A box (P-loop) with sequence motifs (GXXXGXGKS/T) where X stands for any amino acid, Q-loop, ABC-signature motif or C-loop (LSGGQ), Walker B box (ILLDEAT), D-loop and H-loop also referred to as switch regions of the protein [72-74, 78]. All these different motifs perform different functions in the ABC cassette [72-74, 78]. Among these conserved motifs, the P-loops or walker A found in the RecA-like sub-domain, and the ABC signature motif or C-loop found in the helical sub-domain, are most important. Walker A and B partake in binding to Mg$^{2+}$-ATP [72]. The q-loop interacts with the hydrolysable phosphate of the ATP through the water
molecule [73]. With the exception of mutated transporter with only one functioning ATP binding site, example cystic fibrosis transmembrane regulator (CFTR), two ATP molecules are generally thought to be consumed in full transporters with each cycle of transport activity [78]. The ATP-binding domain of ABC transporters is the cytosolic domain of ABC transporters [79, 80]. In some organisms, the four core domains of ABC transporters alternate to form a functional transporter with a typical TMD–ABC–TMD–ABC structure [74].

### 1.10.1 Features of ABC Transporters

The four domains of ABC transporters can be found in a single polypeptide as ‘full transporters’ in two orientations, forward (TMD1-NBD1-TMD2-NBD2) and backward NBD1-TMD1-NBD2-TMD2 [74]. They could also be found in two different polypeptides one polypeptide containing one TMD fused to one NBD while the second polypeptide contain the second TMD fused to the second NBD, example the RED, BROWN and WHITE pigment transporters of *Drosophila* or two NBD fused together in one molecule in one polypeptide and two TMDs fused together in a different polypeptide, for example ribose permease of *E.coli* [74]. When they occur in either of the two latter ways, they are said to be ‘half transporters’. In half transporters, ABC transporters become functionally competent, only after specific dimerization between the two separate polypeptides housing the functionally important domains [74]. Many of the ABC transporters from bacteria have the four core domains distributed in four polypeptides. In this case, they are referred to as a ‘quarter transporter’ [74, 81].

ABC transporters have three salient features: i) energy from the hydrolysis of ATP drives the transport activity. However, in some transporters, nucleotides like guanosine triphosphate (GTP) or uridine triphosphate (UTP) can be used in place of ATP. ii) The activity of an ABC
transporter is independent of the H⁺ gradient across the membrane. Disruption of H⁺ transporters by use of protonophores or ionophores does not affect the transport activity of ABC transporters [72, 74, 82]. iii) Analogs of orthophosphate such as vanadate inhibit the activity of ABC transporters through competitive inhibition. After the hydrolysis of ATP at the NBD and release the hydrolysable phosphate, vanadate binds competitively to ADP pockets thereby preventing another phosphate from binding. This in turn prevents another cycle of hydrolysis from occurring [72, 74, 82].

1.10.2 Types of ABC transporters

ABC transporters are grouped into two classes namely: exporters and importers.

1.10.2.1 ABC exporters

ABC exporters are found in all life forms and they translocate substrates of different sizes from the cytoplasm or inner leaflet of the membrane to outside of the cell or into cell organelles such as vacuoles and mitochondria[73]. ABC exporters traffic drugs, toxins, metabolites and lipids across biological membranes [72, 73]. ABC exporters in their resting state are in an inward facing conformation, with the binding site for the substrates facing the cytoplasmic side of the membranes [72, 74, 82, 83]. Binding of ATP to the NBDs changes the conformation of the transporter to an outward-facing direction. This change in conformation is also able to push the substrates to be secreted outside the cell or inside the organelle. ATP-hydrolysis and release of hydrolysis product restores the transporter to an inward facing conformation completing the transport cycle [73, 74, 82-85] (Figure 3).
Figure 3: Structure and mechanism of transport for ABC exporters. Binding of substrates to the TMDs activates the transport cycle by inducing a change in the conformation of the TMDs. The allosteric interaction between the substrates and TMDs affects the affinity of the NBDs to the ATP causing them to have high affinity for ATP. Binding of ATP cause the NBDs to dimerize and changes the TMDs to an outward facing conformation. ATP hydrolysis and release of products of hydrolysis ADP and Pi disassociates the dimerized protein and resets it to an inward facing open dimer conformation.

1.10.2.2 ABC importers

ABC importers on the other hand are mostly found in prokaryotes, although recently it has been identified in plants. For example, CjMDR1 has been identified as an ABC importer of berberine alkaloid *Coptis japonica* [10]. In typical ABC importers, substrate binding proteins (SBP) mediate transport. The SBD has two domains which normally rotate towards each other when bound by substrates. In ABC importers, the receptor and NBD with bound ATP are in open conformation at resting state. Binding of substrates to the receptor closes the receptor and
triggers the dimerization of the NBD while changing the TMD to an outward facing conformation. The disassociation of the NBD commences with the ATP hydrolysis which further cause substrates to be released thereby completing the translocation events. Release of hydrolysis product ADP and Pi restores the importer back to an open resting state conformation [73, 83] (Figure 4).
Figure 4: structure and mechanism of transport for ABC importers. Substrate binding proteins serves as a delivery tool for the ABC importers. Binding of ATP to the NBDs triggers the dimerization of the NBDs coupled to the change in the conformation of the TMDs to face outwards, the creation of the binding pocket for the substrate and the release of the substrate from the substrate binding protein. ATP hydrolysis disassociates the dimerized NBDs, which is coupled to the change in the conformation of the TMDs to an inward facing and the release of substrates into the cytoplasm.

1.10.3 Classification of ABC transporters

Based on the amino acid sequence of the NBDs, ABC transporters can be grouped into eight subfamilies (A-H) independent of the originating organism [73, 80]. In plants, some families are mainly composed of full transporters while others such as the ABCGs have both full and half transporters. Plant ABC family members have been localized to different subcellular organelles. For example; many members of the plant ABCG family localize to the plasma
membrane, vast majority of the ABCCs localize to the tonoplast while the ABCDs are mostly localized to the peroxisome [86, 87].

A number of ABC transporters have been shown to be involved in the transport of secondary metabolites in plants [31]. For instance, CJMDR1 and CjABC2 has been implicated in the transport of berberine in *Coptis japonica* while CrTPT2 mediates the transports of catharanthine in *Catharanthus roseus* [71].

### 1.11 Conclusion

Most secondary metabolites, specifically alkaloids are found in specialized tissues and/or cell types within the plant. Oftentimes, they are transported from the site of synthesis to the storage site in plant [88]. For instance, morphine synthesis occurs first in phloem, before being transported to laticifers (4).

These translocation events indicate that these compounds are transported within cells, tissues and/or organs. While, mechanisms of membrane transport for these alkaloids remain unclear, Adenosine triphosphate binding cassette (ABC) transporters have been implicated in the transport of plant hormones (6), waxes (5) and secondary metabolites such as berberine and sclareol (7, 1, 6). For example, *CjMDR1* is an ABC transporter that takes up berberine when expressed in frog oocytes (7).

Renewed interest in the structure of California poppy root has largely been driven by the medicinal properties of its alkaloids and suggestions that active transport processes could be involved in the trafficking of the alkaloids. This proposition stemmed from the knowledge gained from the arrangement of the California poppy root cells which is similar to the opium poppy root cell arrangement (see detailed discussion in chapter five). Knowledge of the cell-
specific accumulation of these compounds will help to decipher the potential routes of the alkaloids making it easier for bioengineers to manipulate the accumulation of alkaloids for better and commercial production.

ABC transporters are membrane proteins that require energy in the form of adenosine triphosphate (ATP) to move substrates across cell membranes. Finding these ABC transporters and other transporters will have profound biotechnology implications. Understanding the mechanisms that plants use to produce these compounds can be exploited to better produce secondary metabolites of high commercial value.

1.12 Objectives

I. Molecular cloning and functional characterization of a candidate transporter

II. To test the potential involvement of active processes in alkaloid trafficking by means of inhibition experiment with ABC transporter inhibitor drugs

III. To describe the anatomy of the *Eschscholzia californica* root

1.12.1 Identification of ABC transporter candidates

*E. californica* was used as a model system in this study because of the high accumulation of alkaloids its root. There is also high accumulation of alkaloids mostly the benzophenanthridines in its cell cultures [7, 9, 46, 47, 49, 54, 55, 89, 90]. In addition to this, the cells when maintained as a suspension culture secretes the alkaloids into the media and takes back some of the secreted alkaloid into the cells [55].

In order to identify candidate ABC transporter(s), total RNA will be extracted from cell suspension cultures. The RNA will be reverse transcribed to generate cDNA. A homology search of the California poppy database (http://www.phytometasyn.ca/) with the *CjABCB1* sequence
will be done. Hits with the highest percentage homology will be chosen and primers will be
designed from those sequences. cDNAs generated from the total RNA will serve as a template
for the amplification of the candidate gene. Putative ABC transporter(s) will be cloned into
bacteria. Positive clones will be confirmed by sequencing. Characterization and further studies
will be done using a yeast transport assay.

**1.12.2 Cloning and functional characterization of putative transporter**

Putative ABC transporter(s) will be cloned into double mutant (*mam1 pdr1*) *Schizosaccharomyces pombe* yeast strain. Expression will be confirmed by western blotting. Yeast cells expressing the candidate transporter will be used as a model system to study the
function of the candidate transporter.

MAM1 and PDR1 are yeast ABC transporters localized to the endosomes, endoplasmic reticulum (ER) and plasma membrane [91, 92]. MAM1 is involved in the transport of mating pheromone, M-factor in *S. pombe* [91, 93]. The double mutant strain is used to minimize background during the characterization study. It has been used successfully in an auxin transport assay of Arabidopsis *thaliana* ABCB protein which functions in auxin export [92].

We hypothesize that EcABCB1 will take up alkaloids from the media considering the sequence similarity to CjABCB1 which also mediates uptake. Amounts taken up will be quantified using LC-MS and compared to the control.

**1.12.3 Transient expression of EcABCB1 in Nicotiana tabacum**

We hypothesize that EcABCB1 is localized to the plasma membrane. Subcellular localization of the EcABCB1 will be determined by transiently expressing the gene in tobacco leaf. GV 3101 *Agrobacterium tumefascien* strain will be used to transform YFP-EcABCB1
construct. Positive transformants will be used to infiltrate *Nicotiana tabacum* leaves. Successful expression of the transgene in the tobacco leaf will be confirmed using the confocal laser scanning microscope.

**1.12.4 Confirmation of the accumulation of alkaloids in the *Eschscholzia californica* media**

The cell suspension cultures of *Eschscholzia californica* (California poppy) biosynthesize benzylisoquinolines and secrete them into the media [7]. The amount biosynthesized increases when they are treated with elicitor preparation from fungal cell walls [9].

In order to verify the accumulation of alkaloids in the cells and the media, extraction of alkaloids from the cells and the media will be performed. Successful extraction will be confirmed by use of thin layer chromatography (TLC) [94]. Identification and relative quantification of the extracts will be done using liquid chromatography-mass spectrophotometry (LC-MS).

**1.12.5 qPCR of selected BIA biosynthetic enzymes to check that the inhibited transport does not affect de novo synthesis of selected gene transcripts**

The transcripts of some of the enzymes involved in the biosynthesis of alkaloids in opium poppy can be induced by yeast extract elicitor [53]. Cell suspension cultures of California poppy will be incubated with yeast extract elicitor and ABC transporter inhibitor drugs. Total RNA will be extracted from the treated cells and reverse transcribed. The transcripts of selected BIA biosynthetic genes will be quantified using quantitative Real Time-Polymerase chain reaction (qPCR).

The aim of quantifying some of these mRNAs is to check for any increase in steady state level of transcripts after elicitation and treatments with drugs. This quantification will be useful
in checking the possibility that the drug treatment is blocking *de novo* synthesis of BIA biosynthetic genes as opposed to secretion.

**1.12.6 Testing the potential involvement of active processes in alkaloid secretion by means of inhibition experiment with ABC transporter inhibitor drugs**

To test the potential involvement of active processes in alkaloid excretion into the media, elicited cell suspension cultures of California poppy will be treated with sodium orthovanadate, cyclosporine A and thioridazine. Inhibition will be observed by use of TLC [94] and confirmed by use of LC-MS. LC-MS will also be used to identify and quantify the inhibited compounds.

In *Coptis japonica*, sodium orthovanadate and cyclosporine A has been used to inhibit the uptake of berberine [39] while in *California poppy*, sodium orthovanadate has been used to block the secretion of alkaloid into the media [9]. However in animals, thioridazine and cyclosporine A has been used as multi-specific inhibitor of three human ABC transporters P-glycoprotein (P-gp/ABCB1), Breast cancer resistant protein (BCRP/ABCG2) and multidrug resistance–associated protein MRP2 (ABCC2). Throughout this report, sodium orthovanadate will be referred to as vanadate.

**1.12.7 California poppy root anatomy**

Extensive studies have been done on the anatomy of the opium poppy a distant relative of California poppy but not much has been done on the anatomy of California poppy root. Understanding the basic anatomy of the California poppy root could help in delineating the possible transport route of the alkaloids in the root as well as the subcellular location of the alkaloid’s biosynthetic enzymes. The root anatomy of California poppy will be discussed using fixed and embedded serial sections and the free hand sections of the root.
Chapter Two: Materials and methods

2.1 Introduction

All the experimental materials and methods used to achieve the research objectives will be highlighted in this chapter. Restriction maps and vector maps were also shown in this chapter. Unless stated, all the chemicals used were purchased from Sigma Aldrich (Sigma, St Louis, MO, USA, http://www.sigmaaldrich.com). All the primers used were purchased from integrated DNA technologies (IDT) (http://www.idtdna.com/site), unless stated otherwise.

2.2 Viability test

Viability test was done according to the protocol outlined on Common Access to Biological Resources and Information website (http://www.cabri.org/guidelines/plant/405.html). The aim is to determine the growth curve and the viability of *E. californica* cells in suspension cultures. 10 ml of 7 days old cells were inoculated into 50 ml of fresh plant media in 250 ml flasks. The viability of the cells over 18 days period. Cells were harvested every 3 days for the viability and growth curve analysis. 1 ml of cells treated with elicitor and/or drug and incubated for 2 hours was aliquoted into a 1.5 ml microcentrifuge tube and incubated on ice. 5 μl of 5 mg fluorescein Diacetate (FDA) dissolved in 1 ml acetone was added and incubated for 5 minutes on ice. A small aliquot of the solution containing FDA stained cells is placed on a microscope slide and covered with a cover slip. The greenish fluorescence of the cells is observed at 100x to 400x magnification under a light microscope with fluorescence equipment (filter set 09 ZEISS Co., λex = 450 – 490 nm, λem = 520 nm).
2.3 Alkaloid extraction

2.3.1 Alkaloid extraction from the cells

Alkaloids were extracted according to [95]. Briefly treated and/or untreated cell cultures were separated from the media by vacuum filtration. Cells were dried using a freeze dryer. Dried tissues were extracted with Bieleski extraction solution comprising of methanol, formic acid and water in a ratio of 15:1:4, v/v at 20 mL/g dry weight [95]. The cells in the solution were thoroughly ground using a ceramic mortar and pestle. Alkaloids were passively extracted at -20°C for 12 hours. To pellet debris, the extracts were centrifuged for 10 min at 14,000g and supernatants were transferred into a clean 1.5 mL tube, and reduced to dryness with no heat using speed-vac (Thermo fisher scientific Savant, Ramsey, MN). The dried extract was re-suspended in Bieleski solution.

2.3.2 Alkaloid extraction from the media

Alkaloids were extracted from the media according to [51]. Cells were separated from the media using vacuum filtration. 1:1 v/v sodium carbonate/bicarbonate buffer (Sigma, St Louis, MO, USA), pH 10.0 was added to the media and transferred to a separatory funnel. 2:1 v/v ethyl acetate (www.fisher.sci) was added to the media/buffer solution. The entire mixture was inverted 10 times without shaking for proper mixing of the solvent with the media/buffer solution. The mixture was left to stand for five to ten minutes to allow for the separation of the aqueous phase from the organic phase. The organic phase is preserved while the extraction process is repeated two more times using the aqueous phase. The ethyl acetate extract was vacuumed to dryness under reduced pressure using a rotary evaporator (Heidolph Hei-Vap Value, http://www.heidolphna.com) set to 50 degrees Celsius. The resulting alkaloid residue was
dissolved in methanol, transferred to 1.5 ml eppendorf tube and vacuumed to dryness using the speedvac. The final residue was re-suspended in 200 µl Bieleski solution. Extracts used for liquid chromatography mass spectrometry was filtered and diluted to 1 ml using Bieleski solution.

2.4 Elicitor preparation

Yeast elicitor was prepared according to [96]. 200 g of yeast extract was dissolved in 1 liter of deionized distilled water. 80% (v/v) ethanol was added and stirred. The solution was allowed to settle for 4 days at 4-6 degrees Celsius. The supernatant was carefully decanted and discarded leaving behind the precipitate. The precipitate was again dissolved in 1 liter of deionized H₂O and precipitated for the 2nd time with 80% ethanol at 4-6 degrees Celsius for another 4 days. At the end of 4 days incubation, the supernatant was carefully removed and the second ethanol precipitate was dissolved in 400 ml of deionized dH₂O. This preparation is henceforth referred to as crude fraction in this report. This crude fraction was the elicitor used for all the elicitation work done in this project.

2.5 Induction of alkaloid accumulation using yeast cell wall extract

6 days old cell suspension cultures in 250 ml flasks were induced with 1.25 ml of fungal elicitor prepared according to [96]. The elicited cells were harvested at 1day intervals for a total of three days. Alkaloids extracted from the media were analyzed by TLC. Berberine and sanguinarine standards (Sigma, St Louis, MO, USA) were used to track the retention point of berberine and sanguinarine on TLC plates for annotation.
2.6 RNA extraction

Total RNA was prepared using the chloroform / trizol method modified from [http://animal.ifas.ufl.edu/hansen/lab_protocol_docs/extraction_purification_rna_trizol_tri_reagent.pdf](http://animal.ifas.ufl.edu/hansen/lab_protocol_docs/extraction_purification_rna_trizol_tri_reagent.pdf). Plant cells harvested and frozen at – 80°C for 1-2 days were homogenized in liquid nitrogen with mortar and pestle to a fine powder. An equal volume of trizol or tri reagent Sigma, St Louis, MO, USA) was added (1 ml/mg of cell), and ground together with the powdered cells. The samples were incubated at room temperature for 5 minutes and then transferred to a labelled 1.5 ml tube. 0.2 ml of chloroform was added; the tubes were capped and shaken vigorously by hand for 15 seconds, then incubated at room temperature for 5 minutes and centrifuged for at 12,000x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube. 500 μl of isopropyl alcohol was added and mixed together to pellet the RNA. The mixture was incubated for 5 minutes at room temperature, and centrifuged at 12,000 xg for 10 minutes at 4 degrees. The supernatant was discarded. 1 ml of 75% ethanol was used to wash the RNA pellet. The pellet was either air dried at room temperature, dissolved in RNase free water and stored at -80°C freezer or stored as a pellet in ethanol at -80°C freezer until needed for use. Before use RNA quality was confirmed by measuring the UV absorption ratios at 260/280 nm and 260/230 nm and by agarose gel electrophoresis. All the RNA samples used in the reverse transcription had UV absorption of about 1.9 and 2.1 at 260/280 nm ratio and 2.0 to 2.4 at 260/230 nm ratio. The concentrations of the RNA samples were determined using nanodrop spectrometer.

2.7 Reverse transcription

1 μg of total RNA was reverse transcribed using iscript cDNA synthesis kit (BIO RAD. CA. USA) or RNA to cDNA EcoDry Premix from Clontech (Clontech, Palo Alto, CA, USA).
Both kits contained a mix of oligo dT and random primers. For iscript cDNA synthesis kit, the reaction was set up as follows, 4 μl of 5x iscript reaction mix, 1 μl iscript reverse transcriptase, up to 20 μl with nuclease free water and incubated for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C and Hold at 4°C in an eppendorf master cycler pro 8 vapo protect or Eppendorf master cycler gradient PCR machine. For cDNA EcoDry premix, 1 μg of total RNA diluted in 20 μl of nuclease free water was used to dissolve one tube of the cDNA EcoDry Premix by either vortexing or pipetting up and down. The reaction mixture was incubated at 42°C for 60 minutes, 70°C for 10 min and hold at 4°C.

2.8 Homology search and EcABCB1 cloning

2.8.1 Amplification of EcABCB1

1 μg of total RNA extracted from 6 days old *E. californica* cells in a suspension culture was reversed transcribed using iscript cDNA synthesis kit (BIO RAD. CA. USA). The resultant first strand cDNA was used to amplify EcABCB1. The sequence of CjABCB1 publicly available in [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/) (ncbi) was blasted over transcripts assembly in the *E. californica* database [97, 98]. The database is composed of next generation sequence data reads assembled using MIRA, and Illumina sequence data reads assembled using Velvet Oasis [97]. Primers were designed from sequences with the closest identity to CjABCB1 particularly EcartPF1 c656 and EcartPF1 c332 (Table 2). Amplicons were confirmed by the use of agarose gel electrophoresis. Gels contained ethidium bromide for visualization under Ultraviolet (UV) light.

The optimal annealing temperature of the primers were determined using gradient PCR (G = 53°C ± 5°C) EcABCB1 was amplified using Phire Hot Start II DNA Polymerase (Thermo
Fisher Scientific) in a 0.2 ml tubes containing 4 μl of 5x reaction buffer, 200 μM dNTP, 0.5 μM forward and reverse primers, 2 μl of cDNA, and 0.5 μl of phire hot start II DNA polymerase, volume to 20 μl.

2.8.2 Linearization of pSTBlue Vector

pSTBlue vector (Figure 5) used in this study contained kanamycin and ampicillin resistance with a functional lacZ gene spanning across the multiple cloning site (MCS). pSTBlue was linearized by digesting with 10 units EcoRV purchased from New England Biolabs (NEB, Ipswich, Suffolk, USA). A 20 μl reaction contained: 13.2 μl of milli q H₂O, 2 μl of 10x Buffer 4, and 4.3 μl of Plasmid DNA. The reaction was mixed briefly by gentle vortexing and given a quick spin to concentrate the mixture at the bottom of the tube. 0.5 μl of EcoRV enzyme was added to the tube and mixed briefly by gentle tapping. The reaction mixture was incubated at 37°C for 1 hour. Linear pSTBlue was confirmed by agarose gel electrophoresis.

2.8.3 Blunt end ligation of EcABCB1

PCR product of EcABCB1 was ligated into the linear pSTBlue vector using 4:1 insert: vector ratio. A 10.5 μl reaction contained: 4.5 μl of milli q H₂O, 2 μl of 10X ligation buffer, 0.8 μl of linear pSTBlue vector, 3.2 μl of EcABCB1 insert and 0.5 μl of T4 DNA ligase (NEB). Ligation reaction mixture was mixed briefly and incubated at 16°C for overnight. Ligation reaction mixture was mixed briefly and incubated at 16°C for overnight. 1 μl of proteinase K was added to the reaction mixture to stop the reaction.
Figure 5: Blunt end cloning of EcABCB1 into pSTBlue. The primers ‘EcartPF1 c656 FWD’ and ‘EcartPF1 c656 REV’ were used to amplify the EcABCB1 before it was ligated and cloned into pSTBlue. The EcABCB1 amplicon was inserted into the multiple cloning site of pSTBlue linearized with EcoRV.
2.8.4 Transformation of JM109 cells

JM109 (Promega) chemically competent cells was used to propagate 5 µl of the ligation reaction according to manufacturer’s protocol. Transformed cells were spread on kanamycin (50 µg/ml) plate supplemented with 100 µl of 100 Mm isopropyl-beta-D-thiogalactopyranoside (IPTG) and 20 µl of 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG) also referred to as X-gal to select for white positive clones.

2.8.5 Cloning Confirmation

Following the transformation, single white colonies were picked from the transformed plate and grown overnight on 5 ml of Luria Broth (LB) liquid media composed of 10 g/L bacto-tryptone, 5 g/L bacto -yeast extract, and 10 g/L NaCL, pH 7.0 supplemented with kanamycin (50 µg/ml). Plasmids were extracted using E.Z.N.A plasmid mini kit following the manufacturer’s instructions (http://www.genex.cl/stock/EZNAD694501.pdf). Final elution was done with 40 µl of milli-Q H₂O. Inserts were confirmed by a double restriction enzyme digestion using BamHI and HindIII restriction enzymes according to manufacturer’s instructions (New England Biolabs, Ipswich, Suffolk, USA) and using the manufacture’s buffer recommendations (New England Biolabs, Ipswich, Suffolk, USA).

2.9 Sequencing and alignment analysis of EcABCB1

To further confirm the presence of the insert and obtain its sequence, purified recombinant pSTBlue [EcABCB1] plasmid was sent to Quintara biosciences (http://www.quintarabio.com/) for complete sequencing. Sequencing was done using M13 forward and reverse primer, SP6 forward primer and T7 Reverse primer. The rest of the sequence of the entire insert was
acquired by primer walking. Primers used for primer walking were synthesized and provided by the Quintara group.

2.10 EcABCB1 sequence and alignment search

Following primer walking, the EcABCB1 contigs was assembled and aligned using codon code aligner and DNastar software. The nucleotide sequence of EcABCB1 was translated using the expasy translate tool [99]. Basic Local Alignment Search (BLAST) of the nucleotide and amino acid sequences was performed using the NCBI BLAST tool [100].

2.11 Primer efficiency

To determine the efficiency of the primers, qPCR was performed in a 10.5 µl reaction using SsoFast™ EvaGreen® Supermix (BIO RAD. CA. USA.). A 10.5 µl reaction was composed of 5 µl of Sso fast evergreen supermix, 1 µl of 10 mM forward and reverse primers, 1 µl of template (PCR product) and 3.9 µl of milli q H2O. Unless stated, all qPCR assay was performed in a Multiplate™ Low-Profile 96-Well Unskirted PCR Plates (BIO RAD. CA. USA.) using CFX96 Real Time PCR machine (BIO RAD. CA. USA.). Initial denaturation was done at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds, primer annealing and extension was done at 57.3°C for 5 seconds. To check for the amplification of desired fragments, melt curve analysis was done at 65°C to 95°C in the increment of 0.5°C for 5 seconds. It is expected that PCR products of a given primer pair should have the same melting temperature if they are not contaminated, mis-primed, and have no primer-dimers.

Primer pairs for PCR and qPCR were designed to generate products not longer than 200 bp. The above primer sequences were designed using the PRIMER-BLAST tool described in [101]. PCR products of the various primers were confirmed by gel electrophoresis. The PCR
products were then purified using the E.Z.N.A cycle pure kit according to the manufacturer’s protocol. Purified PCR product was confirmed by agarose gel electrophoresis. To get the primer efficiencies and standard curves, a five replicate of 100 fold serial dilution of the purified PCR products was done to five orders of magnitude. Linear standard curves generated by plotting the cycle threshold against the log of the starting amount of DNA template and primer efficiency was automatically generated at the end of the run. The relative expression ratio of all the genes studied were calculated based on the Pfaffl method [102].
Table 1: Table of primers used for the semi-quantitative-PCR and qPCR analysis of selected alkaloid Biosynthetic enzymes. (S)-N-methylcoclaurine-3’-hydroxylase EcCYP80B1, berberine bridge enzyme (BBE), protopine 6-hydroxylase (P6H) The internal control primer sequence was derived from a homologue of an ubiquitin-conjugating enzyme e2 ubc 28 (At4g27960) in the California poppy, The following primer sequences have been published by [46] and they include S-adenosyl-L-methionine:3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4'-OMT,) stylopine synthase (CYP719A2), stylopine synthase (CYP719A3).

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<th>Template</th>
<th>Strand</th>
<th>Sequence 5’ to 3’</th>
<th>Amplicon length (bp)</th>
<th>Accession numbers</th>
<th>Primer efficiencies (%)</th>
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<td>Accession numbers</td>
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Table 2: Primers used to amplify the EcABCB1 and confirm vectors and inserts. EcartPF1 c656 was used to amplify EcABCB1 while the M13, SP6, T7 and primers provided by the Quintara group at [http://www.quintarabio.com/](http://www.quintarabio.com/), were used to generate the EcABCB1 nucleotide sequence. EcABCB1 GWF and EcABCB1 GWR Stop were used to amplify EcABCB1 flanked by the *att* sites for gateway recombination reaction.

<table>
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<th>Template</th>
<th>Strand</th>
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2.12 Expression profile of EcABCB1 in whole plant

Total RNA was extracted from the leaf, stem, root, flower and flower buds using the chloroform / trizol method already described in paragraph (2.5) of this chapter. 1 μg of total RNA were reverse transcribed using RNA to cDNA EcoDry Premix from (Clontech). 2 μl of the resultant cDNA was used to amplify EcABCB1. A 20 μl reaction contained 10.5 μl of water, 4 μl of 5x reaction buffer, 200 μM dNTP, 0.5 μM forward and reverse primers, 2 μl of cDNA, and 0.5 μl of phire hot start II DNA polymerase.

To check the expression profile of EcABCB1 PCR reaction mixture was incubated in an eppendorf vapo-protect thermocycler using the following PCR cycling conditions: Initial denaturation at 98°C for 30 seconds, followed by 25 cycles of denaturation at 98°C for 30 seconds, annealing at 57.3°C for 15 seconds, and extension at 72°C for 15 seconds. Final extension was done at 72°C for one minute. The reaction was cooled down at 4°C before running on a 1.2 % agarose gel electrophoresis. The internal control primer sequence was derived from a homologue of Arabidopsis thaliana ubiquitin-conjugating enzyme e2 (UBC28) (Table 1).
Gateway cloning

Gateway cloning was performed as per manufacturer’s instructions [103-105]. Briefly, attB-flanked EcABCB1 was amplified from the recombinant pSTBlue [EcABCB1] vector using the EcABCB1 GWF as the forward primer and EcABCB1 GWR as the reverse primer. A 50 µl PCR reaction contained 5 µl of 10x pfx Amplification Buffer, 10mM dNTP to 0.2mM Final concentration, 2 µl of 50 mM MgSO₄, 0.4 µM forward and reverse primers, 250ng of pDNA template and 0.2 µl of high fidelity platinum taq polymerase (Invitrogen, Carlsbad, CA; http://www.invitrogen.com). The reaction was volume to 50 µl with milli-Q water. The cycling parameters used for amplification include 94°C for 2 minutes, 94°C for 30 seconds, 60.1°C for 30 seconds, 68°C for 4 minutes, and 68°C for 2 minutes (35cycles).

For the positive control, a sequence of Streptoavidin binding peptide (SBP) flanked by attB sites was used. The SBP sequence (5’- /5Phos/ ACA AGT TTG TAC AAA AAA GCA GGC TCC ATG GAC GAG AAG ACC ACT GGT TGG CGA GGT GGA CAC GTT GTT GAA GGA CTG GCT GGG GAA CTT GAA CAA CTT CGT GCA CGA CTG GAG CAT CAC CCA CAA GGT CAA CGT GAA CCA GAC CCA GCT TTC TTG TAC AAA GTG GT - 3’) was synthesized from integrated DNA technologies (IDT DNA Tech., Coralville, IA, USA).

2.12.1 Gel purification of PCR product

The attB EcABCB1 PCR product was purified according to the protocol on GE Healthcare illustra™ GFX™ PCR, DNA and gel band purification kit, according to the manufacturer’s protocol. Elution of the purified product was done with 25 µl of Buffer 4. Concentration of the purified PCR product was determined using a nanodrop spectrophotometer.
2.12.2 BP reaction

A BP reaction is a recombination reaction that mimics the site specific recombination event that occurs between bacteria and the phage. Integration of the phage genome into the bacteria’s genome during lysogeny occurs in a site specific manner, involving the attP site of the phage and the attB sites of the bacterium to yield a hybrid of two sites attL and attR flanking the phage [103-105].

The technique referred to as BP reaction involves simple multiple steps with recently introduced single step [105] of gene transfer, all occurring in a parallel fashion without altering the reading frames of the gene of interest. The first step of this reaction involves a recombination between the attB sites of PCR product or expression vector and attP sites of donor vector to yield entry clone and by-product. The reaction is reversible. The donor vector (pDONR 221) and BP clonase II enzyme mix used in the BP reaction were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA.).

To obtain the EcABCBI (pENTR EcABCBI) and SBP (pENTR SBP) entry clones, gel purified attB EcABCBI PCR product and SBP concentrate prepared by diluting in 20 µl TE buffer (pH 8.0) were used for the BP reaction. A 10 µl reaction contained 150 ng of gel purified EcABCBI PCR product, 150 ng of donor vector (pDONR 221). The reaction was volumed to 8 µl with Tris/ Ethylenediaminetetraacetic acid (EDTA) (TE, pH, 8.0). 2 µl of BP clonase II enzyme mix (Invitrogen) was added.

SBP entry clone was derived by first dissolving the attB flanked SBP concentrate in 20 µl of TE buffer pH, 8.0. A 5 µl reaction was prepared by adding 2 µl of the SBP solution, 150 ng/µl of pDONR 221, brought to 4 µl with TE buffer. 1 µl of BP clonase II enzyme mix added.
The reactions were vortexed briefly to mix and spun briefly to collect at the bottom of the tube. The complete reaction mixture was incubated at 25°C for ≥ 10 hours. To stop the reaction, 1 µl of proteinase K was added and incubated for 10 minutes at 37°C to stop the reaction.
Figure 6: Construction of SBP entry clone using BP reaction. Linear SBP containing attB1 and attB2 recombine with an entry vector (pDONR 221) which has a gateway cassette (GW) flanked by attP1 and attP2 sites. During the BP reaction attB site react with the corresponding attP site to yield the entry clone (pENTR-SBP). The resultant entry clone are selected based on the antibiotic resistance gene in the destination vector. The by-product of the BP reaction is selected against by the presence ccdB gene which would cause cells bearing it to die.
Figure 7: Construction of EcABCBI entry clone using BP reaction. EcABCBI PCR product containing attB1 and attB2 recombine with an entry vector (pDONR 221) which has a gateway cassette (GW) flanked by attP1 and attP2 sites. During the BP reaction attB site react with the corresponding attP site to yield the entry clone (pENTR-EcABCBI). The resultant entry clone are selected based on the antibiotic resistance gene in the destination vector. The by-product of the BP reaction is selected against by the presence ccdB gene which would cause cells bearing it to die.
2.12.3 Transformation of DH5α with the BP reaction

50 µl of chemically competent DH5α (fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) [106] (NEB), was used to transform the BP reaction. Cells were thawed on ice, 5.5 µl of BP reaction was added to the cells and incubated on ice for 30 minutes. After the incubation, they were heat shocked for 30 seconds at 42°C, cooled down for 2 minutes on ice. 250 µl of S.O.C (super optimal broth supplemented with glucose) media was added and shaken at 37°C for 1 hour for recovery of the transformed cells. Transformed cells were spread on kanamycin (50 µg/ml) plate to select for entry clones or chloramphenicol (30 µg/ml) + ampicillin (50 µg/ml) plate to select for clones containing the destination vector.

2.12.4 Plasmid miniprep

Single colony from entry clones and destination vector clones were inoculated into 5 ml LB media supplemented with kanamycin (50 µg/ml) and ampicillin (50 µg/ml) + chloramphenicol (30 µg/ml) respectively. The culture was grown overnight at 37°C. An E.Z.N.A miniprep kit was used in plasmid extraction according to the manufacturer’s protocol. Final elution was done with 40 µl Tris/EDTA buffer (pH 8.0).

2.12.5 Confirmation of pENTRs

EcABCB1 pENTR vector was confirmed by digestion with EcoRV. EcoRV has one cut site in pDONR 221 vector backbone and one cut site in EcABCB1. The reaction was set up as stated earlier using the manufacturer’s buffer recommendation. SBP pENTR was confirmed by restriction enzyme digestion with BsrGI.
2.12.6 Generation of destination vector

A BP reaction was performed to switch the gateway (GW) cassette of a donor vector, pDONR 221 into an expression vector with the desired backbone construct (pREP41 3XHA) in order to generate the destination vector pREP41HAGW (a kind gift from Angus S. Murphy, Purdue University, U.S.A). The pREP41GW empty vector has ampicillin resistance gene as a selectable marker in *E. coli* and *Leu2* as a selectable marker in yeast. It also has a medium-strength thiamine repressible nmt1 promoter useful for activation and regulation of foreign gene expression in the host organism.

Expression clone pREP41HA WBC11 was linearized with *Bgl II* (NEB) according to the method stated earlier following manufacturer’s buffer recommendation. Linearization was done to disrupt the WBC11 insert so as to minimize false positives. *Bgl II* has one cut site in WBC11. Linear plasmid was confirmed by agarose gel electrophoresis. BP reaction was done with the linear vector and pDONR 221 using the same protocol as stated earlier.

2.12.7 Confirmation of destination vector

pREP41 3XHA GW destination vector was confirmed by sequencing with attB1 and attB2 primers (Quintara Bioscience) and by restriction enzyme digestion with BamHI (NEB) restriction enzyme. Restriction enzyme digestion reaction was prepared as reported earlier and according to the manufacture’s buffer specification. BamHI has one cut site in 3X HA and one in pREP41 backbone.

2.12.8 LR reaction

LR reaction is the second step of the gateway gene cloning reaction. It involves the reaction between the attL sites of the entry vector and attR site of destination vector to yield expression clone. To generate the expression clone containing the expression vector with N-
terminal 3XHA tag, LR reaction was done with the destination vector pREP41 3XHA GW and pENTR EcABCB1, CjABC1 and SBP. A 10 µl reaction contained 150 ng/µl of entry vector and 150 ng/µl of destination vector, volumed to 8 µl with TE buffer. The reaction was mixed briefly by vortexing and given a quick spin to concentrate at the bottom of the tube. 2 µl of LR clonase II enzyme mix (Invitrogen) was added and mixed by vortexing briefly. The reaction mixture was incubated at 25ºC for overnight.

The LR reaction was transformed into NEB express competent *E. coli* (fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 Δ(mcrC-mrr)114::IS10) (NEB) following the manufacturer’s instructions and protocol. Transformants were spread on ampicillin (50 µg/ml) plate. Positive transformants were picked and grown on 5 ml liquid media for plasmid extraction.

### 2.12.9 Confirmation of expression clones

Plasmids were extracted using Axyprep plasmid miniprep kit according to the manufacturer’s instructions and protocols (Axygen Biosciences, CA, USA). The SBP expression clone was confirmed by restriction enzyme digestion with BgII, while EcABCB1 was digested with EcoRV. Further confirmation of the SBP, EcABCB1 and CjABCB1 expression clones was done by sequencing with attB1 and attB2 primers provided by the Quintara group ([http://www.quintarabio.com](http://www.quintarabio.com)).
Figure 8: Construction of CjABCB1 expression clone using LR reaction. Entry clones containing attL1 and attL2 recombine with a destination vector which has a gateway cassette flanked by attR1 and attR2 sites. The attL site of entry clone reacts with the corresponding attR site of the destination vector to yield the expression clone. The expression clones are selected based on the antibiotic resistance gene in the destination vector. The by-product of the LR reaction is selected against by the presence ccdB gene which would cause cells bearing it to die.
Figure 9: Construction of SBP expression clone using LR reaction. Entry clones (pENTR-SBP) containing attL1 and attL2 recombine with a destination vector (pREP41-3XHA-GW) which has a gateway cassette and ccdB gene flanked by attR1 and attR2 sites. During the LR reaction, attL site react with the corresponding attR site to yield the expression clone (pREP41-3XHA-SBP). The expression clones are selected based on the antibiotic resistance gene in the destination vector. The by-product of the LR reaction is selected against by the presence ccdB gene which would cause cells bearing it to die.
Figure 10: Construction of EcABCB1 expression clone using LR reaction. Entry clones (pENTR-EcABCB1) containing attL1 and attL2 recombine with a destination vector (pREP41-3XHA-GW) which has a gateway cassette and ccdB gene flanked by attR1 and attR2 sites. During LR reaction attL site react with the corresponding attR site to yield the destination clone (pREP41-3XHA-EcABCB1). The expression clones are selected based on the antibiotic resistance gene in the destination vector. The by-product of the LR reaction is selected against by the presence ccdB gene which would cause cells bearing it to die.
2.13 Transformation of fission yeast

Rapid lithium acetate protocol used to transform double mutant strain of *Schizosaccharomyces pombe (mamI pdrI)* was according to [107]. The *mamI pdrI* strain was a kind gift from Angus S. Murphy (Purdue University, U.S.A). 10 ml of Edinburg minimal media (EMM) low glucose (pH, 5.80) supplemented with adenine, leucine and uracil (225 mg/l) was grown to a density of 0.5-1 x10^7 cells/ml (OD600 = 0.2-0.5). Cells were washed once in milli-Q H$_2$O and suspended in 1 ml of milli-Q H$_2$O. Washed cells were transferred to a sterile 1.5 ml eppendorf tube and microfuged at 1000 xg for 5 minutes to pellet the cells. The supernatant was discarded and pelleted cells were washed once in 0.2 ml of lithium acetate (LiOAc)/EDTA (0.1 M LiOAc pH, 4.9 /1 mM EDTA) and re-suspended in fresh LiOAc/EDTA. 1 μg expression clones volumed to 30 μL with TE buffer was added to the cells. Cells were completely re-suspended in 300 μL of pre-warmed PEG4000 solution and incubated with agitation at 30°C for 30 minutes. After the incubation, cells were heat-shocked without agitation in a water bath at 42°C for 15 minutes and cooled down to room temperature for 10 minutes, followed by 1-2 minutes centrifugation to pellet the cells. Pelleted cells were re-suspended in 1 ml of TE buffer (pH, 8.0). 200 ul aliquots was plated on EMM plate supplemented with 225 mg/l of adenine and uracil (lacking leucine) and grown at 30°C for 3 to 4 days. EMM media lacking leucine was used to select for positive transformants.

2.14 Protein extraction

Protein extraction was done according to [108] with slight modification. 3 ml of transformed yeast cells were grown overnight, harvested and washed once in 1 ml of 1x PBS buffer to get rid of salt from the media. Washed cells were re-suspended in 200 μl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol). Just before extraction, 20 mg
of protease inhibitor cocktail mix (Sigma, St Louis, MO, USA) was dissolved in freshly made 1 mM phenylmethylsulphonylfluoride (PMSF). 50 µl of protease inhibitor cocktail mix (Sigma, St Louis, MO, USA) was added to the cells in suspension and transferred to screw cap tube to avoid leakage of samples during lysis. A scoop of cold glass beads was added and vortexed at maximum speed for 10 minutes at room temperature. Lysed cells with beads were immediately placed on ice. Cell lysates were transferred to new 1.5 ml tubes on ice, glass beads were rinsed with 200 µl of lysis buffer and added to the lysates. The lysates were spun down @ 4°C for 10 minutes at 9,000 xg. Supernatant which is the whole protein extract were transferred to a new 1.5 ml tube on ice, the extract was stored at -20°C freezer until ready for use.

For crude membrane protein preparation, the method used was modified from [109]. 1 ml of sucrose solution (0.35 M sucrose, 0.18 g HEPES, 0.006 g MgSO4, volume to 50 ml in 1x PBS buffer) was added to the supernatants generated after pelleting the cell lysates. The sucrose-lysate mixture was transferred to a glass grinder, grinding was done 10 times on ice, one turn equals one grind. The whole mixture was transferred to a new 1.5 ml tube and centrifuged @ 4°C for 10 minutes at 9,000 xg. The supernatant was saved as membrane fraction.

2.15 Sample preparation for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

5X SDS-PAGE loading buffer was prepared according to [110]. 1 ml of 5x SDS-PAGE contains 250mM Tris-cl (pH, 6.8) 8% SDS, 0.1% bromophenol blue, 40% (v/v) glycerol, 100 mM Dithiothreitol (DTT) to 1 ml with milli-Q water. 1 ml of 5x SDS-PAGE loading buffer was supplemented with 14 mg of SDS and warmed up in 37°C water bath for 15-20 minutes. The prepared SDS loading was added to the protein extracts to 1X final concentration and heated for 30 minutes at 37°C water bath before loading on the gel.
2.16 SDS PAGE

SDS PAGE was performed according to [111]. Recipe used for the SDS-PAGE gel was according to [110]. 10 ml of 8% resolving gel contained 4.6 ml of milli-Q H2O, 2.7 ml of 30% Acrylamide/Bis (Biorad), 2.5 ml of 1.5M tris (pH 8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% APS and 6 µl of Tetramethylethylenediamine (TEMED). 6 ml of 5% stacking gel contained 4.1 ml of milli-Q H2O, 1 ml of 30% Acrylamide/Bis (Biorad), 0.75 ml of 1.5M tris (pH 6.8), 0.06 ml of 10% SDS, 0.06 ml of 10% APS and 6 µl of TEMED. 1 mm gel was casted using mini-protean® tetra cell system (Biorad). ColorPlus prestained protein marker, Broad range 7- 175 KDa (NEB) was used. Also used was Mini-PROTEAN® TGX™ Precast Gels (Bio Rad Laboratories, Richmond, CA, USA). 20 µl of prepared protein samples was loaded into the wells. Electrophoresis was done at 80V uninterrupted power supply using 1x protein running buffer. 1x running buffer was prepared from 5x running buffer according to [110]. 1000 ml of 5x running buffer contained 50.1 g tris-base, 94 g glycine and 50 ml of 10% SDS.

After the electrophoresis, gel and a slice of 0.2 µm nitocellucose membrane (Bio-Rad) cut to gel dimension were equilibrated for 15-30 minutes in 1x transfer buffer (2.9 g glycine, 5.8 g tris and 0.37 g SDS to 1 L with milli-Q H2O). Transfer of proteins to nitrocellulose membrane was done using trans-blot® sd semi-dry electrophoretic transfer cell (Bio-Rad) according to the manufacturer’s procedure. Transfer was done at 25V for 30 minutes. Successful transfer was confirmed by staining the membrane for 5 minutes with ponceau (1 g Ponceau S, 50 ml acetic volumed to 1 L with milli-Q H2O) and washing in 1x phosphate buffered saline (PBS), made by dissolving 50 mM tris-base, 150 mM Nacl, in 1 L of milli-Q H2O, pH 7.5. 0.1% tween 20 was added to 1 ml of PBS to make PBST [110].
2.17 Western blotting

After transfer, membranes were blocked with 5% non-fat dry milk in PBST for 1 hour at room temperature. 1:2000 of anti-HA tag antibody developed in mouse (Thermo Fisher Scientific) and diluted in Starting Block (PBS) Blocking Buffer (Thermo Fisher Scientific) was used. Diluting the primary antibody in starting block allows for multiple use of one dilution, up to 10 times or more without losing its sensitivity. Incubation with an anti-HA tag antibody was done overnight at room temperature. Bound anti-HA tag antibody was detected using 1:4000 peroxidase-conjugated mouse anti-goat IgG (Rockland, Gilbertsville, PA, USA). Peroxidase activity was detected by staining with the chemiluminescence luminata crescendo (Millipore, USA). Imaging was done using Bio-spectrum® 600 imaging system (UVP, Upland, CA, USA).

2.18 N-terminal YFP tagged fusion construct

To generate a N-terminal YFP fusion construct of EcABCB1 [10], the pEarleyGate104 vector [112] was used. EcABCB1 pENTR vectors were first linearized with restriction enzymes because both pENTR and pEarleyGate104 vectors have kanamycin resistant gene as their selectable marker. pENTR EcABCB1 was digested with NruI (NEB) because it has one cut site on pDONR 221 backbone and no cut site in EcABCB1. Linear plasmids were confirmed by agarose gel electrophoresis. LR reaction was performed as earlier stated using linear pENTR [EcABCB1] and pEarley Gate 104 vector. pEarley Gate 104 vector is a gateway vector with an N-terminal YFP tag under the control of cauliflower mosaic virus 35S promoter. The 35S promoter allows for the ectopic expression of the recombinant plasmid.
Figure 11: Construction of pEarleyGate104-(N-YFP) EcABCB1-YFP expression clone using LR reaction. Linear entry clone (pENTR [EcABCB1]) containing attL1 and attL2 recombine with a destination vector (pEG104-(N-YFP) which has a gateway cassette flanked by attR1 and attR2 sites. During the LR reaction attL site react with the corresponding attR site to yield the destination clone (pEG104::EcABCB1-(N-YFP) expression clone. The resultant expression clones are selected based on the antibiotic resistance gene in the destination vector. The by-product of the LR reaction is selected against by the presence of ccdb gene which would cause cells containing it to die.
2.18.1 Transformation of DH10B with the LR reaction

10 µl of chemically competent DH10B chemically competent cell, genotype: F\(^{-}\) mcrA \(\Delta(mrr-hsdRMS-mcrBC)\) \(\Phi80dlacz\Delta\text{M15} \Delta\text{lacX74 endA1 recA1 deoR} \Delta(\text{ara,leu})7697 \text{araD139 galU galK nupG rpsL } \lambda^- \) [113, 114] (Invitrogen, Carlsbad, CA; \url{http://www.invitrogen.com}), kindly provided by Lindsay Leahul of Mount Royal University, was used to transform the LR reaction. Cells were thawed on ice, 5 µl of LR reaction was added to the cells and incubated on ice for 30 minutes. After ice incubation, cells were heat shocked for 30 seconds at 42\(^\circ\)C, cooled down for 2 minutes on ice. 250 µl of S.O.C media was added and shaken at 37\(^\circ\)C for 1 hour for recovery. Transformed cells were spread on kanamycin (50 µg/ml) plate to select for expression clones.

2.18.2 Plasmid miniprep

Single colonies from the plates were inoculated into 5 ml of LB media and grown overnight at 37\(^\circ\)C. Plasmids were extracted using Axyprep plasmid miniprep kit according to the manufacturer’s protocol (Axygen Biosciences, CA, USA). Final elution of the plasmids was done with pre-warmed milli-Q H\(_2\)O and concentration was determined using nanodrop spectrophotometer.

EcABCB1 expression clone was confirmed by restriction enzyme digestion with EcoRI (NEB) using the same protocol as stated earlier and manufacturer’s buffer recommendation and it is expected to be about ±14.7 kb. This was confirmed by digesting the pEG104-EcABCB1 with EcoRI (NEB) restriction enzyme. EcoR1 is expected to digest the pEG104-EcABCB1 into 2 places to produce three bands of about ±1.7 kb, ±2.16 kb and ±10.85 kb on agarose gel.
2.19 *Agrobacterium tumefaciens* transformation

1 µg of uncut N-terminal YFP tagged expression clone (pEG104-EcABCBI) was added to 50 µl of *A. tumefaciens* strain GV 3101 thawed on ice. Cells with the recombinant plasmids were frozen in liquid nitrogen for 30 minutes and thawed in water bath set to 37°C for 5 minutes. 1 ml of S.O.C media was added and incubated for 2 hours at 28°C with gentle rotation of about 150 rpm. Cells were centrifuged for 30 seconds, and supernatant was decanted. Pelleted cells were re-suspended in 50-100 µl of S.O.C media and spread on kanamycin (50 µg/ml) and gentamycin (100 µg/ml) plate. Plates were incubated at 28°C for 3 days.

2.20 Transfection of Tobacco plant

Colonies from the transformed plate were inoculated into 5 ml LB media supplemented with kanamycin (50 µg/ml) and gentamycin (100 µg/ml) and grown for 16 hours with shaking (250 rpm) at 28°C. 1.5 ml of culture was transferred to a new 1.5 ml microfuge tubes and spun down at 3.5 xg for 5 minutes. Supernatant was discarded and pelleted cells were re-suspended in 1.5 ml of milli-Q H₂O. Acetosyringone was added to the cell in suspension to 100 µM final concentration. Cells were mixed by inverting up and down severally. 10 µl pipette tip was used to poke a hole on the tobacco leaf to be transfected. These tobacco leaves used are at four-eight leaf stage of growth. 5 ml syring was used to suck up and inject the cells into the leaf.

Transfected area was circled and labelled with sharpie permanent marker. Transfected leaves were incubated at room temperature with 8 hours every day for 3 days.

2.21 Confocal microscopy

Portions of transfected leaves were carefully excised using a single edge blade razor. The excised leaf was placed on glass slide containing a drop of milli-Q H₂O and covered with cover
Laser scanning confocal images were collected using Fluoview FV1000 laser scanning confocal microscope (Olympus). Parameters used in the acquisition include: XY scan mode, 20X objective, 1X zoom, sampling speed of 40 µs/pixel and laser excitation wavelength of 515 nm, 23% laser transmissivity and 527 nm emission wavelength. Images were formatted using Imagej [115].

2.22 Functional characterization of EcABCB1 in yeast cells

To test the function of EcABCB1, single colonies of freshly streaked transformed double mutant yeast cells mam1pdr1 were inoculated into liquid EMM media and grown at 30°C for ≤ 24 hours. At this stage the yeast cells are in exponential growth phase. Berberine, sanguinarine, morphine base, scoulerine and cryptopine alkaloid standards or ethidium bromide were independently added to the cells at 3 µg/ml final concentration, while berberine hydrochloride was added at 13 µg/ml final concentration and incubated for 2 hours. After 2 hours of incubation, cells were harvested by spinning down at 4000 rpm using the Allegra 6 benchtop centrifuge (Beckman coulter). Liquids were decanted and pelleted cells were washed initially with 100 ml of milli-Q water supplemented with a drop of acetic acid. 2 additional wash steps were performed with milli-Q water. Fresh weight of the cells were taken, then washed cells were re-suspended in 200 µl of Bieleski solution, sonicated and kept overnight at -20°C freezer. Afterwards, sonicated cells were spun down at maximum speed using the benchtop microcentrifuge (Thermo Fisher Scientific). All the sample extracts were resuspend to a final volume of 200 µl with Bieleski solution before running on the LC-MS machine.
2.23 Thin layer chromatography

Semi-quantitative analysis of the alkaloids extracted from the cells and the media of treated and untreated California poppy cell suspension cultures was done using the thin layer chromatography (TLC). Briefly, 5 µl of the extracts were applied as a single dot for each sample to silica Gel 60 F254 TLC plates (EM Separations, Darmstadt, Germany). The plates plus extracts were developed in a 4:1:1 v/v n-Butanol: acetic acid: water solvent mixture as the mobile phase [116]. Plates were dried inside a flowhood and then exposed to UV Illumination at 365 nm for observation and imaging. Image was taken with a canon digital camera.

2.24 Quantitative PCR (qPCR) of transcript levels of selected key biosynthetic enzymes

1 µl of cDNA was amplified using SsoFast™ EvaGreen® Supermix (BIO RAD. CA. USA.) in the presence of 0.3 µM forward and reverse primers in a total volume of 10.5 µl reaction. Initial denaturation was done at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds. Primer annealing and extension was done at 57°C for 5 seconds. The internal control primer sequence was derived from a homologue of Arabidopsis thaliana ubiquitin-conjugating enzyme e2 (UBC28).

2.25 LC-MS of alkaloid standards and standard curve

The following alkaloid standards were used to generate standard curves: berberine, sanguinarine (Sigma, St Louis, MO, USA), scoulerine, noscapine standards (Toronto research chemicals, Downsvie, ON, Canada), morphine base and noscapine hydrochloride (gifts from Dr. Peter Facchini). All the compounds were dissolved in high performance liquid chromatography (HPLC) grade methanol (Fisher Scientific, Nepean, Ont.).
1 mg/ml stock solution of the sanguinarine, ethidium bromide and 4 mg/ml berberine standards were used to make a two-fold serial dilution of up to 5 orders of magnitude starting with 18.75 pg/ul. However, the starting concentration for scoulerine, morphine and cryptopine are 2.34 pg/µl from a 1 mg/ml stock solution. Each dilution was injected into the autosampler 3 times to generate 3 replicate data points per dilution. Sample analysis was done using the LTQ-XL linear ion trap mass analyzer (Thermo Fisher Scientific, San Jose, CA, USA) set to target the metabolites of interest for optimal quantification using a non-data dependent method trapping the precursor ion of interest based on retention time and mass/charge ratio. MS² Data were acquired by collision-induced dissociation of the parent ions of interest. Acquired data were processed using the Xcalibur where quantification was based on the abundance of the most abundant product ion. Standard curves generated were used to quantify alkaloids extracted from yeast while noscapine was used as an internal standard to quantify the amount of alkaloids in the cell culture extracts.

2.26 Liquid chromatography mass spectrometry (LC/MS)

Complex alkaloid mixtures and alkaloids extracts from the yeast were separated, identified, and quantified using liquid chromatography coupled to linear ion trap mass spectrometer (LTQ XL MS, Thermo Fisher Scientific, San Jose, CA, USA). Alkaloid mixtures extracted from plant cell cultures were prepared according to [95]. Following extraction, samples were re-suspended in 1 ml of Bieleski solution in a vial. Samples in vials were capped and sealed with crimper to minimize evaporation and contamination. 25 µl of sample mixture was injected and separated using a reversed phase C18 column hypersil gold, Particle Size: 1.9µm (50 x 2.1 mm i.d) loaded with 10% carbon, pore size 175Å (Thermo Fisher Scientific). The mobile phases used for
alkaloid separation were (A) 0.5% formic acid (Fisher Scientific) and (B) 100% acetonitrile. The method consisted of a linear gradient at 50% A for 10 minutes, gradient at 99% B for 2 minutes, and continued for 1 minute of 99% B and then isocratic at 100% A for 4 minutes (total 17 minutes). As sample components elute from the column, they first enter the ion source. Ions were generated by means of electrospray ionization (ESI). The electrospray conditions are as follows: capillary temperature, 350°C; sheath gas flow (stabilizes the sample) 30; auxiliary gas flow (keeps the source dry) 15; source voltage, 4.70 KV, source current, 100 uA, capillary voltage, 26 V, normalized collision energy 35%. The spectrometer was operated in a non-data dependent mode where one scan event was put on MS/MS (MS\textsuperscript{2}). Using this method, a specified precursor ion with known m/z ratio is selected and fragmented to get the product ions. See Appendix B for a list of all the precursor and product ions. For all the statistical analysis done in this section, \( p \)-alpha (0.05) was corrected for multiple comparison

2.27 Detection of alkaloid fluorescence

Fresh hand sections of California poppy root were prepared according to [117]. Fresh hand sections on slides were exposed to UV light using a filter combination of 340-380 nm excitation and 425 nm emission wavelength. Autofluorescence examinations were done according to [117] under a Leica Aristoplan fluorescence microscope. Imaging of all the histological sections was done using Leitz Aristoplan microscope with a Leica DFC480 digital camera set to bright field. Images were assembled using the image processing software ‘ImageJ’ [115]
2.28 Plant material

*Eschscholtzia californica* (California poppy) seeds were a kind gift from the Dr. Peter J. Facchini lab located at the University of Calgary, Alberta, Canada. The seeds were germinated and maintained in the greenhouse at 25°C under natural light conditions augmented with incandescent fluorescent light. Soil premix with vermiculite and fertilizer (N. P. K. 17:17:17) was used as plant nutrients.

2.29 Plant cell suspension cultures

Cell cultures of *Eschscholzia californica* were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany; [http://www.dsmz.de](http://www.dsmz.de)) E. californica Cham. (Catalog number: PC-1096). The cells were maintained as a suspension culture according to the method of [51]. The 1B5C medium contained 3.21 g/l Gamborg B5 (Phytotechnology laboratories), 20 g/l sucrose, 1g/l casein hydrolysate, and 1mL/l of 1mg/mL 2, 4-D. (pH 5.6) and shaken at a gyratory shaker at 125rpm. Cells were sub-cultured every 7 days using 1:5 dilution of inoculum to fresh medium.

2.30 Free hand sections

California poppy root of different ages ranging from 15 day old seedlings to 7 month old plants were used in the free hand sections. Plants were carefully uprooted and washed in running tap water. Free hand sections were done according to [117]. Briefly, a fresh double-edged razor blade masked one side with tape was rinsed in warm tap water to get rid of grease on the surface of the blade. The razor wetted in water was used to make sections between 200-500 μm. The sections were transferred to water with a small paint brush. The thinner sections were selected and stained with toluidine blue O (TBO) stain or iodine-potassium iodide (I-KI). TBO stain
solution contained 0.1 g of TBO dissolved in 100 ml of 0.1 M benzoate buffer, pH 4.4. The benzoate buffer consists of 0.25 g of benzoic acid, 0.29 g of sodium benzoate dissolved in 200 ml of water. I-KI solution is composed of 2 g of KI in 100 ml of water, supplemented with 0.2 g of iodine [117]. TBO binds to negatively charged molecules because of its cationic properties.

The reaction of TBO with carboxylated polysaccharides such as pectins gives a pinkish purple color; with phenolic substances such as lignins and tannins, the resultant colour will be green, greenish blue or bright blue; while purplish or greenish blue will result with nucleic acid [118]. I-KI stains alkaloid brown red [119]. TBO or I-KI stain was done by flooding selected sections placed on a clean slide with either TBO or I-KI solution for few minutes, followed by a gentle wash with flood of water until excess stain is removed. A drop of water is added to the slide and covered with a cover slip for microscopic observation.

2.3.1 Plastic embedding method

Plastic embedding method was done according to [120]. E. californica roots of different age bracket ranging from 2 weeks to 4 months was carefully harvested and washed in running tap water to get rid of soil contaminants. The entire root was fixed so as to retain exudates from the laticifers. The fixative is a mixture of 1.6% paraformaldehyde and 2.5% glutaraldehyde in a 0.05 M phosphate buffer, pH 6.8. Tissues were fixed overnight at 4°C and dehydrated in methyl cellosolve for 24 hours before they were passed through 2 changes of 100% ethanol every 24 hours. Dehydrated tissues were infiltrated with a methylacrylate-type resin, historesin (Leica Canada, Toronto, Ontario), and passed through 2 changes of 100% historesin, every 24 hours. Tissues were embedded in Technovit 7100 (Sigma, St Louis, MO, USA) embedding medium prepared according to the manufacturer’s instructions. The embedding solution was prepared and
tissues were embedded according to [121]. Long chains of serial sections 2-3 μm thick were obtained with Ralph glass knife fitted into Reichart-Jung 2040 Autocut retractable return stroke microtome. These long ribbons were reduced into shorter ribbons, short enough to fit straight on a glass slide. The ribbons were then placed on a glass slide wetted with water and dried on a heating block set at 40°C.

2.32 Staining for plastic sections

Periodic acid-schiff’s reaction (PAS) and Amido black 10B solution used for staining was prepared according to [122]. Periodic acid-schiff’s reaction stains the cell wall and starch grains to give a red colouration, while Amido black 10B stain proteins blue.

Dried slides were carefully arranged in 24-place slide holders (VWR international) prior to staining. For Periodic acid-schiff’s reaction: dried slides in a 24-place slide holders were placed into a 0.15% of periodic acid solution made fresh and left to stain for 15 minutes. Stained slides were gently washed in slow running water for about 15-20 minutes and rinsed in de-ionised distilled water. PAS stained slides were counter-stained with amido black staining solution for 2 minutes. Stained slides were gradually rinsed in slow running tap water, rinsed in de-ionized water. Excess water was removed by blot drying with paper towel, and placed on top of heat block set at 40°C to dry completely before they were mounted. New set of dry slides were stained with aniline blue to confirm the presence of the sieve tube elements. Aniline blue stains for callose and was prepared according to [123-125].
Chapter Three: Molecular cloning and functional characterization of EcABCBI

3.1 Introduction

In this chapter, results from the molecular cloning, sequence, expression and functional analysis of EcABCBI in yeast cells are presented. The significance of these findings and its implications is discussed.

The California poppy cell culture biosynthesize mostly the benzophenanthridine subclass of alkaloids, notably sanguinarine and marcapine. Also abundant is protopine from protopines class. [7, 95, 126]. Furthermore, when the cells are maintained as a suspension culture, they secrete the alkaloids, mostly the benzophenanthridines and protopines into the media [7]. Biotic elicitors such as that prepared from fungal extract have been shown to induce the production of the benzophenanthridine subclass [7, 9, 47, 49, 89, 127, 128]. ATPase inhibitors such as vanadate have been used previously to block the secretion of alkaloids in California poppy cell suspension cultures [9], thus prompting suggestions that active transport process is likely involved in the secretion of alkaloids into the media. The above-mentioned properties have been the reason why the cell suspension cultures of California poppy have been used extensively to study the biosynthetic pathway of benzophenanthridine alkaloids [7, 129, 130]. We hypothesized that ABC transporter(s) is involved in the transport of alkaloids in the California poppy cell suspension cultures because of the cells’ ability to secrete alkaloids into the media and takes up the secreted alkaloid(s) back into the cell [55].

ABC transporters have been implicated in the transport of primary and secondary metabolites in many species of metabolites producing plants. For example, ABC transporters have been implicated in the transport of auxin [60-64], wax [131], cuticular lipid [65, 66],
diterpenes [67], phenolics [68-70], auxins [68], and alkaloids such as berberine [10, 11] and catharanthine [71]. Among the ABC transporters implicated in the transport of the above mentioned metabolites, CjABCB1 [10] and CjABCB2 [11] from Coptis japonica are the only known ABC transporters involved in the transport of BIA alkaloids notably berberine [10, 11]. More recently, another ABC transporter CrTPT2 belonging to the ABCG subfamily member from Catharantus roseus has been implicated in the transport of the indole alkaloid catharanthine [71]. Here we describe an ABC transporter belonging to ‘B’ subfamily from California poppy, EcABCB1. Functional analysis of EcABCB1 suggests that it is involved in the transport of a subset of benzylisoquinoline alkaloids.

The aim of this study was to amplify, clone and characterize an ABC transporter involved in the transport of alkaloid(s) in California poppy. The experimental approach involved the search for homologous of CjABCB1 in E. californica in the E. californica database, verify its expression in E. californica cell cultures and plants, clone and express it in S. pombe mutant strain (mam1 pdr1) for functional analysis.

3.2 Growth and viability of California poppy cell suspension cultures

Typically suspension cultures are about ≥98% viable from the day 0 to 10 days after sub-culturing. Within the days the cells are highly viable (Figure 13), there appears to be a progressive increase in cell bio-mass (Figure 13). Decrease in cell bio-mass sets in from twelve days after sub-culturing (Figure 12) and at this point, the viability of the cells is also on the decline (Figure 13).
Figure 12: The growth curve of California poppy cells in suspension cultures. Data were collected over 18 days period of incubation. Scale bars represent standard error of means (SEM) from three biological replicates.

Figure 13: Viability curve of California poppy cell suspension cultures over 18 days period of time. Scale bars represent standard deviation from three biological replicates.
3.3 Validation of alkaloid accumulation in the media

To confirm that the cells secrete alkaloids into the media, California poppy cell suspension cultures were harvested over 18 days period of time at 3 days interval. Alkaloids were extracted from the media and cells and analyzed by TLC (Figure 14).

![TLC image of alkaloids accumulated in the media and cells of E. californica.](image)

Figure 14: TLC image of alkaloids accumulated in the media and cells of *E. californica*. Cells were maintained as a suspension culture of single cells over 18 days incubation time. (A) Represents an image from a TLC plate showing alkaloids extracted from the media while (B) represents an image from a TLC plate showing alkaloids extracted from the cells.

Results obtained (Figure 14) confirmed our previous knowledge that the California poppy cells in suspension culture secrete alkaloid into the media [9]. Within few hours after the inoculation of cells into fresh liquid media for maintenance and regeneration, alkaloids begin to accumulate as can be seen at day zero (Figure 14). From three days post sub-culture, the amount of compounds accumulated in the media, most notably sanguinarine seem to increase (Figure 14 A). Induction continued until nine days post sub-culture, at this point reduction in the amount of alkaloid accumulated starts to occur. Twelve days after sub-culturing, sanguinarine could barely
be seen on the TLC plate (Figure 14). By eighteen days after sub-culturing no sanguinarine could be seen on the TLC plate (Figure 14).

To ramp up the accumulation of alkaloids within hours, the suspension cultures were incubated with fungal elicitor. California poppy cells in suspension culture have previously been reported to respond to fungal elicitation by accumulating more sanguinarine, chelerythrine and macarpine [89]. According to Collinge and Brodelius (1989), chelerythrine and sanguinarine attain maximum accumulation five to eight hours after elicitation with about 75% of the accumulated compounds found in the media.
Figure 15. Elicitation induced more accumulation of alkaloids in the media and cells. Six day old cultures were elicited and harvested at 0, 24, 18 and 72 hours. 0.2% ethanol was added to a control sample and harvested 72 hours after elicitation. Alkaloids were extracted from the media and cells. (A) Represents alkaloids extracted from the media; (B) represents alkaloids extracted from the cells. Orange arrow points to sanguinarine migration point while green arrow points to protopine migration point. ‘+’ sign indicates addition while ‘-’ sign indicates no addition.

From 24 to 72 hours after elicitation, there was a visible and more likely, a steady increase in the accumulation of alkaloids, notably sanguinarine in the cells and the media extracts (Figure 15). Sanguinarine was annotated by running the extracts side by side with the sanguinarine authentic standard (Sigma) on the TLC plate. Further confirmation was done by re-extracting the compound thought to be sanguinarine from the TLC plate, analyzing it using LC-MS and comparing its fragmentation pattern and retention time to that of the authentic standard and published data [95].
As the cells were incubated with elicitor, the viability of the cells were tested while simultaneously extracting alkaloids from the media and the cells. Results obtained showed that ≥80% of the cells remained viable over these three days. This suggests that the elicitation did not affect the cell’s viability and thus, rules out the possibility that the observed alkaloid accumulation in the media was due to cell lysis. Additionally, it has previously been reported that the lower elicitor concentration does not have a significant effect on the viability of *E. californica* cells in suspension culture [89]. Based on this observation, the incubation time for the elicited cells were reduced to a maximum of 12 hours. Elicited cells were harvested at 1, 2, 6, and 12 hours post elicitation.

![Figure 16: Reduction in alkaloid accumulation 6 hours after elicitation. 6 days old cell suspension cultures were elicited. The elicited cells were harvested at 0, 1, 2, 6 and 12 hours. (A) Represents alkaloids extracted from the media; (B) represents alkaloids extracted from the cells. Arrow points to sanguinarine migration point.](image)
Two hours after elicitation, there was a visible increase in the accumulation of sanguinarine in the media compared to the amount in the media of the unelicited control (Figure 16 A). Six hours after elicitation, there was a reduction in the amount of sanguinarine accumulated in the media of elicited cells compared to the amount accumulated in the media of cells elicited for two hours thus contrasting what was expected. It is expected that with longer incubation, more sanguinarine will be accumulated in the media.

In the cell extract, more sanguinarine was observed in the extracts from all the unelicited control cells (Figure 16 B) compared to extracts from the elicited cells. Batches of cells were elicited and harvested after every hour to see when the reduction in the accumulation of sanguinarine starts. It is predicted that the observed decrease in sanguinarine six hours after elicitation is not a rapid but gradual event.
Based on what was observed in Figure 17 A, decrease in sanguinarine accumulation starts 4 hours after elicitation, and continues till 6 hours thus supporting the hypothesis that the observed decrease in sanguinarine accumulation is a gradual rather than fast event (Figure 17 A). However, there seems to be a slight increase in the amount of sanguinarine accumulated in the cell 2 hours after elicitation (Figure 17 B) while no other difference in accumulation was observed for the rest of the extracts from the cell (Figure 17b). This observation supports the previous report that uptake of sanguinarine occurs in the media of California poppy cells in suspension culture [55]. Based on this observation we proposed that ABC transporter(s) is involved in the uptake of alkaloid(s) in the California poppy cell suspension culture.
3.4 Database search of CjABCB1 homologous in *E. californica* database

The motivation to focus on EcABCB1 was mainly stemmed from the report that CjABCB1 [10] and CjABCB2 [11] mediates the uptake of berberine in *Coptis japonica* [10, 11]. To check if *CjABCB1* homologue can be found in California poppy, *CjABCB1* nucleotide sequences was extracted from the publicly available nucleotide database [http://www.ncbi.nlm.nih.gov/]. Basic Local Alignment Search (BLAST) of *CjABCB1* nucleotide sequence with the seequence data reads available in *E. californica* database [97, 98] was done.

As detailed in [97], cDNA sequences from California poppy deposited in this database originated from the root. The sequence datas were generated by use of 454 next generation and illumina sequencing method. Sequencing with Illumina generates shorter reads compared with 454 next generation sequencing method. Illumina sequence data was assembled using version 0.1.16 Velvet Oasis tool while 454 next generation sequence data were assembled using MIRA. The length of reads produced after assembling next generation data reads with MIRA is longer compared to Illumina reads assembled with Velvet Oasis. There are 472,167 raw reads generated using 454 next generation sequencing method, after cleaning, the number reduced to 423,743, while the number raw reads generated from Illumina is 6, 270, 4080 which decreased to 53, 746,798 after cleaning. On the average, 454 next generation read length is 428 bp while Illumina read length is 37.3bp. Redundant transcripts were removed using CD-HIT-EST clustering tool. Transcripts less than 300 bp were removed. Assembled transcripts were annotated using the Magpie Automated Genomics Project Investigation Environment (MAGPIE). MAGPIE automatically performs a homology search of the sequences against the
available sequences in public database and functions were assigned to each contig after validating each contig as described in [97].

Following the database search, these two sequences

(a) gnl|MAGPIE|eca.ECARTPF1_assembled--ECARTPF1_rep_c332 (e-144) and
(b) gnl|MAGPIE|eca.ECARTPF1_assembled--ECARTPF1_rep_c656 (4e-90) produced significant alignments with CjABCB1. However, the overlapping sequence data of (a) had more gaps basepair mismatch compared to (b). In overall, both contigs had fewer gaps and basepair mismatch compared to the other hits in the database thus making their sequences more reliable. The two sequences (a) and (b) exhibited a blastn expected values of e-144 and 4e-90 and were believed to be originating from a single unigene owing to the fact that they clustered together in the phylogenic tree and also because of high percentage identity (>90 %) they share when aligned and assembled using either DNASTar alignment tool. Primers (Table 2) were designed from these two sequences and used to amplify the EcABCB1. Successful amplification of PCR product of ca ± 4.5kb was confirmed by the use of agarose gel electrophoresis.

3.5 Molecular cloning of EcABCB1

EcABCB1 double digest with BamHI and HindIII, produced two bands of about ± 3.9 kb and ± 4kb as predicted by the restriction enzyme map (See Chapter two, Figure 5). To further confirm the presence of the insert and obtain its sequence, purified recombinant pSTBlue/EcABCB1 plasmid was sent to Quintara biosciences (http://www.quintarabio.com/) for complete sequencing. See appendix A for the nucleotide sequence of EcABCB1.
3.6 Sequence analysis of *EcABCB1*

The EcABCB1 contiguous sequence read is 4209 base pairs. BLASTn analysis of *EcABCB1* using the blast analysis program described in [132] revealed a significant match to some sequences in the www.ncbi.nlm.nih.gov database. However, none of the sequences from the hit is from *E. californica* suggesting that EcABCB1 sequence is novel and have not been previously reported. Top 7 hits with the highest match and query coverage is shown in (table 1).

Translation of the EcABCB1 nucleotide sequence was done using the expasy translate tool [99] and the predicted amino acids is 1290 (table 2). The conserved sequence motifs of ABC proteins are underlined and colored. Notable in the EcABCB1 amino acid sequence are following motifs: the Walker A (gold), Walker B (blue), Walker C or signature motif (red) and D-loop (green).

BLASTP analysis of EcABC1 using the http://blast.ncbi.nlm.nih.gov/Blast.cgi [132, 133] tools revealed ABC proteins similar to EcABC1. Amino acids from these sequences were used to build the phylogenic tree (Figure 20).
MAEANGLNLDPEATASVHPPLTKTDMSATNVEKKEETLKRVSKYLFSFADSTDVVLVMVGTLGAVANGLAM
PVMTLLLGDINAFQTAGTDNVHQQVSKVALRFVYLAIGAGTASFFQVACWMTGERQSAGRSLYLQTILTQDVAFFD
METNTEGVGRMSGDTVLQIAIKEVGKFIQLLLSTFIGGFVIALIKGWLTTVMLTPQSLAGAAMSIVIGKMASRQQA
AYSQAGNVVEQTIGSIRTVASFTGEQKAIANYNSITKAYSVHEGLAAGLGLGALMFVIFCSYALAIWYYGGKLILDKGY
TGGDVINVIIAVLTGSMMSGAQASPCLGSFAQAGQAAAFFKMFETINRKPEIDAYDPNVRVLSDIRGDIELREVFRYPARPDEQI
FSGFSLSIQPSTTAALVGQSGSKSTVISLIERFYDPQAGEVLIDGVNLKEILKWIREKIQLVSQEPVLFASIKENIAYGKD
GATLAEIRNAELANAAKFIDKLPQGDLTVGEHGTQMMSGQKQRPAARAILKDPRILLLDEATSALEAESERIVQEAOLRD
IMINRTTVAVHRSLTRVNRMDIAVHRSKVEKGSTELLKDSGPGYCLRLQEINRPVELHQVINDKGSNSMVEFARQS
SQPLSLPRISRGSSRNSSHLSVSFGQLPTGLNQENVQIEDPASLPPPEAQAEVPVRRLAALNKEIPVLLLGVISAVVNGVI
FPLFGLLFSAMIKTFYPEPPSEKKNRSFWALIYIFGVISFVSPARTYFFVGAGSRLIKIRSMCFEKVYMAGEWFDEPEH
SSGAIGARLSADAAAVRGLVDALALLVQNLATAISGGLIAFTASWELALIIIVLPLLNGWVGWVMKFGFSGDAKMK
MYEESQVANDAVGSRTVASFCAEKVMKYKKCEGPVSISKGRVLGVSIGFGLSFFLLFCVYATSFYAGARFVASGKI
TFTDVFRRVFFALTMTAIGISQSSSAPDSSKASKASASIFALLDRKSLIDASDDSGTTLENVKGEIEFRHSFVKYPTRPIDIQIFR
DLCLTIRAGKAVALVGESGSKSTAISLLQRFYDPDQGVTLDGVEIQKFQLRWLRQQMGLVSQEPALFNDTIRANVAYG
Figure 18: The amino acids of EcABCB1 (1290). The conserved ABC motifs are colored. Walker A (yellow); walker B (turquoise); walker C (Red) and D-loop (green).

Table 3. BLASTP analysis of EcABCB1 showing top 7 sequences with significant alignments. Description of the accession numbers, percentage region and identity of the EcABCB1 sequence covered and the ncbi accession numbers are shown.

<table>
<thead>
<tr>
<th>Description</th>
<th>Query cover (%)</th>
<th>Identity (%)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CjMDR1 [Coptis japonica]</td>
<td>99</td>
<td>78</td>
<td>BAB62040.1</td>
</tr>
<tr>
<td>hypothetical protein POPTR_0002s18860g [Populus trichocarpa]</td>
<td>95</td>
<td>77</td>
<td>XP_006386686.1</td>
</tr>
<tr>
<td>multidrug resistant ABC transporter family protein [Populus trichocarpa]</td>
<td>95</td>
<td>77</td>
<td>XP_002301547.1</td>
</tr>
<tr>
<td>hypothetical protein VITISV_029557 [Vitis vinifera]</td>
<td>97</td>
<td>77</td>
<td>CAN76787.1</td>
</tr>
<tr>
<td>PREDICTED: ABC transporter B family member 11 [Vitis vinifera]</td>
<td>100</td>
<td>76</td>
<td>XP_002273987.1</td>
</tr>
<tr>
<td>ATP binding cassette subfamily B4 isoform 1 [Theobroma cacao]</td>
<td>100</td>
<td>76</td>
<td>XP_007051281.1</td>
</tr>
<tr>
<td>ATP binding cassette subfamily B4 isoform 2 [Theobroma cacao]</td>
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<td>76</td>
<td>XP_007051282.1</td>
</tr>
<tr>
<td>ABC protein [Coptis japonica]</td>
<td>99</td>
<td>76</td>
<td>BAM11098.1</td>
</tr>
<tr>
<td>hypothetical protein CICLE_v10018532mg [Citrus clementina]</td>
<td>97</td>
<td>76</td>
<td>XP_006444609.1</td>
</tr>
<tr>
<td>multidrug resistant ABC transporter family protein [Populus trichocarpa]</td>
<td>100</td>
<td>75</td>
<td>XP_006375419.1</td>
</tr>
<tr>
<td>P-glycoprotein 11 [Arabidopsis thaliana]</td>
<td>96</td>
<td>74</td>
<td>NP_171753.1</td>
</tr>
</tbody>
</table>
Figure 19. EcABCB1 membrane topology. Representative six α-helix of the transmembrane domain (TMD1 and TMD2) and nucleotide binding domain (NBD1 and NBD2) are represented. The TOPCONS online server (http://topcons.cbr.su.se/) was used to predict the TMDs, while the conserved domain search tool in the national centre for biotechnology information (http://www.ncbi.nlm.nih.gov/) was used to predict the NBDs.
The topology of EcABCB1 amino acids were determined using topcon (http://topcons.cbr.su.se/); a consensus membrane topology prediction tool. The topology analysis of EcABCB1 supports the hypothesis that it is a full transporter with twelve transmembrane alpha helixes. Also based on the membrane topology (Figure 19), the N-termini and C-termini of EcABCB1 is orientated in such a way that they are inside (cytoplasm) with the structural arrangement of EcABCB1 predicted to be TMD1-NBD1-TMD2-NBD2.
Figure 20: Phylogenetic analysis of ABCB proteins and CrTPT2. MUSCLE align tool available in the advanced phylogeny analysis program [134-140] was used to align and build the phylogenetic tree. Sequences used in building the tree was pulled out after TBLASTn pairwise alignments of EcABCB1 in the ncbi database [132]. Branch support values are shown in red. Using this program, the maximum allowable bootstrap 100 was used.
3.7 Expression profile of EcABCB1 in Cell cultures and whole plant

To check the expression profile of EcABCB1 in the cell cultures, EcABCB1 F1 (Table 2) primer efficiency was first determined using qPCR. Standard curve and primer efficiency was automatically generated after running the samples in the CFX96 Real Time PCR machine (BIO RAD. CA. USA.).

3.7.1 Expression profile of EcABCB1 in cell cultures

To determine whether EcABCB1 transcripts could be induced by the elicitor preparation from fungal extract, 12 ml cells in suspension culture were inoculated with 290 µl of elicitor preparation and incubated with shaking for 2 hours. Total RNA was extracted from the elicited cells. 1 µg of total RNA was reverse transcribed and used in the expression profiling. Fungal elicitor preparation induced about 5 fold increase in the transcript level of EcABCB1 compared to the transcript level of EcABCB1 in the untreated cells in suspension culture (Figure 21).
3.7.2 Expression profile of EcABCB1 in whole plant

EcABCB1 was found to be expressed in all organs of the plant tested (Figure 22). However, more expression was recorded in the root. The level of EcABCB1 transcripts expressed in the root is five times more than the level expressed in the flower bud, 6 times more than the level expressed in the flower, four times more than the level expressed in the leaf, and two times more than the level expressed in the stem.
3.8 Heterologous expression of EcABCB1 in yeast cells

Recombination reaction was done according to the method already described in Chapter two to get a functional expression clone fused with HA tag.

3.8.1 SDS PAGE and Western Blot

Expression of EcABCB1 and CjABCB1 recombinant protein in the yeast (mam1 pdr1) was confirmed by probing the western blot with anti-HA tag antibody. The predicted size of both EcABCB1 and CjABCB1 is 139 KDa. Bands of expected size were seen in the whole protein extract (Figure 23 A) and the membrane extracts (Figure 23 B).
Figure 23. Western blot of recombinant proteins, EcABCB1, SBP and CjABCB1. (A) Whole protein extracts; lane 1 represents CjABCB1, lane 2 represents SBP, lane 3 represents EcABCB1. (B) Membrane protein extracts; lane 1 represents CjABCB1, lane 2 represents SBP, lane 3 represents EcABCB1. Arrow points to the position corresponding to about 139 Kda.
3.9 Functional characterization of EcABCB1

To functionally characterize the ability of EcABCB1 to transport alkaloid, EcABCB1 was transformed into mampdr1 and exposed to different alkaloid backbone of the benzylisoquinoline type. Yeast cell expressing EcABCB1 accumulated a significant amount of berberine up to 40% more than the amount accumulated by yeast cells expressing the SBP control \(p<0.05\) (Figure 24).

**Figure 24.** LC-MS analysis of the total amount of berberine accumulated in pREP41-3XHA-SBP control and pREP41-3XHA-EcABCB1 expressing in mam1pdr1 yeast cells. EcABCB1 mediated the influx of berberine. Scale bars represents standard deviation from 7 biological replicates. Asterisks show that the amount of berberine accumulated in EcABCB1 is significantly different from the amount accumulated in the SBP control \(p<0.05\).
Figure 25. LC-MS analysis of the total amount of sanguinarine accumulated in pREP41-3XHA-SBP control and pREP41-3XHA-EcABCB1 expressing in mam1pdr1 yeast cells. Scale bars represents standard deviation from 7 biological replicates sanguinarine uptake assay and 9 biological replicates for sanguinarine uptake inhibited with vanadate. Asterisks shows that the amount of sanguinarine accumulated in EcABCB1 is significantly different from the control ($p<0.05$).

In a similar manner, yeast cells expressing EcABCB1 accumulated about 50% more sanguinarine, an amount which is significantly higher than the amount of sanguinarine accumulated by yeast cells expressing the SBP control ($p<0.05$) (Figure 25). To test whether the uptake of alkaloids in yeast cells expressing EcABCB1 is dependent on ATP, yeast cells expressing EcABCB1 and the SBP control were incubated with the same amount of sanguinarine to a final concentration of 3 µg/ml, vanadate was added to a final concentration of 2 mM. The amount of sanguinarine accumulated in the vanadate treated yeast cells expressing EcABCB1 were significantly low compared to the amount accumulated in the non-vanadate treated yeast.
cells expressing EcABCB1 (p<0.05) (Figure 25). Vanadate-treated yeast cells expressing EcABCB1 accumulated about 48% less sanguinarine than non-vanadate treated yeast cells (Figure 25).

Figure 26. LC-MS analysis of total the amount of morphine base accumulated in pREP41-3XHA-SBP control and pREP41-3XHA-EcABCB1. Scale bars represents standard deviation from 8 biological replicates. Asterisks shows the difference in the amount of berberine accumulated SBP is significantly different from the amount accumulated in EcABCB1 (p<0.05).

Other benzylisoquinoline alkaloid such morphine base, scoulerine and cryptopine were tested also tested for transport. Yeast cell expressing morphine-base accumulated 21% more morphine base than control cells. This amount is statistically significant compared to the amount accumulated in the yeast cells expressing the SBP control (p<0.05) (Figure 26). However not all the alkaloids tested were transported. Yeast cells expressing EcABCB1 were not able to take up scoulerine at an amount greater than the control (Figure 27). The amount of cryptopine
accumulated in yeast cells expressing EcABCB1 and SBP were not quantifiable with the available standard curve implying that there was no influx of cryptopine (data not shown).

A Non-alkaloid, ethidium bromide, was also tested for transport. Yeast cells expressing EcABCB1 did not accumulate ethidium bromide at a level past the level detected in the yeast cells expressing the SBP control (Figure 28).

Figure 27. LC-MS analysis of total the amount of scoulerine accumulated in pREP41-3XHA-SBP control and pREP41-3XHA-EcABCB1. Scale bars represents standard deviation from 8 biological replicates.
Figure 28. LC-MS analysis of total the amount of ethidium bromide accumulated in pREP41-3XHA-SBP control and pREP41-3XHA-EcABCB1. Scale bars represents standard deviation from 8 biological replicates. The difference between the control and EcABCB1 ($p>0.05$) is not statistically significant.

3.10 Subcellular localization of EcABCB1 in *Nicotiana tabacum* leaf

To determine the expression pattern of EcABCB1 in the subcell, N-terminally YFP fusion construct of pEarley Gate 104 Vector (pEG104) was used to perform a LR recombination reaction with an aim to fuse the N-terminus of EcABCB1 with YFP.

As can be seen in Figure 29, EcABCB1 seem to localize to the plasma membrane, and the golgi bodies around the nuclear envelope. However, some weak fluorescence seem to be seen in
the nucleoplasm thus requiring further confirmation. Further confirmation was done by incubating the transfected leaves in 5 mM NaCl for few minutes and then imaging. Figure 29 C is an image of plasmolyzed leaf showing the Hechtian strands fluorescing.

Figure 29. YFP-EcABCB1 localization in tobacco leaf. (A) and (B) are images from the transfected leaf of tobacco expressing YFP-EcABCB1. (C) is an image from plasmolyzed transfected leaf of tobacco expressing YFP-EcABCB1. White arrow points to a Hechtian strand.
3.11 Discussion

Eliciting the California poppy cell suspension cultures with yeast extract induced more accumulation of alkaloids in the media (Figure 15). Sanguinarine was one of the induced compounds (Figure 15, and Figure 16). It has been previously reported that about 40% of the alkaloid produced in the yeast extract treated California poppy cell suspension cultures get secreted into the media after 6 hours of incubation [9].

Evidence have shown that sanguinarine uptake occurs in the California poppy cell suspension cultures [55]. Weiss et al., 2006 reported that sanguinarine reductase catalyze the conversion of sanguinarine taken up from the media to dihydrosanguinarine [55]. Based on this report, the reduced amount of sanguinarine observed 6 hours after elicitation (Figure 16 and Figure 17) was thought to be due to uptake and maybe further conversion into dihydrosanguinarine. Although no transporter has been associated with this particular uptake event, ABC transporters have been implicated in the transport of alkaloids in several other plants such as the *Coptis japonica* (CjABCB1) [10] and (CjABCB2) [11] and *Catharanthus roseus* (CrTPT2) [71] where they function in import and export respectively. CjABCB1 and CjABCB2 belong to the ABCB subfamily of transporters the same family to which EcABCB1 belong.

The ABCB subfamily members are the largest subfamily of ABC transporters in plants. Majority of them have been identified as full size ABC transporters with a characteristic two nucleotide binding domains [61]. The structural arrangement of ABCB’s is typically TMD-NBD-TMD-NBD [61] and their occurrence is not restricted to any plant part.

Topcons was used to predict the topology of the EcABCB1. It was the tool of choice because its prediction is based on the consensus prediction from 5 different prediction tools
making the prediction result more reliable. Like many ABCB members, EcABCB1 is a full transporter with twelve transmembrane helixes. The C domain of EcABCB1 has a sequence identity of MSSGQ as opposed to LSSGQ found in CjABCB1 and CjABCB2.

Evidence available suggests that, the direction of transport for plant ABCB subfamily members are not unidirectional. They could function as importers as well as exporters of a wide range of substrates. For instance, AtABCB1 [60, 141], LjABCB1 [142], ABCB4[62] and AtABCB19 [61, 64] function in auxin export while CjABCB1 and CjABCB2 function in berberine import [10, 11, 39].

Not many plant ABCB subfamily members have been tested for specificity. Among those tested are CjABCB1 and CjABCB2 with evidence suggesting that they have low substrate specificity. CjABCB1 [10]and CjABCB2 [11] mediated the influx of berberine and 4-Nitroquinoline N-oxide but not cycloheximide [10, 11]. CjABCB1 also mediated the accumulation of ajmalicine and tetrahydroalstonine [143]. Also tested is AtABCB1, which showed specificity to auxin [60].

Unlike most ABCB proteins tested, evidence from this report suggests that EcABCB1 is a multi-specific ABCB influx transporter most probably localized to the plasma membrane and the ER. Cells were plasmolyzed to confirm its localization to the PM. Localization to the ER was suggested because fluorescence was observed in the membrane around the nucleus (Figure 29).

Significantly higher amounts of berberine (Figure 24), sanguinarine (Figure 25) and morphine (Figure 26) accumulated in yeast cells expressing EcABCB1 compared to the amount accumulated in yeast cells expressing SBP control. Yeast cells expressing EcABCB1 did not
accumulate ethidium bromide compared to yeast cells expressing SBP suggesting that EcABCB1 does not mediate the uptake of ethidium bromide (Figure 28). Yeast cells expressing EcABCB1 also did not accumulate scoulerine (Figure 27) and cryptopine (data not shown) at an amount higher the basal level detected in yeast cells expressing SBP control suggesting a type of selectivity in the compounds transported. However, nothing is known about the determinants of substrate specificity.

Alkaloids were found in all parts of California poppy plant so is the transcript of EcABCB1 thus strongly supporting the evidence it is a multi-specific ABCB transporter. However, this expression pattern of EcABCB1 transcript could also suggest that it is involved in other function(s) besides being involved in the trafficking of alkaloids. It could also be possible that its localization is not restricted to one cell type because alkaloid stains and fluorescence as observed in histology sections (see chapter three) suggest that more than one cell type accumulates alkaloid in the root of California poppy. However, this could be addressed in future studies.

Although the evidence available for subcellular localization suggest that EcABCB1 is plasma membrane localized (Figure 29), replicates of that experiment showed some mis-localization to the nucleoplasm. This was thought to be due to the location of the YFP tag at the N-termini of the protein (cytoplasm) (Figure 19). However, further confirmation using a more robust technique of subcellular localization may be required.
Chapter Four: **Pharmacological studies in California poppy cell suspension cultures**

4.1 **Introduction**

In this chapter, all the results from the pharmaceutical studies and expression analysis done in California poppy cell suspension cultures are presented.

The California poppy plant produces various classes of isoquinoline-derived alkaloids such as tetrahydroproberberine (escholidine) [144], benzophenanthridine (sanguinarine) and protopines (protopine). California poppy at the flowering stage of growth accumulates the largest amount of different subgroups of alkaloids [145] such as the aporphines example $N$-methylaurotetanine; pavines example californidine and eschscholtzine; protopines example protopine and benzophenanthridines example sanguinarine, marcapine and chelerythrine [145, 146]. Although different alkaloids are found in different parts of California poppy plant, the dominant ones in select plants parts such as the root are allocryptopine, protopine, sanguinarine chelerythrine, escholidine, magnoflorine or escholine, escholinine and canadine. In the leaf and stem, the most abundant are californidine, lauroscholtzine, protopine, eschscholtzine, and allocryptopine. Also in smaller quantities are the benzophenanthridines, while eschscholtzine is mostly found in the flower [147]. The California poppy cell suspension cultures have been reported severally to biosynthesize benzylisoquinoline alkaloids notably the antimicrobial sanguinarine and secrete them into the growth media [7, 9, 47, 49, 89, 127, 128].

The aim of this study is to determine whether an active transport process is involved in the secretion of alkaloids into the media of California poppy. The experimental approach involved the use of thin layer chromatography to confirm the presence of alkaloids in the media and the cells. Fungal elicitor preparation will be used to induce the accumulation of alkaloids. Successful induction will be confirmed by extracting alkaloids from the induced cells and their
media and analyzing by use of thin layer chromatography. To check for the involvement of active transport processes in the secretion of alkaloids into the media, induced cells will be treated with ABC transporter inhibitor drugs. Inhibition will be confirmed by extracting from the drug treated cells and media and analyzing them by thin layer chromatography. Effect of the drugs on transcript level of selected alkaloid biosynthetic enzymes will be determined using qPCR.

4.2 Pharmacological studies

To check if active transport is involved in the secretion of alkaloids into the media of California poppy cell suspension cultures, ABC transporter inhibitor drugs were used to attempt to block the secretion of alkaloids into the media.

One of the drugs used was vanadate. Vanadate is a broad spectrum inhibitor of tyrosine phosphatase and P-type ATPase such as H⁺ ATPase. It is an analogue of free phosphate and engages in competitive binding with free phosphate for binding to ADP to form ATP. Treating the cells with this drug will directly inhibit ABC transporters and proton pumps and also cause depolarization of membrane potential usually required for active transport of solute across the membrane [9]. 2 mM vanadate has been reported to inhibit the accumulation of alkaloid in the media of California poppy cells [9].

The second used is thioridazine hydrochloride. Thioridazine hydrochloride has been shown to inhibit three major human ABC transporters namely; P-glycoprotein (P-gp/ ABCB1), Breast cancer resistance protein (BCRP/ABCG2) and Multidrug Resistance Associated Protein 2 (MRP2/ABCC2) [148]. It was chosen because it is a lipophilic multi-specific inhibitor and does not bind to the catalytic site of the human MRP1 ATP-binding domain [148] suggesting that it acts as an inhibitor of substrate-binding, rather than ATP.
Thioridazine is also a Ca\(^{2+}\) antagonist. Calcium-antagonist drugs principally interfere with the entry of calcium into cells through voltage-sensitive channels \[149\]. Cross reaction of this drug with other pumps such as Ca\(^{2+}\) channel is expected.

The third ABC transporter inhibitor drug used was cyclosporine A (CsA). CsA has been shown to inhibit three major human ABC transporters namely: P-glycoprotein (P-gp/ABCB1), Breast cancer resistance protein (BCRP/ABCG2) and Multidrug Resistance Associated Protein 2 (MRP2/ABCC2) \[148\]. These three transporters mediate the efflux of drug-like compounds to the lumen of the intestine, limiting the absorption of exotic substances by the intestine \[148\]. In addition to the above activities, it was also reported not to affect H\(^+\)/K\(^+\)-ATPase transport activity in MDCK cells and two subtypes C7 and C11 \[150\]. The above reported properties are the reasons why these drugs were chosen for use in this study.

4.2.1 The effect of sodium orthovanadate (Vanadate) on the accumulation of alkaloids in the media of suspension cells

In an independent drug inhibition trial, six days old cell suspension cultures were incubated with 500 \(\mu\)M vanadate and elicitor and 2 mM vanadate and elicitor for 2 hours. After 2 hours of incubation, cells were harvested and alkaloids were extracted from the cells and the media using the method described in chapter two. The results obtained showed that 500 \(\mu\)M vanadate had little or no observable inhibitory effect in the usual accumulation of sanguinarine in the media of elicited cells but it seem to have induced more accumulation of sanguinarine in the cells (Figure 30).

On the contrary, 2 mM vanadate had obvious inhibitory effect in the usual induced accumulation of sanguinarine in the media of elicited cells compared to the control (Figure 30)
A). In the extracts from the cells of 2 mM vanadate-treated cultures, sanguinarine accumulated at an amount about the same as that seen in the extracts from the elicited cultures (Figure 30 B).

![Image 1](image1.jpg)

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Figure 30: Reduction in the accumulation of mostly sanguinarine in the media of elicited cell cultures treated with 2 mM Na$_3$O$_4$V. 6 days old cell suspension cultures were elicited and at the same time treated with 500 μM and 2 mM vanadate. Treated cells were incubated for 2 hours with shaking at 125 rpm at room temperature. At the end of 2 hours incubation, cells were harvested and alkaloids were extracted from the media and the cells. (A) Represents alkaloid extracted from the media; (B) represents alkaloid extracted from the cells. Arrow points to sanguinarine migration point.

4.2.2 The effect of thioridazine hydrochloride on the accumulation of alkaloids in the media of suspension cells

Two other ABC transporter inhibitor drugs thioridazine hydrochloride and cyclosporine A were also used to try to block the secretion of alkaloids into the media of elicited and unelicited cells in suspension culture. 50 μM thioridazine hydrochloride was added at the same time as the elicitor to six days old cell suspension cultures and incubated for 2 hours. There was
a reduction in the accumulation of mostly sanguinarine in the media and cells of suspension cultures treated with 50 μM thioridazine hydrochloride (Figure 31).

![Figure 31: Reduction in the accumulation of sanguinarine in the media of elicited cells treated with 50 μM thioridazine hydrochloride. 6 days old cell suspension cultures were elicited and at the same time treated with 50 μM thioridazine hydrochloride. Treated cells were incubated for 2 hours with shaking at 125 rpm at room temperature. At the end of 2 hours incubation, cells were harvested and alkaloids were extracted from the media and the cells. (A) Represents alkaloid extracted from the media; (B) represents alkaloid extracted from the cells.](image)

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4.2.3 The effect of cyclosporine A (CsA) on the accumulation of alkaloids in the media of suspension cells

500 μM CsA was added at the same as the elicitor to 6 days old cell suspension cultures and incubated for 2 hours. Cells were harvested after 2 hours and alkaloids were extracted from the media and the cells. The presence of 500 μM CsA in the media of cell cultures caused a visible reduction in the accumulation of mostly sanguinarine in the media of elicited cells (Figure 32).
Just like what was observed in the extracts from the media of cell cultures treated with Vanadate, where reduction of mostly sanguinarine was observed (Figure 30), reduction of mostly sanguinarine alkaloid was observed when cell cultures were treated with 50 µM thioridazine hydrochloride suggesting that the secretion of alkaloids into the media of E. California cell suspension cultures involves ATP hydrolysis (Figure 31 A).

Figure 32: Reduction in the accumulation of sanguinarine in the media of elicited cells treated with 500 µM cyclosporine A. 6 days old cell suspension cultures were elicited and at the same time treated with 500 µM cyclosporine A. Treated cells were incubated for 2 hours with shaking at 125 rpm at room temperature. At the end of 2 hours incubation, cells were harvested and alkaloids were extracted from the media and the cells. (A) Represents alkaloid extracted from the media; (B) represents alkaloid extracted from the cells. Arrow points to sanguinarine migration point on TLC plate.
4.3 Expression profile of transcripts of selected BIA biosynthetic enzymes in elicited and drug treated cells

qPCR of transcripts of selected enzymes were done for the following reasons (i): to determine the effect of elicitation on the transcripts of selected key enzymes (BBE, P6H, 4’OMT, CYP80B1, CYP719A2 and CYP719A3) involved in the BIA biosynthesis. These enzymes were selected because they play key roles in the biosynthetic pathway of the BIAs. Some of these enzymes 4’OMT [47], P6H [129] and BBE [127] have been reported to be induced by fungal elicitor, however, their response to Vanadate, thioridazine hydrochloride and cyclosporine A have not been reported. We propose that elicitation with fungal extract elicitor preparation will induce the up-regulation of the other genes. (ii) Upon confirming the induction of the transcripts of the selected genes, qPCR will be performed using samples treated with drugs to check for the effect of the drugs on the steady state mRNA level of these genes. Considering that these drugs could have other side effects on the cells, it is important to check that one of the effects is not inhibition of the transcription of these genes which in turn leads to inhibition gene expression and alkaloid biosynthesis. This confirmation is important in understanding the reasons why the amount of alkaloids accumulating in the media decreased following treatment with these drugs.

To do all these, total RNA was extracted from the elicited cells and drug treated cells. 1 µg of total RNA was reverse transcribed and used in the transcript level expression profiling of the selected enzymes: Outliers in this experiment were detected and removed using the interquatile range method.
4.3.1 BBE response to vanadate, thioridazine hydrochloride and cyclosporine A in elicited cells

Elicitation induced about 22 fold increase in the BBE transcript level in California poppy cell suspension cultures compared to the level of BBE transcript in the untreated cell cultures (Figure 33 A). The observed increase in the BBE transcript level is significant compared to untreated, p<0.05. In a similar manner Vanadate and elicitor treated cell cultures induced about 45 fold increase in the level of BBE transcripts compared to the level of BBE in untreated cell cultures which is about two times the level of induction in the elicitor treated suspension cultures (Figure 33 A).

A 165 fold increase in the BBE transcript level was found in suspension cultures incubated with elicitor and thioridazine hydrochloride compared to the level of BBE transcript in elicited cell suspension cultures (Figure 33 A) suggesting that thioridazine hydrochloride which appeared to block the secretion of alkaloid into the media of elicited suspension cultures (Figure 31) did not affect the signalling process leading up to the biosynthesis of the benzophenanthridine alkaloids in the California poppy suspension cultures.

On the hand, CsA significantly reduced the level of BBE transcript level in the CsA and elicitor treated cell suspension cultures by about ½ fold compared to the elicitor-treated cell suspension cultures, p<0.05 but a 13 fold increase in the BBE transcript level was observed in the CsA and elicitor treated cell suspension cultures compared to the level of BBE transcript level in the untreated cell suspension cultures (Figure 33 A).
Figure 33: The qPCR of transcripts of selected BIA biosynthetic enzymes incubated with elicitor, vanadate, thioridazine hydrochloride and cyclosporine A. Scale bars represents standard error of means (SEM) from twenty three to twenty nine biological replicates of untreated, twenty five to thirty biological replicates of elicited, nine to ten biological replicates of vanadate and elicited, thioridazine hydrochloride and elicited and CsA and elicited cell suspension cultures. Asterisks show statistically significant difference between the following pair of treatments: elicited and untreated, elicited and vanadate and untreated, elicited, elicited and vanadate and elicited, elicited and thioridazine hydrochloride and elicited, elicited and CsA and elicited (p<0.05); one asterisk equals single comparison, two equals double comparison.
4.3.2 Protopine-6-hydroxylase response to Vanadate, thioridazine hydrochloride and cyclosporine A in elicited cells

The response of protopine-6-hydroxylase transcripts to Vanadate, thioridazine and CsA were similar to the response of BBE to these three drugs. However, P6H transcript responded more to elicitor compared to BBE transcript (Figure 33 A). Yeast extract elicitor induced the P6H transcript level to about 75 fold compared to the P6H transcript level in the untreated cell suspension cultures (Figure 33 B).

P6H transcript level in the elicited and Vanadate treated cell suspension cultures increased significantly to about two times the level induced by fungal extract elicitor, p<0.05 (Figure 33 B). However, compared to the untreated cell suspension cultures, P6H transcript level increased significantly to about 147 fold in the elicited and vanadate cell suspension culture, p<0.05.

Similar to vanadate, cell suspension culture treated with elicitor and thioridazine hydrochloride, showed a significant increase of about 113 fold in the level of P6H transcripts compared to the level of P6H in the untreated cell suspension cultures, p<0.05 (Figure 33 B) but when compared to the elicited cell suspension cultures, P6H transcript level increased to about 37 fold, p<0.05 (Figure 33 B).

However, CsA significantly inhibited the transcript level of P6H although some level of induction was still observed, p<0.05 (Figure 33 B). The P6H transcript level in the CsA and elicitor treated cell suspension cultures decreased to about one-third fold compared to the elicitor-treated cell cultures but increased to about 25 fold compared to the level in the untreated cell suspension (Figure 33 B).
4.3.3 The response of CYP80B1 to Vanadate, thioridazine hydrochloride and cyclosporine A in elicited cells

Similar to the responses of BBE (Figure 33 A) and P6H (Figure 33 B) transcript levels to yeast extract elicitor, CYP80B1 (Figure 33 C) and 4’OMT (Figure 33 D) responded to yeast extract elicitor by increasing the level of their transcripts but at a lower level compared to the former two. Elicitation induced CYP80B1 transcript level to about 2.5 fold compared to the level of CYP80B1 transcripts in the untreated cell suspension cultures. The CYP80B1 transcript level increased significantly to about 4 fold in the elicited and Vanadate treated cell suspension cultures compared to the basal level in the untreated cell suspension cultures (Figure 33 C). Compared to the elicited cell suspension cultures, the level of CYP80B1 transcripts increased significantly to about 1.7 fold in the elicited and Vanadate treated cell suspension cultures, p<0.05.

On the contrary, the level of CYP80B1 reduced significantly by about 2.1 fold in cell suspension culture incubated with elicitor, p<0.05 and thioridazine (Figure 33 C) compared to the transcript level in the elicitor treated cell suspension cultures (Figure 33 C). The transcript level of CYP80B1 elicitor and cyclosporine A treated cells were about the same as the level induced by yeast extract elicitor (Figure 33 C).

4.3.4 The response of 4’OMT to Vanadate, thioridazine hydrochloride and cyclosporine A in elicited cells

Elicitation induced the transcript level of 4’OMT to about 3.8 fold compared to the level detected in the untreated cell suspension cultures (Figure 33 D). Compared to the level of 4’OMT in the elicited cell suspension cultures, none of the drugs used in the study caused a further significant increase or decrease in the transcript level of 4’OMT (Figure 33 D).
4.3.5 The response of CYP719A2 and CYP719A3 to Vanadate, thioridazine hydrochloride and cyclosporine A in elicited cells

Upregulation of CYP719A2 transcript level could not be detected in the elicitor treated cell suspension cultures. However, significant level of induction was detected when cell suspension cultures were incubated with elicitor and thioridazine and elicitor and cyclosporine A compared to the incubation with just elicitor (Figure 33 E). CYP719A2 transcript level increased to about 25 fold in the presence of elicitor and thioridazine and to about 2 fold in the presence of elicitor and cyclosporine A (Figure 33 E). Incubation of cell suspension with elicitor and vanadate caused a significant 0.8 fold reduction in the transcript level of CYP719A2 compared to the levels detected in the untreated and elicited cell suspension cultures, p<0.05 (Figure 33 E).

CYP719A3 transcript level responded differently to treatments with yeast elicitor compared to BBE (Figure 33 A), P6H (Figure 33 B), CYP80B1 (Figure 33 C) and 4’OMT (Figure 33 D) which were all induced at the transcript level by yeast extract elicitor. A 0.7 fold reduction which is statistically significant was recorded for the transcript level of CYP719A3 in the elicitor treated cell suspension cultures compared to the untreated, p<0.05 (Figure 33 F). About 0.8 fold further reduction was detected in the transcript level of CYP719A3 when cell suspension cultures were incubated with vanadate and elicitor compared to the level of transcripts in the untreated cell suspension cultures (Figure 33 F). Compared to the level of transcripts in the elicitor treated cell suspension cultures, a 0.1 fold decrease was recorded for CYP719A3 in vanadate and elicitor treated cell suspension cultures. However, in the presence of CsA and elicitor CYP719A3 transcript level reduced by about 0.2 fold compared to the level in
the untreated while it significantly increased by about 0.5 fold in the elicited cell suspension cultures, p<0.05 (Figure 33 F).

4.4 Quantification of alkaloids by use of LC-MS

Absolute quantification of analytes by use of TLC is usually very difficult. Part of the reason is because the solvent system used does not always separate all the compounds very well on the TLC plate thus co-migration of compounds is anticipated. Hence, the migration of more than one compound together on a TLC complicates the interpretation of result. In order to overcome this TLC shortfalls, LC-MS was used. LC-MS is a very reliable technique used in the quantitation of small molecules like the alkaloids. Cells in suspension culture treated with vanadate was used in this analysis because, first, vanadate blocked the secretion of alkaloid into the media. Secondly, of the three drugs used in this study, vanadate was the only one without any inhibitory effect on the induction of the transcripts of select enzymes studied (see Figure 33).

4.4.1 The effect of Sodium orthovanadate on the total amount of select alkaloids

The cell suspension cultures of *E. californica* biosynthesize a wide variety of structurally diverse benzophenanthridine subclass of alkaloids [95]. However, for the purpose of this report, the response of four major alkaloids namely sanguinarine, marcapine, chelirubine and dihydrosanguinarine to treatments will be discussed. (See Chapter One: for their positions in the pathway). Incubation with either elicitor, vanadate or elicitor and vanadate was done for 2 hours.
Similarly, significant increase in the total amount of alkaloids was seen in cell suspension cultures incubated with vanadate together with elicitor for 2 hours compared to the untreated, \( p<0.05 \) (Figure 34). No significant difference was observed in the total amount of characterized alkaloids accumulated in the untreated and vanadate treated cell suspension cultures but, the alkaloid content in vanadate treated cultures seem to be non-significantly higher compared to the control (Figure 34).
Cell suspension cultures treated with elicitor accumulated significantly more alkaloid compared to the untreated cell suspension cultures \( p<0.05 \) (Figure 34). This also confirms the observation made in Figure 16 that the elicitation of cell suspension culture of *E. californica* cause an increase in the accumulation of total amount of alkaloids within 2 hours of incubation.

**Figure 35**: The LC-MS analysis of the response of total amount of sanguinarine, marcapine, chelirubine and dihydrosanguinarine to elicitor and drug. Scale bars are standard error of means from 3 biological replicates for untreated and four biological replicates for elicited, vanadate and elicitor, and vanadate treated cell suspension cultures. Asterisks show statistically significant difference compared to the control (\( p<0.05 \))

The total amount of sanguinarine present in the elicited cell suspension cultures is significantly higher compared to the untreated, \( p<0.05 \) (Figure 35). Also, when cells are treated
with vanadate and elicitor, the total amount of sanguinarine present increase at a significant level compared to the amount present in the untreated, \( p<0.05 \) (Figure 35). Other compounds also seem to respond to elicitor but their level of accumulation were not found to be significantly higher after statistical analysis, \( p>0.05 \).

**4.4.2 The ratio of the total amount of characterized alkaloids accumulated in the media compared to the cells**

![Bar graph showing the ratio of alkaloid accumulation in media compared to cells](image)

*Figure 36: The LC-MS analysis of the ratio of response of individual alkaloid evaluated to treatments with elicitor, vanadate, and vanadate and elicitor in the media compared to the cells. Scale bars are standard error of means from 3 biological replicates for untreated, four biological replicates for elicited, vanadate and elicitor and vanadate treated cell suspension cultures. Asterisks show statistically significant difference compared to the control (\( p<0.05 \)).*

Generally speaking, incubation of the cell suspension cultures with vanadate alone appeared to cause an increase in the amount of alkaloid accumulated in the cell thereby leading to a decrease in the amount accumulated in the media (Figure 36): This observation falls in line
with previous report that vanadate induce more accumulation of alkaloid within the cells of *E. californica* [9]. However, a statistically significant difference was only observed for the amount of macarpine in the media of vanadate treated cells compared to the amount in the media of untreated cells, p<0.05. Although it has previously been reported that elicitation cause an increase in the total amount of alkaloids accumulated in the media [9], we observe that this response seems to vary from compound to compound (Figure 36).

Dihydrosanguinarine is barely detected in the media of untreated and elicited cell suspension cultures (Figure 36). Its amount increase in the media compared to the cells though not at a significant level when cell suspension cultures are incubated in the presence of either vanadate or with elicitor and vanadate to about 0.1 fold, p<0.05. There was about 1.3 fold increase in the amount of sanguinarine accumulated in the media of elicited cells compared to the media of untreated cells. However the amount of sanguinarine in the media of vanadate treated cells reduced with about about 0.3 fold compared to the amount in the media of untreated cells (Figure 36) suggesting that vanadate inhibited the secretion of sanguinarine into the media, thus supporting the observation made using TLC (Figure 16). A slight increase of about 0.4 fold was observed in the media of cells incubated with elicitor and vanadate. This slight increase might be due to more production of alkaloid induced by the elicitor which may have undermined the effect of vanadate on the secretion of alkaloid into the media (Figure 36). However this result further suggests that more sanguinarine accumulated in the cell of elicitor and vanadate treated cells. In contrast to the amount of sanguinarine accumulated in the media of elicited cells and in support of the observations using TLC, marcapine and chelirubine does not seem to accumulate more in the media of elicited and vanadate and elicitor treated cells compared to the media of
untreated cells (Figure 36). However the amount chelirubine in the media of vanadate treated cells reduced by about 0.9 fold compared to the amount in the media of untreated cells. While the amount marcapine in the media of vanadate treated cells reduced by about 0.6 fold compared to the amount in the media of untreated treated cells.

4.5 Discussion

Elicitation also induced the transcript level of BBE (Figure 33 A), P6H (Figure 33 B), CYP80B1 (Figure 33 C) and 4’OMT (Figure 33 D). Increase in the level of BBE and CYP80B1 proteins have been reported in yeast extract elicitor treated opium poppy cell cultures [53].

The total amount of alkaloids accumulated in the cell and media when cell suspension cultures are incubated with elicitor and vanadate are significantly high compared to the untreated, p<0.05 (Figure 34). According to Villegas et al., 2000, incubation of California poppy cell suspension culture with either vanadate or elicitor caused an increase in the pH of the medium and inhibited the transport of protons from the cytoplasm to the external medium thus leading to the acidification of cytoplasm. The acidification process then in-turn signals for the production of more alkaloid as a defense response. They also reported that treatment with either elicitor or vanadate led to an increase in alkaloid production but most of the alkaloid produced remain in the cell while a small amount about 10 % get transported into the medium [9]. At this time, the pH of the medium is the same as the untreated or just the elicited [9]. This is similar to what was observed in this assay, the pH of the untreated, elicited, and vanadate treated cell cultures remained the same within the time of the incubation (data not shown).

The transcript level of P6H (Figure 33 B), BBE (Figure 33 A) and CYP80B1 (Figure 33 C) increased in suspension cultures incubated with elicitor and vanadate strongly suggesting that
induction of these genes’ transcripts still occurred in the presence of vanadate. It also suggest
that the decrease in the amount of alkaloid secreted into the media of suspension cultures treated
with vanadate is not due to the interference of vanadate to the signalling events leading up to the
biosynthesis of alkaloids in elicited cell suspension cultures but more likely to be due to
inhibition of one or more ATP dependent transporter. It has also been reported that the California
poppy cell suspension culture incubated with vanadate showed an increase H⁺ATPase activity
[9].

Vanadate and elicitor can independently induce the production of Tyrosine decarboxylase
(TDC) involved in the BIA biosynthesis in California poppy [9] and this induction could be
detected for as early as 2 hours after elicitation [9].

Incubation of cell suspension culture with vanadate caused a reduction in the amount of
sanguinarine secreted into the media and an increase in the amount accumulated in the cells.
However, the amount of dihydrosanguinarine secreted into the media increased after incubation
with vanadate compared to the amount retained in the cells suggesting that the transport of
sanguinarine into the media is blocked (Figure 36). These result falls in line with previous
reports [7, 9, 47, 49, 89, 127, 128], that incubating the California poppy cell suspension cultures
with fungal elicitor preparations induces the accumulation of more alkaloids in both the media
and the cells. Sanguinarine accumulation is the most visible on the TLC plate showing alkaloids
extracted from the media and cells of elicited cell suspension cultures (Figure 15 and
Figure 16). These observations were further confirmed using the LC-MS technique (Figure 36).
The decrease in the amount of sanguinarine observed on TLC plates containing alkaloids
extracted from the media of elicitor and vanadate treated cell suspension cultures (Figure 30) was
also confirmed using LC-MS (Figure 36). The non-significant response of other compounds studied could be due to “type II error” which usually arise when the number of biological replicates used in an experiment is low. Increasing the number of the replicates might improve on the data reported in Figure 35.

A good number of the alkaloids produced such as sanguinarine, protopine chelirubine, dihydrosanguinarine and marcapine were annotated using the LC-MS (see appendix B for a list of their precursor masses and product ions). More sanguinarine accumulation observed in extracts from the cells of untreated cell suspension culture (Figure 16 B) compared to extracts from the cells of elicited cell suspension culture suggests that elicitation induced more biosynthesis and secretion of alkaloid into the growth media of suspension cultures. Therefore, in the absence of elicitor more sanguinarine is retained and less is secreted into the media suggesting that less is biosynthesized.

The transcript level of P6H (Figure 33 B), BBE (Figure 33 A) and CYP80B1 (Figure 33 C) increased in suspension cultures incubated with elicitor and thioridazine hydrochloride strongly suggesting that induction of these genes’ transcripts still occurred in the presence of thioridazine hydrochloride. It also suggest that the decrease in the amount of alkaloid secreted into the media of suspension cultures treated with thioridazine hydrochloride is not due to the interference of thioridazine hydrochloride to the signalling events leading up to the biosynthesis of alkaloids in elicited cell suspension cultures, rather it is more likely to be due to inhibition of one or more ATP dependent transporter

Cyclosporine A significantly reduced the transcript level of BBE (Figure 33 A) and P6H (Figure 33 B) but had no significant effect on the transcript level of CYP80B1, 4’OMT,
CYP719A2 and CYP719A3, \( p<0.05 \). This suggest that CsA might be affecting the signaling event leading up to the biosynthesis and secretion of alkaloids into the media or the active transport of alkaloid into the media because some level of induction of the two genes repressed were still detected.

Based on the evidence presented in this chapter, there is a strong indication of the involvement of ABC transporters in the transport of alkaloids. Incubation with vanadate or any of the drugs used did not change the pH of the medium for the duration of the incubation, suggesting that the reduced amount of alkaloid accumulated in the media is not because the pH of the medium was altered. Vanadate is known to be specific for the inhibition of plasma membrane ATPase [151]. Therefore, its ability to inhibit secretion of alkaloids into the media without affecting the transcript level of the selected enzymes suggest that it is affecting ATP hydrolysis at the plasma membrane. It also suggest that the ATP hydrolysis affected is linked to export of alkaloids into the media. However, it remains to be determined if the ATP being affected is that of the EcABCB1.
Chapter Five: Anatomy of the California poppy root

5.1 Introduction

The structure of California poppy root as well as the structure and formation of the laticifers in the root will all be discussed in this chapter.

5.2 Structure of root

Plant roots perform numerous functions. The principal role of roots includes the provision of support and absorbance of nutrient from the soil, while the secondary role includes storing food and conductance of water and minerals in the plant. Plant roots undergo two stages of growth, namely: primary and secondary growth stage and at each growth stage, the plant root exhibit unique structural organization [152].

5.2.1 Root structure at primary growth stage

The primary root is the first root to emerge from a germinating embryo. There is relatively little difference between tissues at primary stage of growth. Three most prominent tissues at primary growth stage are the dermal tissues, example the epidermis; the ground tissues, example the cortex and the vascular tissues, example the vascular cylinder [152].

5.2.1.1 The epidermis

The epidermis provides surface for the emergence of the root hairs which aid the uptake of water and nutrient from the soil. In some roots, the absorbing part of the epidermis have a thin layer of cuticle while in some others, the cell wall of the epidermis are suberized [152].

5.2.1.2 The cortex

A large portion of the primary root is composed of the cortex. Found in the cortex are the plastids which store starch grains but lack chlorophyll. Angiosperms only have their cortex at the
primary growth stage. As the plant root progress from the primary to the secondary growth stage, the cortex is lost. The cortex has a lot of air spaces required for aeration of the root cells. The flow of materials from one cortical cell to the other can occur through the plasmodesmata in a process known as the symplastic pathway, or through the cell walls in a process known as the apoplastic pathway, but movement of materials can also occur through both pathways [152]. The arrangement of the cortex is not uniform from inside and outside. The innermost part of the cortex is called the endodermis and it is composed of closely packed cells with no air spaces. The anticlinal walls of the endodermis have the Casparian strips laden with suberin and lignin sometimes. This is why the Casparian strip does not permit the passage of water and ions. Because of this feature, materials entering or leaving the vascular cylinder pass either through the plasma membrane, symplastic pathway or through the plasmodesmata connecting the endodermal cells to the adjacent cells [152].

5.2.1.3 The vascular cylinder

In the root, the vascular cylinder comprise of the xylem, the phloem and the pericycle. The pericycle originates from the procambium and this is the reason why it is component of the vascular cylinder. The pericycle of primary root comprise of parenchyma cells which has primary walls while that of matured root has secondary walls [152].

5.2.2 Root structure at secondary growth stage

As the root matures, it enters into secondary stage of growth which is characterized by the formation of secondary xylem and phloem and the periderm. Secondary growth in root start with the division of the meristematic procambial cells found in between the primary xylem and phloem [152].
At the secondary growth stage, the pericycle grow rapidly and divide to give rise to more pericycle cells which furnishes the vascular cambium opposite the protoxylem. When this happens, the vascular cambium facing the phloem divides to give rise to secondary xylem on the inside. As the vascular cambium cells continue to divide on the outside and the inside, layers of secondary xylem and phloem are added to the root [152].

With the formation of the secondary xylem and phloem comes the formation of periderm; a protective layer that replaces the epidermis. The periderm is comprised of the cork, cork cambium and the phelloderm, all of which are derived from the pericycle cells. The formation of the periderm leads to the separation of the cortex, the epidermis and the endodermis from the rest of the root cells before they finally die and are shed off [152].

5.3 Plant secretory structures

Vast array of plants’ secretory structures have diverged into even more useful forms to aid plants interact with its environment. Some evolved from those plant structures that do not originally perform secretory functions [153]. Secretory structures in plants have been classified based on the type of secretory products. For instance, nectaries secrete nectar; a sugar-rich liquid [153, 154], osmophores secrete scents that attracts pollinators [153, 155] while laticifers produce latex; the milky fluid latex mostly secreted by angiosperms which consist of many biomolecules [2, 4, 153, 156].

Latex is usually found in plants like the milkweed, euphorbias and poppies [2]. Latex is mostly secreted when plant is injured, there it exudes as sticky emulsion-like fluid [157]. There have been suggestions that latex may be performing non-defensive functions such as conduction and food storage [2, 158]. However, evidence has shown that they perform a wide
array of defensive roles. For instance, latex when secreted prevents herbivores from attacking the plants, and can also have negative effect on the growth of attacking herbivores. Some of the secondary metabolites contained in the laticifers have been reported to be toxic and detrimental to animals, implying a role in plant defense [158, 159]. Latex when secreted can immobilize biting and boring insects perched on it, due to its sticky nature [158]. Biomolecules like non-protein amino acids, hydrates, lipids, tannins, terpenes, salts, sterols, and alkaloids are found in the latex [160, 161]. Some of these biomolecules have been used as anticoagulant, anti-inflammatory, antioxidant and antiproliferative agents [157]. The latex of opium is the source of opiates and some other medicinally important alkaloids [2, 157]. Latex has been referred to as the future raw material for bioengineering and biotechnological industries because of the vast number of industrially important compounds it contains [2, 157]. For example itaconic acid is a copolymer of styrene butadiene polymers which has the potential to be used as a nitrile latex [162]

5.4 Laticifers

Laticifers are specialized secretory cells, known to occur in about 12,500 species, 900 genera and over 20 latex producing plant families [2, 163-165]. Their origins are evolutionarily diverse, so also is the type of latex [163, 164]. Recently they have received more attention because they accumulate compounds of biological and medicinal importance. Accumulation of various compounds of important medicinal and pharmacological values in the laticifers have been reported [2]. For instance, the monoterpenoid indole alkaloid such as vinblastine and vincristine used in chemotherapy to fight breast cancer accumulates in the laticifer of Madagascar periwinkle (*Catharanthus roseus*) [2, 30]. Benzylisoquinoline alkaloids such as
morphine used as an essential analgesic, accumulates in the laticifers of opium poppy (*papaver somniferum*) [2]. The accumulation of toxic secondary metabolites in a separate compartment like the laticifers and idioblasts could be plant’s evolutionary means of protecting itself from its own poisons [2, 31]. Laticifers are classified into articulated and non-articulated laticifers.

Non-articulated laticifers are uniquely long, unbranched in some cases, and invades newly formed stem and root tissues. They occur mostly in soft part of the plant like the cortex and pith, but can also invade wood and phloem [166, 167]. They form by intrusive cell growth which further undergoes karyokinesis to become even longer. Non-articulated laticifers, when mature, seem to have few or no plasmodesmata. However, when very large, non-articulate laticifers can be multinucleate. They can be branched, for example in the genera *Nerium* and *Ficus*; and unbranched, for example in the genera *Cannabis* and *Vinca* [165, 168].

Articulated laticifers first occur as single laticiferous cells, and can connect to the neighboring cell through the plasmodesmata or perforations of the shared walls. They differentiate downward in the root by continuously recruiting adjacent parenchyma cells to form laticifer cells as opposed to intrusive growth of the non-articulated. As the newly recruited laticifer divides, beside the older one, the walls diving them perforates and the new laticifers get joined to the older one to form a network of laticiferous cells. Anastomosing and non-anastomosing are terms used to describe different structures of laticifers. When the laticifers occur as a row of cells without joining with one another, they are referred to as non-anastomosing, for instance, the *Ipomea* and *Musa* laticifers [165, 168]. Anastomosing laticifers are able to join with other laticifers to form a long network of cells spread throughout the entire plant. Laticifers of *Catharanthus roseus* and *Cannabis sativa* have been reported to exhibit this
feature. So also are the laticifers of *Papaver* and *Carica* [2, 165]. Presence of laticifers has been reported in the secondary phloem of *Hevea brasiliensis* [159, 165]. The latex of laticifers which exudes when wounded as a result of pressure distinguishes it from other cells. Symplastic transport of nutrients from phloem to parenchyma cells has been thought to be responsible for loading of laticifers. Intense studies on end to end transport in intact laticifer cell have not been done [165, 168]. The involvement of laticifer in the long distance transport such as from root to shoot, or shoot to root if any is still very elusive.

5.5 The laticifers of opium poppy

As stated earlier in chapter one, opium poppy (*Papaver somniferum*) has been used for centuries as a medicinal plant [14, 31, 35]. Just like the California poppy, opium poppy is an annual plant belonging to the family papaveraceae. It is the closest relative of the California poppy whose root and laticifers have been studied extensively. In opium poppy, the laticifers are the articulated anastomosing type which originate from the procambium. They are usually found associated with the phloem elements, the sieve tube and the companion cells [169, 170]. The laticifers of opium poppy have been studied greatly because they accumulate the benzylisoquinolines such as the essential narcotic analgesics codeine, morphine and noscapine [2, 4, 156, 160, 169-171].

5.6 Prior knowledge on the structure of the California poppy root

In California poppy, the development of the seedling shoot at the primary stage of development through rosette establishment, inflorescence and flower formation, inflorescence branching, leaf heteroblasty and enlargement of the shoot apical meristem has been described [1]. Very many chemical analyses of the California poppy metabolites have been done but not
many structural studies. For instance, the ontogeny of the root laticifers and the possible correlation of the appearance of the laticifers with the appearance of the alkaloids have not been done. It remains to be found whether the laticifers of California poppy are found in association with the sieve tube elements as it is the case with the opium poppy [4, 156]

This study aims to describe the anatomy of the California poppy root, the location and distribution pattern of the laticifers using the techniques of histology. The presence of alkaloids in cell types of the root system will be confirmed by use of histochemical techniques. This study is in part, the background knowledge required for the studies done in the subsequent chapters. Knowledge gained from this study will form the basis for future studies on the biosynthesis, localization and transport of alkaloids in California poppy.

5.7 The progressive phenotypic changes in California poppy root.

The primary root of *E. californica* is pale yellow and thin at young age (Figure 37 A) which extends into yellowish brown, hard, long root with lots of fibrous root at maturity (Figure 37 B and C). Light red coloration is first seen at the junction between the hypocotyl and root tip of the emerging young root (Figure 37 A), appearing even brighter at the point of initiation of the fibrous root (Figure 37 B), before the yellowing of the entire root in mature root (Figure 37 C). Root elongation is more rapid during the first four weeks of seed germination. After one month, root elongation slows down, while expansion in root girth continues.

5.8 The structure of California poppy root

The organization of the root system is complex in the sense that the different layers of the root are not readily distinguishable. In the young root of about 2 weeks old, the xylem arm is diarch (Figure 38 A) with two poles of protoxylem exterior to the metaxylem and the primary
phloem surrounding the xylem. A thin layer of epidermal cells fluorescing red (Figure 38 B) surround about five layers of cortical cells in young roots (Figure 38 B). Auto fluorescence of the cell walls was a clear indication of the presence of the epidermis in the young root (Figure 38 B). No other obvious pattern of fluorescence and laticifer formation was observed in the 2 weeks old young root (Figure 38 B). TBO stained young root showed no pattern metabolite accumulation (Figure 38 A) compared to the matured root which trapped some TBO give a blue–black colored group of cells (Figure 38 D; black arrows). In the absence of TBO stain, these group of cells have a yellow sticky exudate (Figure 38 D).
Figure 37. The Root of California poppy showing the progressive changes in the phenotype. (A) 2 weeks old root; (B) 23 days old root; (C) mature root at > 3 months.
Figure 38. Observable features in young and mature California poppy root. (A) Free hand cross section of young root (2 weeks) stained with toluidine blue O; (B) Free hand cross section showing the epidermis and the nucleus fluorescing in young root. (C) Free hand cross section stained with aniline blue showing fluorescence from the periderm, callose (white fluorescent spots) and other cell types in mature root. (D) Free hand cross section of mature root stained with toluidine blue O. Scale bars of 500 µm and 40 µm; Orange lined triangle: collapsed sieve tube members; black triangle: the laticifers; orange stars: the idioblasts; black four point star: the two different fluorescing cell types in mature root. VC, vascular cambium; XY, xylem.
In the mature root (>3 months) exhibiting secondary growth characteristics, the epidermis has been replaced by the suberized periderm giving a golden yellow fluorescence (Figure 38 C). The xylem elements in the mature root show a strong fluorescence due to the lignified wall just as in the young root. Laticifer-like cells which could not be seen in young root was visible in the mature root (Figure 38 C; bright yellow star and Figure 39 A). Other fluorescing cells (Figure 38 C and Figure 39 A; black stars) was also visible in the mature root. Presence of alkaloid in the mature root was confirmed by use of alkaloid histochemical stain I-KI. I-KI stain alkaloids brown red [119, 172]. In the mature root, freshly cut sections flooded with I-KI solution stained specific cells (idioblasts) and laticifer-like cells brown-red (and Figure 39 C and D) suggesting that these cells contain alkaloids. The idioblasts could be seen everywhere in the root except in the cambium and the pith (Figure 40 C and Figure 39).
Figure 39. Mature root showing chains and distribution of laticifers. (A) Free hand longitudinal section of mature stained with aniline blue; (B) longitudinal section of mature root, embedded, fixed, stained with PAS and counter-stained with toluidine blue O; (C) Free hand longitudinal section and (D) free hand cross section of matured root stained with I-KI Scale bars of 40 µm. Green lined white arrow point to the laticifers. Black stars point to the other fluorescing cell.
5.9 Laticifers of California poppy

In thin sections stained with PAS and counter-stained with toluidine blue O, the laticifers were detected easily as cells filled with high density protein particles which appeared as granular materials contained within the cytoplasm. Their walls are darker than the walls of the adjacent cells.

The laticifers were found everywhere in the mature root except in the pith and vascular cambium. However, some are found very close to the vascular cambium, (Figure 40 C and Figure 39 D) among the newly formed secondary phloem cells.
Figure 40. Observable features in mature California poppy root showing the close association of the laticifers with the sieve tube elements. (A and B) Longitudinal section of mature root, embedded, fixed, stained with PAS and counter-stained with toluidine blue O. (C and D) Cross section of mature root, embedded, fixed, stained with PAS and counter-stained with toluidine blue O. Scale bars of 40 µm. Black triangle showing perforation on the side walls of the sieve tube. SP, sieve plate; ST, sieve tube; CC, companion cell; SE, sieve tube elements; Lt, laticifers
Presence of sieve plates and starch grains as observed in sections stained with PAS amido black 10B stain, was used to confirm the sieve tubes. The cell wall of the sieve tube elements stained with PAS amido black are thicker than the surrounding cells thus confirming their identity. Bulk flow of materials within the chains of laticiferous cells could be observed in freshly ruptured laticifers under the light microscope. Laticifers could be seen in the early phloem (Figure 40 C) and in close association with the sieve tube members (Figure 40 A–D). This close association was confirmed by staining freshly cut sections with aniline blue. Aniline blue stains the callose deposited in the sieve tube to give a bright white fluorescence (Figure 38 A). Based on this close association, they were thought to have the same origin as the phloem cells which originated from the procambial cells (Figure 40 B, C and D and Figure 39 D). The newly formed secondary phloem has several articulated laticifers (Figure 40 C) which occur either singly, in groups of two, three or four. They are long and narrow in the early phloem cells (figure not shown) before they expand and get pushed into the pericycle cells as the phloem cells differentiate (Figure 40 C). The laticifers are narrower than the adjacent cells (Figure 40 B). The new laticifers are separated from the older ones by several lines of parenchyma cells.

The sieve tube elements can occur either singly or in groups (Figure 40 A, C and D). When they occur in groups, they have perforations or plasmodesmatal connections on the side walls (Figure 40 A). The sieve tube elements were seen closely associated with the anastomosing articulated laticifers (Figure 40 B & C and Figure 39 A). The sieve tube members associated with the laticifers collapse as the phloem cells differentiate (Figure 40 C).
Figure 41: Observable features in mature California poppy root showing the laticifers recruiting nearby cells.

(A and B) Longitudinal section of mature root, embedded, fixed, stained with PAS and counter-stained with toluidine blue O. Scale bars 100 µm. Black lined white arrow point to the partly-degraded wall.
The laticifers first occur as non-anastomosing articulated laticifers (Figure 39 B) before joining together through the dissolution of the wall of the adjacent laticifer cells (FIG. 5 A) to form un-branched articulated anastomosing laticifers (Figure 41 B). The parenchyma cells adjacent to the laticifer cells have nucleus which is visible after staining with PAS amido black thus confirming the identity of the cells.

5.10 Discussion

The different fluorescent properties observed in laticifers and some group of parenchyma cells (idioblasts) in fresh cut sections of mature California poppy root suggest that they contain different groups of metabolites (Figure 38 C, and Figure 39 A). More so, many fluorescing cells were seen in the mature root compared to the 2 weeks old root thus supporting the report that the flowering California poppy plant accumulate more alkaloid [145]. A fresh cut free hand section is the simplest but very powerful tool in the study of plant structure. This description favors free hand sections because there are fewer artifacts, plants are alive and there is less leakage of plant contents and exudates when the sections are thick enough. By using this simple technique, the contents leaking out in thin sections can be captured and the source of the contents identified. It is a quick and easy method used to screen sections for any interesting observation in the plant structure before going into the intensive and time consuming plastic embedding and sectioning technique. Plastic sections allows for clear observation of structures to the extent that one can say with certainty what was observed. Fixing a whole root prevents leaking out of laticifer contents which can be of great help in interpreting the result.

In *Papaver somniferum*, the laticifers are found in the phloem areas 18-30 hours after seed germination under electron microscope, while it is invisible under the light microscope
Akin to the observations in California poppy, laticifers could not be seen in 2 weeks old root under the light microscope. Lack of detection could be due to the limitations of the light microscope when compared to the electron microscope. Similar to what was seen in this experiment, California poppy laticifers have previously been reported to be articulated and can occur either singly or in groups of two, three and/or four [173]. The laticifers of *E. californica* are found closely associated with each other and so far could be located very close to the vascular cambium (Figure 40 C).

A different report has it that the newly differentiated articulated laticifers of *Papaver somniferum* are separated from the older ones by at least one or two layer of cell [171]. This is similar to what was seen in California poppy root at least as seen under the light microscope. The newly formed laticifers closely associated with the sieve tube members. New laticifer cells are separated from the older ones by few lines of cells (Figure 40 C). The laticifers connect with one another through the dissolution of their end walls by an unknown mechanism. This requires further studies for more understanding of the events leading to the dissolution of the end walls of adjacent laticifers. Nessler and Mahlberg (1976) observed that the laticifers found in the stamen of opium poppy have perforated end or lateral walls. So they concluded that those perforations arise as the walls thin down during the process of articulation as in the end wall or as the laticifer cells anastomose as in the lateral walls [169].

Results from I-KI stain suggest that alkaloids accumulated in two different cell types in the root of California poppy, notably the laticifers and the idioblasts (Figure 39 D and Figure 39 C). In a different study, benzophenanthridines which are the major alkaloids found in the root of California poppy have been reported to accumulate in different cell types in other plants. In
Sanguinaria canadensis, the benzophenthridine accumulates in the idioblasts and while in Chelidonium majus, it accumulates in the laticifers [59].

In the sieve tube members, the perforations observed between them suggest a cross talk among them, and maybe the possibility of flow of material between the sieve tube members. The occurrence of laticifers close to the sieve plate suggests that both of them could be working towards a common goal and may be playing supportive roles for each other. In opium poppy, a related family member of California poppy, the sieve tube members and laticifers are found side by side [156]. The close association of the sieve elements with the laticifers in California poppy suggests that both cell types could be actively involved in alkaloid biosynthesis, but further studies is required to prove that this is the case. Recent evidence show that some of the enzymes involved in the pathway leading to the biosynthesis of the morphine alkaloid are localized to the sieve tube, while some localize to the laticifers [174]. In other words, the laticifers and sieve tube elements are involved in the alkaloid biosynthesis in opium poppy [174].

Benzophenanthridines when exposed to UV can fluoresce red, yellow, and orange depending on the compound. For example, sanguinarine fluoresce yellow under UV excitation. The protopines on the other hand could fluoresce cyan blue under the UV. Based on the fluorescent pattern of these group of compounds, it stands to reason that the benzophenanthridines are probably the compounds fluorescing in the laticifers of California poppy while either the protopines or the protoberberines were the ones fluorescing cyan blue in the idioblast. Therefore, one could propose that some intermediates are biosynthesized in the sieve elements and then gets transported to the laticifers for the biosynthesis of the benzophenanthridines to take place. Another hypothesis is that the biosynthesis of the benzophenanthridines occur in the sieve
elements and immediately after biosynthesis they are transported to the laticifers for accumulation. This proposition was made because it is what happens in opium poppy.

On one hand, it is appropriate to propose that the observed fluorescence is possibly due to accumulation particular alkaloids. Arguably, the autofluorescence may also be due to accumulation of other unidentified metabolites, therefore, further analysis would be required.
Chapter Six: **Discussion**

The benzophenanthridine alkaloids have been shown to be toxic to non-benzophenanthridine producing cell cultures even at micro molar concentrations by inhibiting their growth [55]. In contrast, cell cultures producing these alkaloids have evolved to tolerate their toxicity, even at higher concentrations, using a unique detoxification process [175]. After a maximum tolerant level of benzophenanthridine accumulation occurs in the media, rapid uptake of notably the most toxic of these alkaloid and subsequent detoxification ensues [175]. Data presented in chapter three suggest that elicited cells reach the threshold for the accumulation of the benzophenanthridine alkaloids three hours after elicitation (Figure 17). After three hours rapid uptake of alkaloids into the media starts, which is when we start to see reduction in the accumulation of mostly sanguinarine in the media of elicited cells (Figure 17). However, a tiny or almost non-detectable amount of sanguinarine is found in the extracts from the elicited cell because, the cell has through the process of detoxification converted the sanguinarine taken up into dihydrosanguinarine; a lesser toxic form of the alkaloid. The tiny amount detected are probably coming from those bound to the cell wall as have been previously reported [175]. A cytosolic enzyme sanguinarine reductase has been demonstrated to be the enzyme involved in the reduction of sanguinarine to dihydrosanguinarine, so other that cells are not harmed by their own product [55, 175]. As the accumulation in the media nears threshold, the rate of uptake surpasses the rate of alkaloid production [175] and this is probably the reason why the reduction in the amount of alkaloid accumulated in the media was much more visible after the three hour threshold (Figure 17). On the other hand dihydrosanguinarine irreversibly inhibits sanguinarine reductase after a threshold is reached [175], and this could be the reason why more sanguinarine
in the media twelve hours after elicitation compared to the amount accumulated six hours after elicitation (data not shown). Based on this we believe that somewhere between six and twelve hours after elicitation, a threshold for the accumulation of dihydrosanguinarine is reached. Evidence presented here suggests that EcABCB1 is an ABC importer involved in this uptake of sanguinarine from the media into the cell before it is reduced to dihydrosanguinarine by sanguinarine reductase (Figure 42). Substrate-binding proteins are known to associate with ABC importers in prokaryotes, where many ABC importers have been characterized [176]. In plants where few importers have been characterized and among those characterized, association of a substrate-binding protein with substrates have not been demonstrated [10, 11, 76, 79, 177, 178]. Therefore, it could be that the ABC importers in plants use a different mechanism of operation such as mediating the import of substrates without the need for the binding protein. It could also be that the binding protein is present and active but it is yet to be identified.

Differential sub-cellular localization of enzymes involved in BIA biosynthesis has been reported [179]. For instance, DBOX, P6H, MSH, STS, NMCH and CFS have all been localized to the membrane of endoplasmic reticulum (ER); 4’OMT, CNMT, TYDC, TNMT and 6’OMT have been localized to the cytosol while NCS and BBE localize to the lumen of cytosol [179]. The oxidation of the benzophenanthridines which is the last step in the biosynthetic pathway is catalyzed by DBOX residing in the membrane of ER or the membrane of ER derived vesicles [175, 179]. So it may not be a coincidence that EcABCB1 localized to the ER and plasma membrane (Figure 29). ABC transporters may not only be involved in the import of alkaloids as we have demonstrated, our data and previous work suggest that such transporters may also be
involved in the export of alkaloids or at least intermediates of the benzyl isoquinoline pathway [174].

In this report, a link between an ABC transporter and export of alkaloids was not established. This is partly because of the complexity of plant genomes. For example, there are over 120 ABC transporters in one of the smallest plant genomes, Arabidopsis [73]. Finding a specific transporter with a specific activity is a challenging task. However, it is possible that EcABCB1 is also involved in the export of alkaloids. This could still be tested using, for example, biolistics to transiently express EcABCB1 in California poppy plants or cell cultures. Using this method one could also confirm the sub-cellular location of EcABCB1 in the native cell system [180]. Immuno-fluorescence labelling of EcABCB1 could also be used to confirm the subcellular location of EcABCB1 in plants and cell cultures. Immuno-fluorescence labelling has been used to localize many BIA enzymes in opium poppy [174]. As well, direct involvement of EcABCB1 in the transport of metabolites in plants could also be checked using a type of RNA interference called viral induced gene silencing (VIGS). VIGS has been used successfully to characterize plant ABC transporter in Catharanthus roseus [71].

Fission yeast as a heterologous system for plant membrane proteins is an efficacious one because of its rapid growth, ease of transformation, and the plasma membrane of Schizosaccharomyces pombe is more similar to plant membranes than other microbial systems that have been used for heterologous expression [92]. However, S. pombe has ~11 ABC transporters, which could interfere with transport assays [181]. Characterization of EcABCB1 in a mutant S. pombe system lacking 2 ABC transporter genes, allowed for the determination of the activity of this uncharacterized transporter. Using this system, EcABCB1 was shown to be an
importer with specificity towards certain BIA alkaloids. Furthermore, it was shown that vanadate inhibits this activity (Figure 25). However, this still does not demonstrate that ATP hydrolysis is required for uptake. The ATPase activity of EcABCB1 could be monitored by measuring the amount of phosphate released during the incubation period with substrates. Mutated EcABCB1 can also be used in this experiment and expression of the reporter gene monitored as well. ATP binding sites of the EcABCB1 can be mutated and the mutant cDNA cloned into pREP41 vector to serve as a control. Both mutant and non-mutant constructs will be transformed into yeast and used for alkaloid uptake.

EcABCB1 is expressed in all organs in California poppy, which seems to suggest that it may have other functions besides alkaloid transport (Figure 22). Plant ABC proteins have been implicated in non-transport functions [182]. Some have been reported to be involved in the biosynthesis of chlorophyll, iron-sulphur cluster formation, the movement of the stomata and ions [182]. However, EcABCB1 is most highly expressed in roots, and the other tissues still have alkaloids, albeit not benzophenanthridines [147]

Results from histological studies suggest that alkaloids may accumulate in two different cell types, the idioblasts and the laticifers. Idioblasts emit a blue fluorescence, which may represent the accumulation of protopine or similar precursors, while laticifers appear yellowish-red in colour, suggesting the presence of benzophenthridines (Figure 38 and Figure 39). While it is not clear what relationship the idioblasts have to laticifers, the sieve elements of the root closely associate with laticifers suggesting a synergistic interaction of these cell types working towards the biosynthesis and transport of alkaloids (Figure 40). Synergistic interaction between the laticifers and the sieve elements, working towards the biosynthesis and transport of alkaloid
have previously been proposed in opium poppy [174]. This proposition was made because the laticifers and the sieve elements occur side by side in the opium poppy; and also because some of the enzymes involved in the latter steps of BIA biosynthesis in the opium poppy localize to the laticifers while enzymes involved in the earlier step localize to the sieve elements [174]. We therefore propose that EcABCB1 may be seated at the plasma membrane of laticiferous cells to mediate the import of alkaloids into the laticifers. Alternatively, they may be seated at the membrane of ER-derived vesicles to mediate import of alkaloids into the vesicles for deposition into the vacuole (Figure 43). Plant vacuoles have been reported to store alkaloids, most of which are synthesized in the cytosol [151]. Such a model would imply a difference in BIA composition between cell types within the root. To perform cell-specific alkaloid identification, protoplasts could be extracted from different California poppy plant organs using a dextran gradient. This method has been used in Catharanthus roseus to differentiate idioblast protoplasts from those of mesophyll and epidermis [183]. After dextran gradient separation of protoplasts, alkaloids will be extracted from protoplasts from different cells types. The extracted alkaloids will then be analysed and quantified using the LC-MS.
Figure 42: The theoretical concept of alkaloid importation into the media of California poppy suspension culture. As sanguinarine is secreted into the media, it is transported back into the cell through the EcABCB1 before it then gets converted to dihydrosanguinarine by sanguinarine reductase (SR).
Figure 43: Hypothetical transport route of sanguinarine through the sieve elements into the laticifers. EcABCB1 may be seated at the PM of the vesicles (blue circles represent the vesicles; blue triangles show the direction and position of EcABCB1 in the vesicles) to mediate import of alkaloids into the vesicles. EcABCB1 may also be seated the PM of the laticifers to mediate the import of alkaloids into the laticifers.
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Appendix A

Table 4. Nucleotide sequence of EcABC1B

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## Appendix B

### Table 5: List of all the analyzed product ions with their corresponding precursor ions. The relative intensities of the precursor ions are enclosed in parenthesis.

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<th>References</th>
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<tr>
<td>Morphine base</td>
<td>286</td>
<td>2.5</td>
<td>35</td>
<td>147.07 (5.84), 155.11 (5.21), 162.14 (3.71), 165.1 (4.23), 173.02 (11.43), 178 (4.76), 183.08 (16.49), 185.01 (8.34), 191.12 (3.72), 193.02 (9.88), 201.07 (100), 209.04 (7.47), 211.06 (29.75), 218.99 (7.87), 221.12 (6.36), 227.08 (3.61), 229.05 (57.27), 237.16 (8.41), 239.11 (9.81), 268.13 (43.1)</td>
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<td>Sanguinarine</td>
<td>332</td>
<td>7.65-8</td>
<td>35</td>
<td>244.14 (-1.07), 245.14 (0.48), 246.18 (1.47), 272.15, (1.99), 274.1 (14.11), 275.15 (2.03), 276.19 (3.42), 277.21 (0.39), 289.19 (0.43), 302.09 (23.26), 303.42 (2.2), 304.11 (100), 305.15 (7.64), 314.13 (1.35), 315.18 (0.62), 317.13 (11.31), 318.17 (0.41), 330.14 (23.14), 332.1 (86.81), 333.18 (1.69)</td>
<td>[95] and authentic standard</td>
</tr>
<tr>
<td>Compound</td>
<td>+ESI- [M+H]^+</td>
<td>RT</td>
<td>CID</td>
<td>m/z (Relative intensity, %)</td>
<td>References</td>
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<td>Dihydrosanguinarine</td>
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<td>12.14-12.29</td>
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<td>[95]</td>
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<tr>
<td>Protopine</td>
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<td>6.90-7.32</td>
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<td>Chelirubine</td>
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