ANALYSIS OF *KCS2*, A GENE ENCODING A NEW CONDENSING ENZYME FOR THE ELONGATION OF VERY LONG CHAIN FATTY ACIDS IN *Arabidopsis thaliana*

By

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Abstract

Condensing enzymes for very long chain fatty acid (VLCFA) synthesis catalyze the first of a series of four reactions that elongate the growing acyl chain by two carbons at a time. It has been shown that the activity of the condensing enzyme determines the amount and acyl chain length of the VLCFAs produced by a fatty acid elongation system. My research project focused on the characterization of KCS2, a new putative condensing enzyme of *Arabidopsis thaliana*. Computer database analyses showed that the *KCS2* gene is located directly upstream of the *FAE1* gene, the first condensing enzyme for the elongation of VLCFAs to be studied in *Arabidopsis*, suggesting a gene duplication phenomenon.

Analysis of the expression pattern of *KCS2* showed that the gene was primarily expressed in the anthers of *Arabidopsis* flowers. The ability of the condensing enzyme to elongate VLCFAs was determined by expressing it in yeast and in seeds of CB25, an *Arabidopsis* mutant defective in VLCFA elongation in the seed. In both systems, KCS2 was able to catalyze the elongation of VLCFAs. However, the accumulating products of fatty acid elongation carried out by the KCS2 condensing enzyme differed in the two organisms. Yeast accumulated preferentially saturated VLCFAs from C20:0 to C26:0, whereas *Arabidopsis* seeds accumulated primarily mono-unsaturated VLCFAs, C20:1 and C22:1. Finally, in an attempt to generate co-suppressed plants to determine the function of the KCS2 condensing enzyme, the *KCS2* gene was transformed into *Arabidopsis* plants under the control of the CaMV35S promoter. No visible phenotype was obtained. However, some lines that were over-expressing the gene were able to synthesize more VLCFAs in the seed when compared to the wild type. In contrast, total wax load in stems of 35S-*KCS2* over-expressors was not increased. Moreover, there was no correlation between the level of expression of the transgene and the amount of total wax produced. The specific function of the KCS2 condensing enzyme requires further study.

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CHAPTER I

Introduction

1. VLCFAs in plants – Biological relevance

The synthesis of very long chain fatty acids (VLCFAs), or fatty acids of more than 20 carbons in length, is a very important process (Domergue *et.al.*, 1998). In plants, the major site for the synthesis of VLCFAs is the epidermis, where they are used as precursors for the production of waxes. Waxes can be found embedded in cutin and suberin or as epicuticular waxes, forming crystals on the surface of aerial tissues. They constitute the outermost layer of the plant and have a very important protective role (Kolattukudy, 1980). Waxes are also important components of the pollen coat and are involved in germination of pollen grains (Piffanelli *et. al.*, 1998). In seeds, VLCFAs are used as components of triacylglycerols (TAGs) or seed oils, and represent the main way in which plants store energy reserves (Frentzen, 1993). VLCFAs are also membrane constituents, being components of sphingolipids which, depending on the species, may account for 5-15% of the membrane acyl moieties (Domergue *et. al.*, 1998).

1.1. VLCFAs on plant surfaces

On all plant surfaces, VLCFAs are synthesized as precursors and components of either cuticular and epicuticular waxes in vegetative organs (von Wettstein-Knowles, 1993), or suberin in roots and wounded tissue (Kolattukudy, 1980). Both cutin and suberin constitute a major protective barrier between the plant and its surrounding environment (Kolattukudy, 1980).

1.1.1. Cuticular and epicuticular waxes Aerial tissues of all land plants, including some liverworts and mosses are covered by cutin, which is a polymer derived from C16 and C18 fatty acids (Kolattukudy, 1980). Associated with cutin there is a complex mixture of lipids collectively called waxes. Waxes can either be embedded in the cutin matrix, where they are called cuticular waxes, or form a crystalline structure on the surface of aerial tissues, known as epicuticular waxes (von Wettstein-Knowles, 1993; Kolattukudy, 1996). Biochemically, epicuticular waxes are mainly composed of free very long chain fatty acids (VLCFAs), fatty aldehydes, primary alcohols, alkanes, secondary alcohols, ketones and esters, all of which are derived from VLCFA precursors (Lemieux, 1996). However, wax composition and distribution can vary considerably, even within one species, with age and growth conditions, especially temperature and light. Air pollutants also have an effect on wax physicochemical characteristics (Cape and Percy, 1993). The proportion of the different wax components as well as the total amount of wax vary among organs and tissues (Post-Beittenmiller, 1996). For example, *Arabidopsis* stem and silique have the greatest wax load and their main components are alkanes, ketones and alcohols. In contrast, leaves have a considerably lower amount of total wax, mainly composed of alkanes, alcohols and fatty acids (Hannoufa *et. al*, 1993; Jenks *et. al*, 1996).

Due to their hydrophobic nature, cuticular waxes play a significant physiological role in regulating the water balance of the plant (Lemieux, 1996). When stomata are closed, as during darkness or drought, or missing as in some fruit tissue, the cuticle layers are responsible for controlling water loss (Schreiber and Schonherr, 1992; Jenks *et. al*, 1994). In addition, the reflective properties of epicuticular waxes may reduce the absorption of heat, decreasing leaf temperature and consequently, reducing the loss of water by transpiration (Reicosky and Hanover, 1978). They also protect the plant against damaging effects of UV light (Tevini and Steinmiller, 1987). The hydrophobicity of the cuticular and epicuticular waxes

make them good solvents for organic pollutants and a barrier against foliar sprays without surfactant addition (Lemieux, 1996).

The cuticle also plays an important role in the interaction of plants with predators and pathogens. The hydrophobic properties of the cuticle help it prevent the accumulation of water in the leaf surface, which in addition to the presence of specific chemicals, can prevent germination of fungal spores (Jackson and Danehower, 1996; Jenks *et. al*, 1994; Mendgen, 1996). Epicuticular wax crystals can act as a protective barrier against some herbivorous insects, by physically interfering with their movement on the surface of the plant. The chemical composition of epicuticular waxes can also influence insect behavior. Usually, the most common wax components stimulate acceptance, while less common ones act as deterrents for the settling and oviposition of herbivorous insects and their predators and parasitoids (Eigenbrode, 1996).

1.1.2. Suberin In underground parts and wounded tissues as well as in bark, in the endodermis (Casparian band) and in the bundle sheath of grasses, another polymer, suberin, is found (Kolattukudy, 1980; Harwood, 1997). As cutin, suberin is deposited at an extracellular location, between the plasmalemma and the cell wall (von Wettstein-Knowles, 1993).

Suberin matrix consists of a polymer that contains an aliphatic and an aromatic domain. The aliphatic domain consists of fatty acid monomers, which range from C16 to C24, but may be as long as C30 (von Wettstein-Knowles, 1993). This matrix is covalently attached to the cell wall via phenolic residues. Suberin serves many functions. In casparian bands, it helps minimize apoplastic transport of water and solutes and protects the vascular tissue from microbial attack. It forms a layer around the bundle sheath of grasses, that is thought to have a major effect on the concentration of CO_2 and therefore on photosynthesis. It also controls the transport of materials to grains during their development. One of the most important

functions of suberin however, is that it constitutes a defensive barrier to environmental threats involving wounding. For example, fungal attack triggers the deposition of a polymeric structure containing phenolic substances on the cell wall and this prevents the spread of the pathogen. The synthesis of suberin as a response to wounding in all organs, prevents water loss and decay (Kolattukudy, 1980).

1.2. VLCFAs as components of cell membranes

Lipids are essential constituents of plant cells, being components of all membranes. Lipid membranes are major barriers that define the cell and its compartments, and determine sites where essential processes such as photosynthesis take place (Ohlrogge and Browse, 1995).

Most lipids in plants are acyl lipids, meaning that they have fatty acids esterified to a glycerol backbone (Harwood, 1997). In membranes, they usually have fatty acids attached to the *sn-1* and *sn-2* positions of the glycerol backbone and a polar headgroup attached to the *sn-3*, which gives them the amphipatic physical properties, essential to the formation of membrane bilayers (Ohlrogge and Browse, 1995).

In addition to acyl lipids, plant membranes contain sphingolipids, which consist of a long-chain base (amino-alcohol) with a single fatty acid linked by an amide, forming a ceramide. Complex sphingolipids can have polar groups such as phosphocholine or sugar residues linked to the ceramide by a glycosidic bond. Glucosylceramide is the predominant sphingolipid in plant tissues, being important component of the plasma membrane and tonoplast (Lynch, 1993).

The fatty acids of plant sphingolipids are almost exclusively 2-hydroxy fatty acids, normally C16 to C24 in length (Cahoon and Lynch, 1991). Both the base and the fatty acids change considerably within the plant. For example, glucosylceramides from seeds are enriched in C16 to C20 hydroxy fatty acids (Lynch, 1993), whereas in leaves of cereals, very

long chain (>C20) hydroxy saturated and monounsaturated fatty acids are more common (Imai *et. al.*, 1995).

Glycosylceramides are thought to increase stability and decrease permeability of membranes and have been implicated in regulating ion permeability (Cahoon and Lynch, 1991). It has also been sugested that sphingolipids participate in membrane-related phenomena associated with chilling sensitivity, cold acclimation and freezing injury (Yoshida and Uemura, 1986; Uemura *et. al.*, 1995). Sphingolipids also seem to be important regulatory molecules (Mazliak, 1996). Recently, they have been studied as intracellular messengers and they have also been implicated in driving both cell proliferation and cell death by apoptosis (Michell and Wakelam, 1994; Mazliak, 1996).

The proportion of sphingolipids varies among species, from relatively low (6.5 mol%) in potato to up to 27.2 mol% in oat (Uermura *et.al.*, 1995). The first report on the composition of *Arabidopsis* membrane lipids revealed that it had a relatively low proportion of sphingolipids compared to other species, only 7.3 mol% (Uemura *et.al.*, 1995). Furthermore, the majority of the ceramides in the plasma membrane of *Arabidopsis* leaves contain hydroxylated C16:0 as the major acyl moiety, so VLCFAs do not seem to be as frequent in *Arabidopsis* membranes as they are in other species (Uemura *et.al.*, 1995).

1.3. VLCFAs as components of storage lipids

When all three positions of the glycerol are esterified with fatty acids, a triacylglycerol (TAG) molecule results, that constitutes the major form of lipid storage in seeds (Domergue *et. al.*, 1998). Triacylglycerols are found in oil bodies, which are surounded by a monolayer membrane enriched in proteins called oleosins. These proteins seem to function in preventing the coalescence of oil bodies and possibly also act as anchors for the lipases involved in the degradation of TAGs during germination (Harwood, 1997; Ohlrogge and Browse, 1995).

TAG synthesis is carried out by the Kennedy pathway, which initially leads to the formation of diacylglycerol, used for both membrane and TAG biosynthesis. Only one additional enzyme, a seed specific diacylglycerol acyltransferase, is required to complete the synthesis of TAGs (Ohlrogge and Browse, 1995). As opposed to the rather high substrate specificity of the first two acyl transferases that acylate the *sn-1* and *sn-2* positions of the glycerol molecule (Harwood, 1997), DAG acyltransferase is thought to have a broad substrate specificity in most plants. The fatty acid composition of the *sn-3* position is usually dependent on which acyl groups are available in the fatty acyl-CoA pool. This is important because TAGs can accumulate fatty acids that are not normally found in membranes, called unusual fatty acids (van de Loo *et. al.*, 1993). Unusual fatty acids deviate from the structure of fatty acids found in membranes that are C16 and C18 with one to three *cis* double bonds. Instead, their chain length can vary from as few as eight carbons to >22 carbons. The position and number of the double bonds might also change, and functional groups such as epoxy or hydroxy might be added. The reason for this diversity and the question of how plants control which fatty acids are stored in the TAG and which are restricted to membranes are still not clear (Ohlrogge and Browse, 1995; Wiberg *et. al.*, 1997).

VLCFAs are present in seed oils of the *Brassicaceae*, including species such as the mustards *B. juncea, B. carinata* and *B. nigra*, as well as rape (*B. napus*) and *Arabidopsis* (Barret *et. al.*, 1998), which contain primarily eicosanoic acid (20:1) and erucid acid (22:1) in their seed oils. Other species that contain VLCFAs as components of TAGs include nasturtium (*Tropaelum majus*) and meadowfoam (Limnanthes alba) (Kunst *et. al.*, 1992), whereas jojoba seeds (*Simmondsia chiniensis*) contains VLCFAs in wax esters (Lassner *et. al.*, 1996). The seeds of *Arabidopsis* contain approximately 28% (w/w) of total fatty acids of VLCFAs, esicosenoic acid (20:1) being the predominant one (22.1% w/w of total fatty acids) (Millar and Kunst, 1997).

1.4. VLCFAs in the pollen grain

Pollen grains have four major lipidic structures. The outer layers, exine and tryphine, which contain VLCFAs and long chain wax esters, are derived from the sporophyte and are released from the degrading tapetum as the pollen grain matures. In addition, pollen grains have two structures derived from the gametophyte, an intracellular membrane system containing ER and surface-adjacent membrane vesicles and intracellular oil bodies, which TAGs constitute the pollen storage lipids (Piffanelli *et. al*, 1998). Both intracellular lipid structures are based mainly on C18 polyunsaturates, particularly linolenic acid (18:3).

The outer wall of pollen grains, or exine, is mostly derived from acyl lipid precursors, which form a polymer with phenylpropanoids called sporopollenin. The formation of this outer wall involves the synthesis of a cellulosic matrix, the primexine, to which sporopollenin monomers polymerize (Piffanelli *et. al.*, 1998). These monomers are mainly composed of long chain fatty acids and fatty alcohols from C20 to C30, and phenylpropanoids in lesser amount (Gubatz *et. al*, 1993).

The outer suface of the exine is covered by another lipidic structure, the pollen coat or tryphine layer, which is especially important in pollen of entomophilus species (Piffanelli *et.al.*, 1998). The pollen coat has numerous functions such as enabling pollen to adhere to insects or animal vectors or to stigmatic surfaces. It also provides the proteinaceous signaling molecules involved in self-incompatibility responses. In addition, the pollen coat carries lipid-derived volatile compounds that attract pollinators and other components such as carotenoids and flavonoids that can protect against radiation or pathogens. It also protects the pollen grain from water loss during its brief autonomous life, and facilitates the uptake of water once it lands on a receptive stigma (Piffanelli and Murphy, 1998). The lipid composition of the pollen coat is different from the rest of the pollen grain, containing mostly medium (C6 to C18) and long-chained (C20 to C30) saturated fatty acids, flavonoids and terpenes/sterol esters (Piffanelli *et. al.*, 1998).

Analysis of pollen lipid fractions has shown the presence of small but significant amounts of very long chain wax esters, which have been shown to have an essential role in pollen hydration upon landing on the stigma surface (Preuss *et. al*, 1993). In *Arabidopsis,* some *eceriferum* (cer) mutants, defective in wax production, also show male sterility because of the lack of all or part of the pollen coat structure. Many of these mutants have their fertility restored when they are grown at high humidity, which shows that long chain lipids probably have a function in the hydration process of the pollen grain (Preuss *et. al*, 1993; Aarts *et. al*, 1995). Similar results were obtained when studying CUT1, a condensing enzyme involved in the elongation of VLCFAs for wax production (Millar and Kunst, 1999). Co-suppression of *CUT1* gene results, in addition to a waxless phenotype, in conditional male sterility. However, fertility can be restored when plants are grown at high humidity (Millar and Kunst, 1999).

2. VLCFA biosynthetic pathway

2.1. Fatty acid synthase (FAS) and fatty acid elongase (FAE)

De novo fatty acid synthesis in plants occurs in the plastid, and is a primary metabolic pathway. It is found in every cell and is essential to growth. Inhibitors of fatty acid biosynthesis are lethal and there is no mutant defective in this process (Ohlrogge and Browse, 1995). Fatty acid synthesis is carried out by a group of enzymes collectively called fatty acid synthase (FAS). Acetyl-CoA is the primary starting molecule. It acts as a substrate for the generation of malonyl-ACP, and as a carbon donor for the first condensation with malonyl-ACP to form a C4 fatty acid. Each additional condensation reaction is followed by a reduction, dehydration and a further reduction of the *trans*-2 double bond to form a saturated fatty acid. These reactions are catalyzed by the enzymes 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase and enoyl-ACP reductase respectively (Ohlrogge and Browse,

1995). Each cycle extends the acyl chain by two carbons at a time, using malonyl-ACP as the carbon donor (Figure 1) (Post-Beittenmiller, 1996).

The final products of the FAS are C16 and C18 fatty acids (Ohlrogge and Browse, 1995). Three condensing enzymes, called 3-ketoacyl-ACP synthases (KAS), participate in the production of these fatty acids. KAS III is responsible for the condensation of acetyl-CoA and malonyl-ACP to form a four-carbon fatty acid (Jaworski *et.al.* 1989). KAS I elongates C4 to C16 and KAS II, C16 to C18 (Shimakata and Stumpf, 1982).

Once C16 and C18 are formed, they are cleaved from the ACP by an acyl-ACP thioesterase, exported to the cytoplasm and re-esterified to CoA forming acyl-CoA. This creates a pool of acyl-CoAs that can be used for the synthesis of TAGs, membrane lipids or further elongation to VLCFAs (Harwood, 1997; Millar *et. al.*, 2000) by a fatty acid elongase (FAE) system in the ER membrane (Figure 2) (von Wettstein-Knowles, 1979; Voelker, 1996). Analogous to the *de novo* fatty acid synthesis, it is thought that each cycle of FAE involves the following steps: (1) condensation of malonyl-CoA with a long chain acyl-CoA, (2) reduction to β-hydroxyacyl-CoA, (3) dehydration to an enoyl-CoA and (4) reduction of the enoyl-CoA, resulting in an elongated acyl-CoA (Fehling and Mukherjee, 1991).

Fatty acid synthesis and elongation pathways are similar in that they not only involve the same sequence of enzymatic events (condensation, reduction, dehydration and a further reduction), but also use NAD(P)H as the reducing equivalent for the reductase (Post-Beittenmiller, 1996). Despite their similarities, FAS and FAE show structural and biochemical differences. For FAS pathway, malonyl-ACP is used as the carbon donor, whereas FAE uses malonyl-CoA. In addition, the enzymes participating in VLCFA biosynthesis are membrane associated, rather than stromal and soluble as the enzymes that carry out the *de novo* fatty acid synthesis (Post-Beitenmiller, 1996).

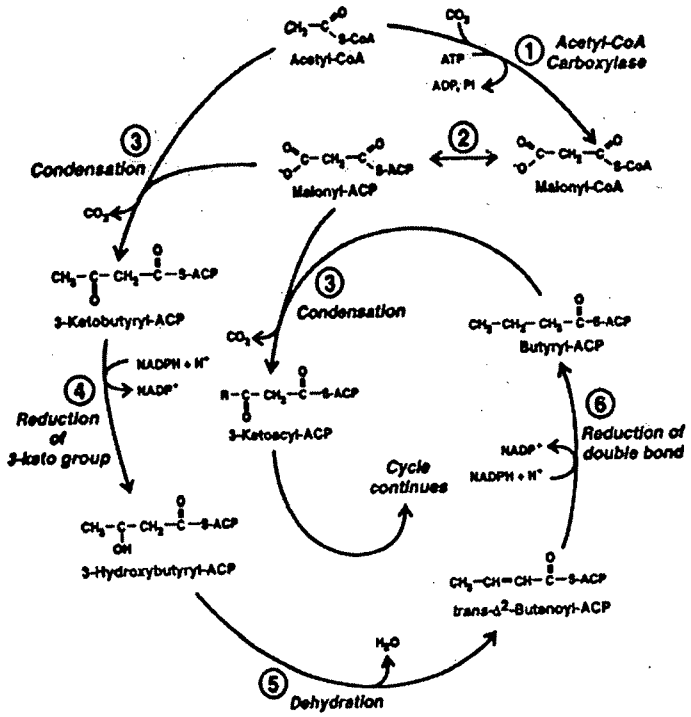

Figure 1. *De novo* fatty acid biosynthesis in the plastid. Acetyl-CoA is the basic bulding block of the fatty acid chain and enters the pathway both as a substrate for acety-CoA carboxylase (reaction 1) and as a primer for the initial condensation reaction. At least three separate condensing enzymes, known as 3-ketoacyl-ACP-synthases are required to produce a C18 fatty acid. KAS III (C2 to C4), KAS I (C4 to C16) and KAS II (C16 to C18) (reaction 3). Reaction 2, catalyzed by malonyl-CoA transacylase, transfers malonyl from CoA to form malonyl-ACP, . Which is the carbon donor for all susequent elongation reactions. After each condensation, the 3-ketoacyl-ACP product is reduced (reaction 4), dehydrated (reaction 5), and reduced again (reaction 6), by 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrogenase and enoyl-ACP reductase respectively (Ohlrogge and Browse, 1995).

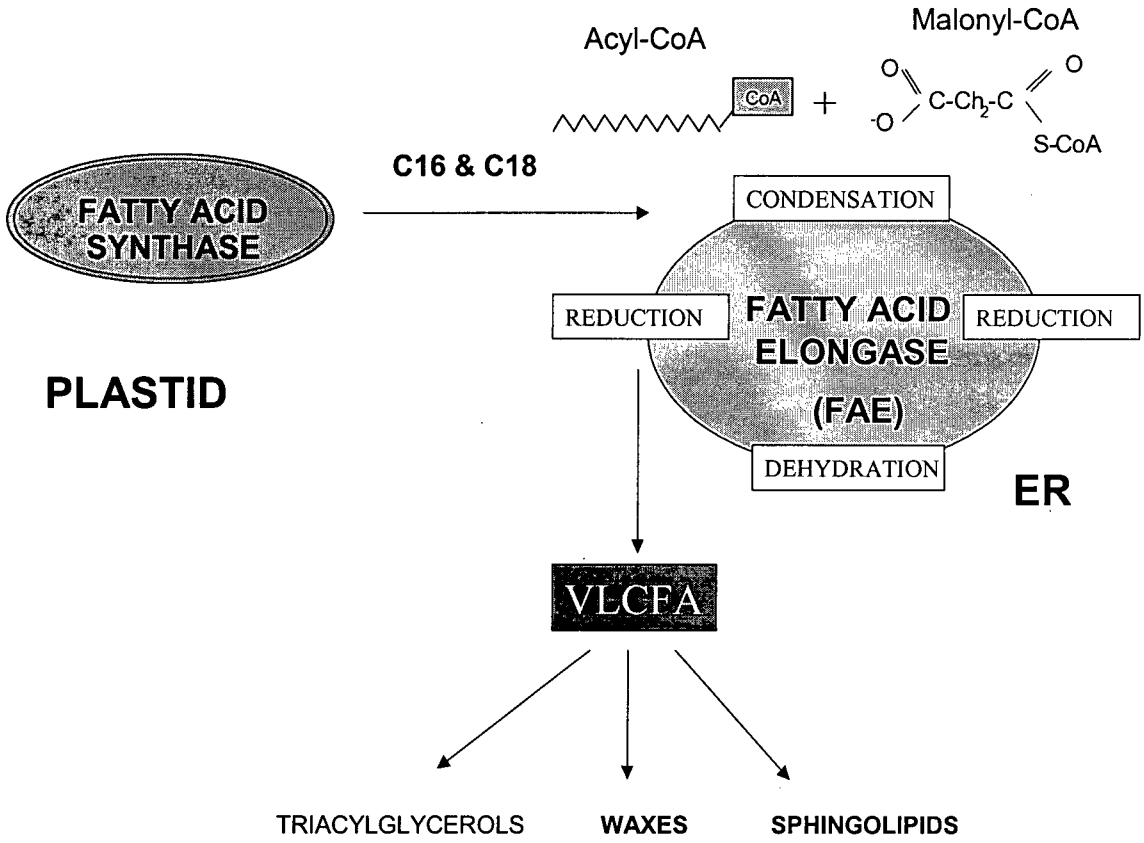

Figure 2. Schematic representation of fatty acid elongation. C16 and C18 fatty acids produced in the plastid can be further elongated in the ER by the fatty acid elongase (FAE) enzymatic complex. Analogous to the FAS, the FAE involves a condensation of the acyl-CoA with malonyl-CoA, a reduction to β-hydroxyacyl-CoA, dehydration to an enoyl-CoA and a reduction of the enoyl-CoA. Very long chain fatty acids (VLCFAs) prduced by the FAE system can then be used as in seed oils or triacylglycerols, as precursors for the synthesis of waxes or in the synthesis of sphingolipids for the plasma membranes.

2.2. The importance of the condensing enzyme in VLCFA synthesis

Even though the biochemical pathways leading to VLCFA biosynthesis are well known, the fact that the enzymes are membrane-bound has made it difficult to isolate and purify them. Genetic analysis has been, therefore, an alternative approach for the identification and characterization of genes and their products involved in VLCFA synthesis.

Usually, when a genetic approach is taken, a mutagenized population is studied in order to find a phenotype characteristic of a mutation in the target process. In the case of VLCFA synthesis, a large *Arabidopsis* population was screened by gas chromatography, in order to identify seeds with a reduced VLCFA content. As a result of this screening, three groups independently identified the *FATTY ACID ELONGATION* (*FAE1*) gene (James and Dooner, 1990; Lemieux *et. al*, 1990; Kunst *et. al*, 1992). A mutation at this locus resulted in <1% w/w of total fatty acids in the seed, when compared to wild type. This reduced content of VLCFAs was the result of a deficiency in acyl chain elongation activities from C18 to C20 and C20 to C22, suggesting that the product of the *FAE1* gene was required for both elongation steps (Kunst *et. al*, 1992). Despite the fact that four enzymatic activities are required for each elongation step, all mutations disrupting VLCFA biosynthesis mapped to the FAE1 gene.

The *FAE1* gene was cloned by transposon tagging, using the maize element *Activator* (Ac) (James *et. al,* 1995). The predicted amino acid sequence of FAE1 protein showed homology to three condensing enzymes, chalcone synthase (CHS), stilbene synthases (STS) and β-ketoacyl-acyl carrier protein synthase III (KAS III - of *de novo* fatty acid synthesis).

FAE1 enzyme was extensively studied *in vivo* by ectopically expressing it in *Arabidopsis* as well as in tobacco and yeast. Each of the transgenic organisms responded to the introduction of *FAE1* by noticeably altering their fatty acid profiles. *Arabidopsis* showed an increase in VLCFA content in all tissues examined. In tobacco seeds, which have almost

no VLCFAs (less than 0.5%), the introduction of *FAE1* increased the amounts of 20:0, 20:1 and 20:2 to 1-2.5% of the total fatty acids. Similarly, when the *FAE1* gene was transformed into yeast, the cells were able to produce 20:1, 22:1 and 20:0 VLCFAs, which were not previously reported in the wild type (Millar and Kunst, 1997). These results suggested that the other three activities of the elongase (two reductases and a dehydrase - see Figure 2), must be constitutively expressed throughout the plant, or induced by the presence of *FAE1* (Millar and Kunst, 1997). The condensing enzyme also appeared to control the acyl length and amounts of VLCFAs made. These results strongly suggested that the specificity of the condensing enzyme is the major activity of each FAE system. This situation is analogous to the *de novo* FAS in the plastid, where there are three condensing enzymes with strict acyl chain length specificity. In contrast, the reductases and the dehydrase are shared between all three elongating systems (Ohlrogge, 1993).

After the isolation of *FAE1*, another putative condensing enzyme (jojoba-KCS), specific for the elongation of VLCFAs destined for the production of jojoba seed waxes, was isolated through protein purification (Lassner *et. al*, 1996). The cDNA was used to transform LEAR (low erucic acid rapeseed) varieties of Canola, which are unable to elongate 18:1-CoA substrates. Jojoba-KCS was able to restore the elongation activity, resulting in the synthesis of 20:1, 22:1 and 24:1 VLCFAs. This further demonstrates the importance of the condensing enzyme in controlling VLCFAs production in the seed (Lassner *et. al*, 1996).

2.3. Structure and function of condensing enzymes

Condensing enzymes comprise a related family of enzymes, found in various metabolic pathways that catalyze carbon-carbon bond-forming reactions. In fatty acid biosynthesis, these enzymes catalyze the condensation of malonyl groups to a growing acyl chain, bound to either acyl carrier protein (ACP) or CoA (Huang *et. al*, 1998). The way in which condensing enzymes act, varies among organisms. For example in animals, the

condensing enzyme for fatty acid synthesis is part of a multifunctional polypeptide chain, the fatty acid synthase, in which the enzymes for each step of fatty acid synthesis are organized as subunits of this large polypeptide (Wakil, 1989). In contrast, in plants and bacteria, the condensing enzymes of the FAS and FAE act as single proteins (Ohlrogge and Browse, 1995).

Site directed mutagenesis experiments allowed to identify a cysteine that is thought to be essential for the functioning of the condensing enzymes chalcone and resveratrol synthases, key enzymes in the biosynthesis of flavonoids and stilbenes respectively. The amino acids that surround the cysteine are highly conserved, being predominantly serine/threonine and small neutral amino acids (glycine or alanine) (Lanz *et. al.*, 1991). All the condensing enzymes identified so far for VLCFA synthesis also have a conserved active site surrounding a cysteine (Siggaard-Andersen, 1993).

During fatty acid and VLCFA synthesis, the SH- group of the cysteine covalently binds to the acyl-ACP or acyl-CoA group, forming a thioester. In parallel, malonyl-ACP or malonyl-CoA gets decarboxylated to form a two carbon anionic molecule. The condensation reaction finishes when the carbanion binds to the growing acyl chain, through the formation of a carbon-carbon bond (Huang *et. al.*, 1998).

2.4. Identification of additional condensing enzymes involved in VLCFA synthesis

Homology searches of the *Arabidopsis* EST database identified multiple cDNAs with open reading frames with high sequence similarity to both the *FAE1* gene and the jojoba-KCS. One of these ESTs showed to be expressed in aerial tissues. In order to study its function, a reverse genetics approach was taken, in which the complete cDNA was fused to the CaMV 35S promoter and used to generate transgenic *Arabidopsis* plants. By co-suppression, a number of plants with a waxless phenotype were obtained. Stem tissue showed a complete absence of wax crystals, implying the lack of an epicuticular wax layer. These

epicuticular wax layer. These results suggested that the gene obtained was a condensing enzyme that participated in stem wax production, and was named *CUT1* for its involvement in cuticular wax biosynthesis (Millar and Kunst, 1999). In addition to the previously mentioned waxless phenotype, *CUT1* co-suppressed plants showed a high level of male sterility. Of the 36 plants examined, four were semi-sterile, only setting 50-100 seeds, whereas the other 32 plants were completely sterile (Millar and Kunst, 1999). Fertility was partially restored when the plants were grown in a highly humid environment.

When analyzing the wax composition of the co-suppressed plants, it was found that a loss of function of the *CUT1* gene resulted in an almost complete absence of the products of the decarbonylation pathway, as well as a decrease in almost 50% of the products of the acyl-reduction pathway. In contrast, C24 products were abnormally accumulating in these plants, which suggested that *CUT1* is responsible for the elongation of the acyl chain beyond C24 (Millar and Kunst, 1999).

Recently, a new condensing enzyme from *Arabidopsis* called *KCS1*, was characterized (Todd *et. al.*, 1999). *In vitro* enzymatic analysis in yeast expressing *KCS1*, using radioactivelly labeled fatty acid precursors showed that *KCS1* could elongate VLCFAs from C18 to C26. Expression studies suggest the gene to be expressed in aerial tissues as well as in roots. Thus, *KCS1* may participate in the production of VLCFAs for cutin and suberin waxes. T-DNA tagged *kcs1* mutants showed no morphological differences when compared with wild type when grown under normal conditions. However, they seem to be less resistant than wild type plants to low humidity (Todd *et. al.*, 1999). In contrast to what was observed with *CUT1*, a loss of function of the *KCS1* gene does not seem to result in significant losses of the major wax components. However, the products of both decarbonylation and acyl-reduction pathways were affected, suggesting the involvement of *KCS1* in the elongation of VLCFAs for both pathways (Todd *et. al.*, 1999).

FAE1, *CUT1* and *KCS1* all share high sequence similarity and some features such as the lack of introns, the presence of the active site cysteine surrounded by six conserved residues and similar hydrophobicity patterns (Millar and Kunst, 1999; Todd *et. al.*, 1999). Recently, the *FIDDLEHEAD* (*FDH*) gene, initially thought to have a regulatory function controlling epidermis-specific developmental signals (Lolle and Cheung, 1993), has showed to encode a new putative condensing enzyme (Yephremov *et. al.*, 1999). The *FDH* sequence is very similar to the above mentioned condensing enzymes in the regions of high sequence similarity among them. However, in contrast to what was observed with other condensing enzymes, it has two introns and a characteristic N-terminal extension of 44 aminoacids, that is not present in any of the FAE-like condensing enzymes studied so far (Yephremov *et. al.*, 1999; Pruitt *et. al.*, 2000). Therefore, it has been postulated that the *FDH* gene could have a specialized role, elongating substrates that differ from the ones elongated by *FAE1, CUT1* or *KCS1.* Possible substrates could be fatty acids with different levels of unsaturation or hydroxylation or different lengths compared to the ones preferred by the other condensing enzymes (Yephremov *et. al.*, 1999).

Based on the research completed to date, it is apparent that there is a number of condensing enzymes participating in sequential elongation events in different tissues, except for the seed, in which *FAE1* is the only condensing enzyme responsible for the production of VLCFAs (James *et. al.*, 1995). A good example of this was presented in the study of *CUT1* co-suppressed plants. Analyses of the total wax load on stems of these plants revealed a reduction to approximately 6% of the wild type. On the other hand, the wax load on leaves was reduced to 50% of that found in wild type leaves (Millar and Kunst, 1999). These data strongly suggests the participation of at least one additional condensing enzyme responsible for the elongation of VLCFAs destined for the production of leaf waxes, and another one in stems, required for the elongation of fatty acids from C18 to C24. *KCS1* specificity seems to overlap with *CUT1*, since *KCS1* is thought to elongate VLCFAs from

C18 to C26. However, the number and degree of overlap and the amount of tissue-specific expression of the different elongases is still unknown.

2.5. Characterization of the KCS2 condensing enzyme

The *Arabidopsis* Expressed Sequence Tags (ESTs) database (Newman *et. al.*, 1994; Cooke *et. al.*, 1996), has provided several sequences that show a high degree of similarity with the sequences of the condensing enzymes for the elongation of fatty acids known so far. This has made it feasible to use reverse genetics for the study of other condensing enzymes. The region of chromosome IV in which *FAE1* gene is located (James *et. al.*, 1995) is completely sequenced and available as a BAC clone. Interestingly, directly upstream of *FAE1* on chromosome IV (Figure 3), there is an open reading frame that shows very high nucleotide sequence identitity to *FAE1*, *CUT1* and *KCS1* (70.9%, 62.3% and 64.8% respectively). This ORF shows the conserved active site cysteine and has no introns in its sequence, a characteristic also shared by *FAE1*, *CUT1* and *KCS1*.

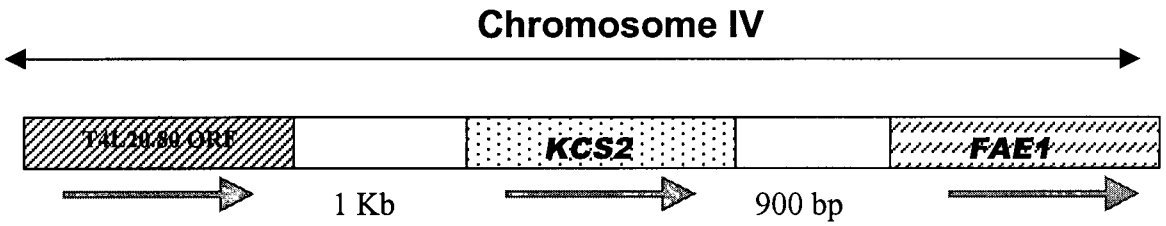

Figure 3. Schematic representation of the position of *KCS2* on chromosome IV, BAC clone T4L20. *KCS2* ORF is located directly upstream of the *FAE1* gene. 5' to 3' orientation is indicated by arrows. The white boxes correspond to the *KCS2* and *FAE1* putative promoters.

This gene and the putative condensing enzyme that it encodes have not been studied so far. I focused my project, therefore, on the characterization of this gene and its product. Consistent with the *Arabidopsis* nomenclature we named it *KCS2*, since *KCS1* was the last condensing enzyme to be characterized.

The specific objectives of my project were to characterize the *KCS2* gene in terms of its expression pattern in tissues of *Arabidopsis* and study the specificity of the condensing enzyme by expressing it in yeast and *in planta*. Finally, by using reverse genetics, I intended to study the function of the KCS2 gene product in *Arabidopsis* plants.

CHAPTER II

Materials and Methods

1. Plant material

Arabidopsis thaliana ecotype Columbia-2 (Col-2) plants were used as wild type material. Seeds of *A. thaliana* were placed on the *Arabidopsis thaliana* (AT) solid minimal salts medium (Somerville and Ogren, 1982), stratified for two days at 4°C and grown for a week in a Conviron growth chamber at 20°C in continuous light [CL: 90-120 $\mu Em^{-2}sec^{-1}$ photosynthetically active radiation (PAR)] for one week. Seedlings were then transplanted to pots using Terra-Lite Redi Earth prepared soil mix (W. R. Grace and Co. Canada Ltd., Ajax, Ontario) and kept in growth chambers under the same growth conditions.

Seeds of transgenic plants were sterilised in a laminar air flow hood, using 75% ethanol for three min and 10% bleach for 10 min, followed by three rinses with distilled water. These seeds were sown on solid AT medium supplemented with 50μg/ml kanamycin. They were also stratified for two days at 4°C, followed by one week at 20°C in a Conviron growth chamber. Kanamycin resistant plants were then transplanted to soil and kept in growth chambers in the same conditions as wild type plants.

2. Isolation and characterization of the *KCS2* gene

The putative sequence for the *KCS2* gene was obtained from the GenBank database. The *KCS2* gene corresponds to the accession number AL023094.

The *KCS2* ORF was amplified from *Arabidopsis* Col-2 genomic DNA by PCR, using the oligonucleotides #81 (5' GTA TCA TCA ACA AAA ATA TC 3') and #82 (CAA AGA TCG ATC TTA ACC 3') (Figure 4). 100 ng of genomic DNA was used as a template for the PCR reaction with 0.5 units of PWO DNA polymerase (Roche Molecular Biochemicals). The conditions of the PCR were 94°C/2 min; 10 cycles of 94°C/15 sec, 50°C/30 sec and 72°C/1min 30 sec; 20 cycles of 94°C/15 sec, 50°C/30 sec and 72°C/1min 30 sec plus an extension of 20sec/cylce; 72°C/7 min.

A 1.5 Kb PCR fragment was obtained and purified from a 0.8% agarose gel using a QIAEX Gel Extraction kit (Qiagen). The fragment was then subcloned into pCR2.1 vector (Invitrogen) in an EcoRI site. The vector generated (pCR-*KCS2*) was transformed into *Escherichia coli* DH5α competent cells by heat shock (Sambrook *et. al.*, 1989). After extracting the plasmid DNA, the presence of the insert was checked by PCR with 0.5 units of Taq polymerase (Gibco-BRL) and the desired orientation was selected by restriction analysis with BamHI.

2.1. DNA sequencing and sequence analysis

All the sequencing reactions were done by the University of British Columbia Nucleic Acid Protein Service Unit, using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and DNA sequencer 373 (Applied Biosystems). The *KCS2* promoter and ORF were sequenced from the DNA isolated from the pGEM-*promKCS2* (see section "construction of transformation vectors") and pCR-*KCS2* vectors respectively, using the plasmid DNA minipreparation kit (Qiagen). Sequences were analysed using SeqEd

version 1.0.3 (Applied Biosystems) and BOXSHADE (Biotoolkit - http://www.biosupplynet.com/cfdocs/btk/btk.cfm).

2.2. Southern blot analysis

10 μg of genomic DNA were digested overnight with EcoRI, EcoRV, HindIII or SstI. The DNA was then separated on a 1% agarose gel at 22 V for about 6 hr. It was then transferred to a Hybond- N nylon membrane (Amersham) according to the protocol for downward Southern blotting described by Koetsier *et.al.* (1993), in which the membrane is placed underneath the gel and the transfer is done by capillary action using paper towels and filter paper as the carrier. The transfer buffer used was 0.4 N NaOH. Once transferred, the DNA was fixed to the membrane by heating for 2 hr at 80°C. A 1.5 Kb probe, corresponding to the full length *KCS2* ORF, was prepared from pCR-*KCS2* vector by cleaving with EcoRI, gel purified using a QIAEX Gel Extraction kit (Qiagen) and radioactively labelled using the Random Primer DNA Labelling System (Gibco-BRL), according to the manufacturer's recommendations. Hybridisation was carried out in 6XSSC, 0.02% Ficoll, 0.02% PVP and 0.1% SDS.

Three washes were done at high stringency, using 2XSSC and 0.1% SDS at 65°C. The membrane was then exposed overnight to X-ray film (Kodak) at -80°C.

The expected banding pattern for each enzyme was deduced by analysis of *KCS2* nucleotide sequence, using Webcutter (Biotoolkit - http://www.biosupplynet.com/cfdocs/btk/btk.cfm).

3. Expression of *KCS2* in plants

3.1. Competent cell preparation and transformation of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain GV3101, containing the plasmid pMP90 (Koncz and Schell, 1986), were grown in liquid LB medium supplemented with 50μg/ml of gentamycin and 25 μg/ml of rifampicin until they reached an $O.D._{550}$ of 0.45–0.55. Bacterial cells were pelleted by centrifugation at 4000 X g for 5 min at 4°C and re-suspended in 20mM $CaCl_2$. 100 μl aliquots were used for transformation with approximately 1μg of plasmid DNA. Bacteria and DNA were incubated at 37°C for 5 min and, after adding 900 μl of LB medium, placed in a shaker at 28°C for 3 hr. After centrifugation to pellet the transformed bacteria, these were plated in solid LB medium, supplemented with 50 μg/ml gentamycin, 50μg/ml kanamycin and 25 μg/ml rifampicin and incubated overnight at 28°C.

3.2. Generation of *Arabidopsis* transgenic plants

15 wild type *A. thaliana* ecotype Columbia-2 plants were grown per pot in 18 cm pots as previously described.

All the transformations were done using the floral dipping method described by Clough and Bent (1998). After transformation, the plants were grown to maturity and when dried, the seeds from each pot were bulk harvested. Approximately 1000 seeds per pot were screened on AT medium containing 50μg/ml kanamycin. The kanamycin resistant plants were transferred to soil and grown under standard conditions. These plants were harvested individually to generate a series of transgenic lines.

4. Construction of transformation vectors

4.1. *pKCS2*-GUS transformation vector

KCS2 promoter region (*pKCS2)* was obtained by PCR using the forward primer 5' CGA TCA CGG AGT AGA GAA 3' (#123) and the reverse primer 5' GGA CAG TTT CTA AAG CAG 3' (#124) (Figure 4).100 ng of genomic DNA was used as a template for a PCR reaction with 1 unit of PFU DNA polymerase (Stratagene) under the following conditions: 94°C/2 min; 35 cycles of 94°C/15 sec, 50°C/30 sec and 72°C/2 min, and 72°C/5 min. A 1001 bp fragment was amplified and purified from a 0.8% agarose gel using the GELEX DNA extraction kit (Quantum).

The fragment obtained was subcloned into a SmaI site of pGEM7z(f) (Promega), resulting in the plasmid pGEM-*promKCS2.* This plasmid was transformed into *E.coli* competent cells by heat shock (Sambrook et.al., 1989). Transformants were selected on LB medium containing 100 μg/ml ampicillin. The presence and orientation of the insert was confirmed by restriction digest using HindIII. pGEM-*promKCS2* was cleaved with BamHI and XbaI and the fragment obtained was subcloned into the BamHI/XbaI site in the vector pBI101.1 (Clontech), containing the GUS gene. This places the GUS gene behind the *KCS2* promoter region (Figure 4-A). The resulting binary vector, *pKCS2*-GUS was then used to transform *E.coli* competent cells by heat shock, and they were selectively grown in LB medium containing 50 μg/ml kanamycin. The orientation and presence of the insert was confirmed by restriction analysis with HindIII, and by PCR, using 10 ng of plasmid DNA and 0.5 units of Taq DNA polymerase (Gibco-BRL). The primers used for the PCR (#123 and #124) were the same ones previously used to amplify the *KCS2* upstream region. Approximately 600 ng of *pKCS2*-GUS plasmid DNA were used to transform *Agrobacterium tumefaciens* competent cells strain GV3101 (pMP90). Transformants were selected on LB

medium containing 25μg/ml gentamycin, 50 μg/ml kanamycin and 25 μg/ml rifampycin. The presence of the insert in *Agrobacterium* was confirmed using PCR analysis of *Agrobacterium* DNA, under the same conditions described previously.

4.2. 35S-*KCS2* transformation vector

DNA from pCR-*KCS2* was cleaved with KpnI and ApaI and directionally subcloned into the KpnI/ApaI site of pSL1180 (Pharmacia), generating the vector pSL-*KCS2*. The presence of the insert was confirmed using PCR and restriction analysis. *KCS2* was again cleaved from pSL1180 using SmaI and SstI and subcloned into the binary vector pBI121 (Clontech), placing *KCS2* ORF under the control of the CaMV 35S promoter. The presence of the insert was checked by PCR and confirmed by restriction digest with SmaI and SstI. The newly created binary vector, 35S-*KCS2* (Figure 4-B) was used to transform *Agrobacterium tumefaciens* competent cells as previously described. After confirming the presence of the insert by PCR, *Agrobacterium* was used to transform *A.thaliana* ecotype Columbia-2 plants, as described previously.

4.3. *pFAE1-KCS2* transformation vector

A construct containing *FAE1* promoter (*pFAE1*) in pRD400 [derived from pBIN19 (Datla *et. al.* (1992)] had been previously generated in the lab (stock number LK 241). *KCS2* ORF was excised from the recombinant vector pCR-*KCS2* using EcoRI. This fragment was subcloned into an EcoRI site of the pRD400-*pFAE1* vector. The presence and orientation of *KCS2* was confirmed by restriction analysis using BamHI. This new vector (*pFAE1-KCS2*) (Figure 4-C) was used to transform *E.coli* competent cells by electroporation (Sambrook *et. al.*, 1989). The colonies were checked by PCR, using primers #81 and #82 (Figure 4). DNA from this vector was used to transform *Agrobacterium tumefaciens* competent cells by heat shock. Again, the presence of both *pFAE1* and *KCS2* were checked by PCR analysis.

Transgenic plants were generated as previously described, using the mutant CB25 of *Arabidopsis,* isolated in Dr. Kunst's laboratory (University of British Columbia, Botany Department). This mutant has a lesion in the *FAE1* gene, which results in a truncated FAE1 protein. Therefore, CB25 does not synthesise VLCFAs in its seeds. Approximately 100 transgenic lines were generated and their seeds were used for gas chromatography analysis.

5. Expression of *KCS2* in yeast

5.1. Construction of pESC-*KCS2* yeast transformation vector

KCS2 coding sequence was amplified by PCR, using primers #210 (ATG GAT GCT AAT GGA GGA C) and #211(TCA AAG ATC GAT CTT ACC C), which amplify the ORF from start to stop codon. PCR was performed using PWO DNA polymerase (Roche Molecular Biochemicals) under the following coditions : 94°C/2 min, 10 cycles of 94°C/15 sec, 51°C/30 sec and 72°C/1min; 20 cycles of 94°C/15 sec, 51°C/30 sec and 72°C/1min plus an extension of 5sec/cycle; 72°C/7 min.

A 1.4 Kb fragment was obtained and subcloned into a SmaI site of pBluescriptIIKS+ vector (Stratagene), creating the new vector pBS-*KCS2*. *KCS2* was then cleaved with EcoRI and NotI and subcloned into pESC-T (allows growth on a selective medium without tryptophan) (Stratagene) yeast expression vector, behind the GAL10 promoter, creating the yeast expression vector pESC-*KCS2* (Figure 4-D).

5.2. Yeast competent cells and transformation

Yeast cells, strain YPH499 were grown in liquid YPD medium containing 5% glucose, until they reached an OD_{600} of 1.0. Competent cells were then generated by sequentially centrifuging for 5 min at 3000 rpm in a OmnifugeRT (Heraeus) centrifuge and

re-suspending cells in 50 ml of distilled water and twice in Li/TE (10 M LiAc pH5, 10 X TE). 100 µl aliquots of these competent cells were used for transformation with 0.2-0.5 µg of plasmid DNA, adding 10µl of DMSO and 600 µl of PEG/Li/TE (50% PEG, 10M LiAc and 10X TE). The cells were incubated for 30 min at 30°C, followed by 15 min at 42°C, and precipitated by centrifugation at 3000 rpm for 5 min. They were then resuspended in 500 µl of distilled water and plated on solid YPD selective medium containing 5% glucose and a mixture of amino acids without tryptophan. Colonies that were able to grow on the selective medium were transferred to a medium containing galactose, to trigger the expression of the gene. The presence of *KCS2* was confirmed by PCR, using Taq polymerase under the following conditions: 94°C/2 min; 30 cycles of 94°C/15 sec, 45°C/1 min 30 sec and 72°C/30 sec; finally, 72°C/5 min.

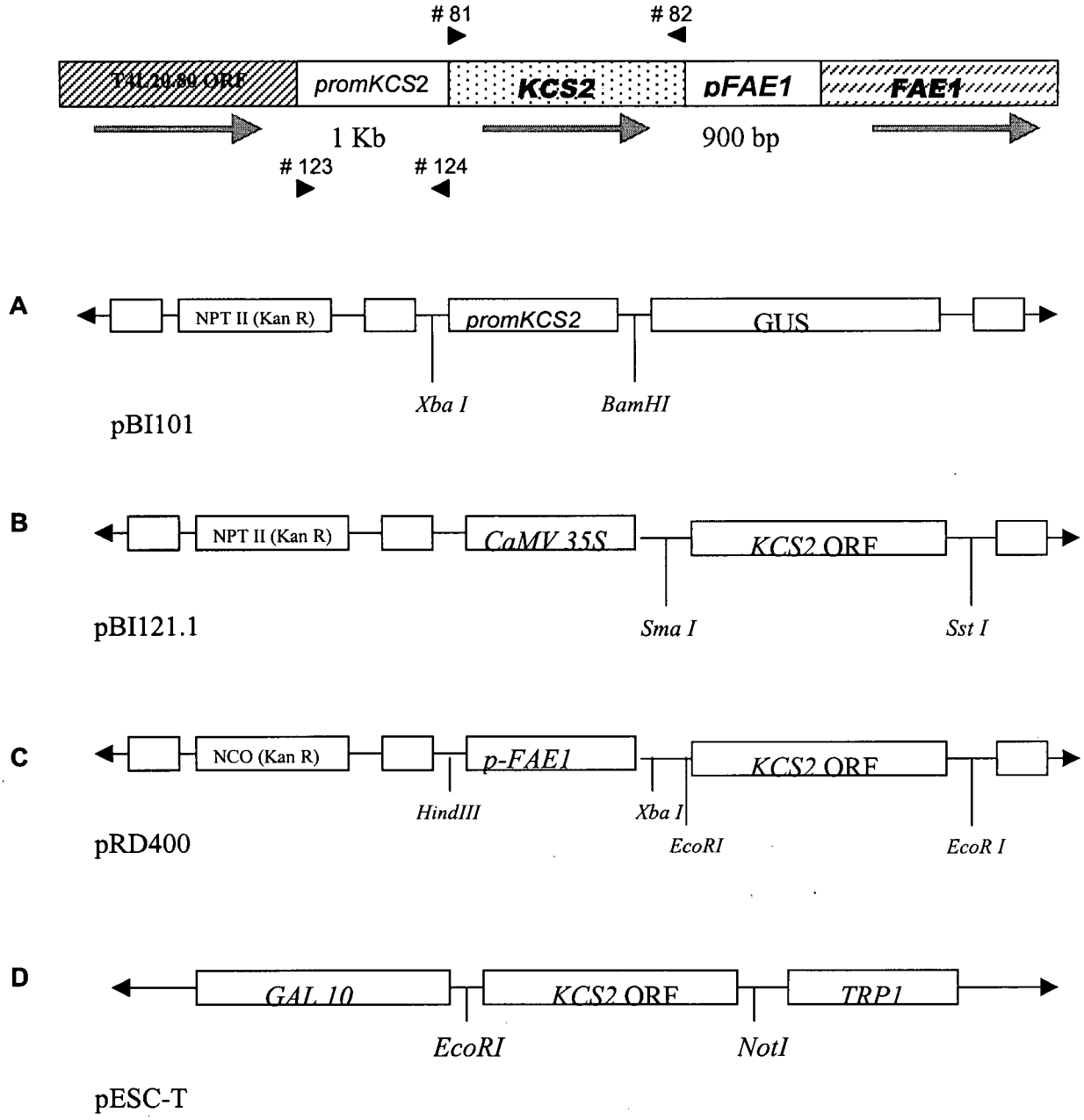

Figure 4. Different transformation vectors made for the expression of *KCS2* in yeast and plants. **A.** Transformation vector pKCS2-GUS. *KCS2* promoter region (p*KCS2*) in pBI101 upstream of the β-glucoronidase (GUS) gene. **B.** Transformation vector 35S-*KCS2*. *KCS2* ORF under the control of the 35S CaMV promoter in the pBI121.1 binary vector. **C.** Transformation vector *pFAE1-KCS2*. *KCS2* ORF under the control of the *FAE1* promoter (*pFAE1*) in the pRD400 binary vector. **D.** Transformation vector pESC-*KCS2*. *KCS2* ORF in the yeast expression vector pESC-T, under the control of the GAL10 promoter.
A schematic of the *KCS2* ORF and putative promoter is shown above. The primers used to amplify the putative promoter and coding sequence are indicated by arrowheads.

6. Expression analysis

6.1. GUS assay

Whole organs were excised from transgenic *pKCS2*-GUS plants and immediately immersed in 1.5 ml Eppendorf tubes containing 0.5 ml of GUS buffer (100mM Na phosphate buffer pH7, 10mM EDTA, 0.1% Triton X-100, 1mM ferricyanide, 1mM ferrocyanide, 1 mg/ml X-Gluc). After vacuum infiltrating for 1 hr, the tissue was incubated in GUS buffer overnight at 37°C. GUS buffer was then replaced with distilled water or 75% ethanol in the case of green tissues. The analysis of GUS expression was done using a dissecting microscope.

6.2. RNA blot analysis

For all RNA blot analyses described, a 1.4 Kb *KCS2* ORF was used as a probe. The fragment was radioactively labelled with ^{32}P-ATP by PCR, using primers #210 and #211. 10 ng of pESC-T-*KCS2* plasmid DNA was used as a template for the PCR, using Taq DNA polymerase (Gibco BRL). The conditions were 94°C/2 min; 30 cycles of 94°C/15 sec, 50°C/30 sec and 72°C/1 min 30sec, and 72°C/5 min.

For the northern blots using total RNA, 100 mg of tissue was harvested from the plant and immediately frozen in liquid nitrogen. Total RNA was isolated from all tissues using Trizol reagent (Gibco-BRL), according to the manufacturer's specifications. The tissue was grounded using liquid nitrogen and placed in Trizol. After mixing by vortexing and incubating at room temperature for 5 min, chloroform was added and the mixture was incubated for another 3 min at room temperature. RNA was isolated from the aqueous phase after precipitation in isopropanol and centrifugation for 15 min. For siliques, an additional precipitation step was performed, using a solution containing 1.2M NaCl and 0.8M Na-

citrate. The additional precipitation step was performed in order to purify the RNA from the polysaccharides, abundant in developing seeds.

For the RNA blots using polyA RNA, 1g of tissue was used for isolating total RNA. The tissue was harvested and immediately frozen in liquid nitrogen. After grinding, the tissue was transferred to a solution containing a 2:1 mixture of NTES buffer (100mM NaCl, 10mM Tris HCL pH 7.5, 1mM EDTA and 1% SDS) and phenol/chloroform/isoamyl alcohol (25:24:1). After mixing by shaking for 15 minutes, the tubes were centrifuged at 4°C. Nucleic acids were precipitated from the aqueous phase using 0.1 volumes of 3M NaAc and 2 volumes of 95% ethanol. The pellet was resuspended in distilled water and a second precipitation using 4M LiAc was performed to isolate the RNA. Finally, the pellet was further purified using 0.1 volume of 3M NaAc (pH 5.2) and 2 volumes of 95% ethanol and washed in 70% ethanol. The RNA obtained was re-suspended in RNAse-free distilled water.

250-500 μg of total RNA were used to obtain poly A RNA using the Oligotex mRNA preparation kit (Qiagen), following the manufacturer's recommendations.

20 μg of total RNA or 1 μg of poly A RNA were denatured by 50% formamide and 2.2M formaldehyde at 55°C for 15 minutes and separated by 5.8% formaldehyde, 1.5% agarose gel electrophoresis at 50V for 4 hours. The RNA was then transferred to a Hybond^{TM}-NX filter (Amersham) in 20 X SSC. After fixing the RNA to the membrane by heating for 2 hours at 80°C, hybridisation with the radioactive probe was performed in Modified Church Buffer, according to the recommendations described in the Hybond-NX User's Manual (Amersham). The blots were washed in 0.1% SDS and 2X, 1X and 0.1X SSC sequentially. Hybridisation and washes were performed at 65°C. Blots were then exposed to X-ray film (Kodak).

To confirm equal loading of the RNA, the 801bp *Arabidopsis* cytosolic cyclophilin (*ROC1*) gene (Lippuner *et. al.* 1994) was used. This gene was obtained from the plasmid

pNB73 [courtesy of Dr. Charles Gasser (University of California, Davis)] by cleaving the plasmid with EcoRI, purifying the fragment from a 0.8% agarose gel using a QIAEX Gel Extraction kit (Qiagen), and radioactively labeling it using the Random Primer DNA Labeling System (Gibco-BRL), according to the manufacturer's recommendations.

7. Gas chromatography (GC) analysis

7.1. GC analysis of yeast cells

Yeast cells were grown on selective YPD medium containing 5% galactose at 28°C for two to four days. Approximately half a 10 cm Petri plate was harvested, re-suspended in 500 µl of 1 N methanolic-HCL and trans-methylated at 80°C for I hour. After cooling to room temperature, 500 µl of 0.9% NaCl and 160 µl of hexane were added. The mixture was vortexed for 30 sec and centrifuged for 2 min at 3000 RPM. After centrifugation, 100 µl of the hexane phase was transferred to conical glass inserts in GC vials. The vials were capped and analysed in a gas-liquid chromatograph (Hewlett-Packard 5890 series II), equipped with a flame ionisation detector, using a 30m DB-23 capillary column [(50%cyanopropyl) methylpolysiloxane], and helium as the carrier gas. The GC analysis was performed at the initial temperature of 180°C, followed by a ramping of 4°C/min to 240°C, which was maintained for 3 min. Peaks were identified by comparison of their retention times to known fatty acid standards.

7.2. GC analysis of seed fatty acids

100 seeds per plant were harvested and placed in 10-ml tubes. 2 ml of 1N methanolic-HCl was added and the samples were trans-methylated at 80°C for 1 hour. After cooling down to room temperature, 2ml of 0.9% NaCl and 150 µl of hexane were added and

mixed by vortexing. The hexane phase was separated by centrifugation at 3000 RPM for 2 min and120 μl per sample were transferred into conical glass inserts in GC tubes. After capping the tubes, the samples were analysed in a gas-liquid chromatograph (Hewlett-Packard 5890 series II), equipped with a flame ionisation detector, using a 30m DB-23 capillary column, and helium as the carrier gas. The GC analysis was performed as described above for the analyses of yeast fatty acids. Peaks were identified by comparison of their retention times to known fatty acid standards.

Total fatty acids were calculated as μg/seed, using 20 μg of 17:1 methylester as an internal standard, added to each sample after the trans-methylation.

7.3. Wax extraction and analysis

Arabidopsis plants that were completely dried and at the end of their life cycle (approximately 3 months old), were used for gas chromatography analysis. The bottom part of the stem (5 cm) was immersed in a 2:1 chloroform:methanol mixture for 10 seconds, to remove surface waxes, as described in Millar and Kunst (1999). Following extraction, the samples were evaporated to dryness under nitrogen and dissolved in 100 μl of N,O-bis(Trimethylsilyl)trifluoroacetamide with 1% Trimethylchlorosilane (Pierce). The tubes were sealed under nitrogen and derivatized for 1 hour at 80°C. Samples were then cooled down for about five minutes at room temperature and placed into conical insert in GC vials. The vials were capped and analysed by gas-liquid chromatography in a Hewlett-Packard 6890 gas chromatograph equipped with flame ionisation detector, using a 30m DB-5 [(5% phenyl)methyl plysiloxane] capillary column, with helium as the carrier gas. The GC analysis was performed at the initial temperature of 150°C, followed by a ramping of 4°C/min to 320°C, which was maintained for 10 min. Peaks were identified by comparison of their retention times to known standards. Quantification of total wax load was based on peak

areas, which were converted to mass units by comparison to an internal standard, 17:1 methylester (60 μg), added to each sample immediately after wax extraction.

CHAPTER III

Results

1. Analysis of the *KCS2* sequence

KCS2 was amplified by PCR using genomic DNA as a template and the primers #81 and #82 (Figure 4), and cloned into an EcoRI site of pCR2.1 vector, generating the vector pCR-*KCS2*. The 1 Kb 5' upstream region of *KCS2* ORF was considered to be the putative promoter of the gene. Primers #123 and #124 (Figure 4) were used to amplify this region by PCR and the product was cloned into a SmaI site of pGEM7z(f), generating the vector pGEM-*promKCS2*. Both the ORF and the putative promoter were sequenced using internal primers.

1.1. Analysis of the *KCS2* putative promoter

The promoter of *KCS2* was defined as the 1001bp segment between the stop codon of the ORF directly upstream of *KCS2* and the start ATG codon of the *KCS2* gene. The putative promoter was analyzed for the presence of elements of a complete minimal promoter, such as a CAAT box and a TATA box (Hillebrand *et. al.*, 1998). The putative TATA box is located at – 171 bp from the ATG start codon of the *KCS2* gene. A putative CAT box, located at – 199 bp and a putative ACGT element, -15 bp from the ATG start codon of *KCS2* were also found (Figure 5). However, the transcription start point has not been determined.

```
                                                                                  #123
TGTTGTGGAG GACTTGTGAG AACCACCACC AGAGTCCGAC ATCGCGATCA CGGAGTAGAG AAAGTCAAAA
CTACTTCTCT CAGACGGATT AGTTTGGTTT GCTGGAGATT GTTCCAAGAA AGAGAAACGT TAGGAGCAAA
CAAACAAAAG AGAAAAGACG ATGATGACTG ATGAGAGCTT TAACAAAAAA ATAAAATGAG AGAGCTCAAC
GGGTAGAATT GTGAGACTTG AGAGAGTGTT TCCTATTTAA GGCATGCGAT TAGTGTTTAT TACGAGAATG
CCACCGAACG AGTACATATT AATGTATAGT ATGTTAATGA TAGTCTAACT AAAATTTGGT TTTTATTGAA
ATAGAATTTT GTAAGAATAA TGAGGATCTG TAATATAGCT GGATTTGCAT TAAATCGTAC GCCGTTGGTA
ATCGAAATTA GTTAAATAAA TGTTTTAGCA TATAATGTTG GTGCTTCCGA CATGTTTATT GGACAATAAT
ACCATATTTT TTCTTTGGGA TCTTAAAAAA ATTGAGGAAG AAAATAGTAA AATAGTCAAA CTTAGGTTAC
ATCATAATGG GCCAATTCTT TGAGTTGTGA TTGATCTCCA AAGATATACA TAGATTTACA CAAGATCAAA
AGAAAAACAA TTGGGCCTAA ACCCCAAGCC CATATCAACG TCCATTATCA TTAAGATTCC TTTTTTTCTT
GAAATTTGAA AATTTGAAAT TCGATTCAAA TCTACTCTCT CTGTTTTTTT CCCATAAAAA TCTGAAAAAC
CAGAAGCTTC TTCATCACTT TTCCTCTTGA TATCTTCCAT TAGTTGGCCG ATACACATGA CGCCAAATAC
ATCAATGGCG ACTCTTCTCT GTTTTTTAGT TATATCAAAC TCCCACCCAA CCTGCAGAAG AAAAAATGGT
GTCTATAAAC ACATCCCCTT ACGATTTCTT CTCTATCTCT CTCACAGTAT CTATATATAC GCACACAAAC
# 81                                                                              #124
CCAGATTCAG TTTCTCATCA GTATCATCAA CAAAAATATC AAAGATTCTG CTTTAGAAAC TGTCCATGGA
TGCTAATGGA GGACCTGTAC AGATCCGGAC CCAAAACTAC GTCAAGCTTG GTTATCACTA TCTGATCACT
CACTTTTTTA AACTCATGTT CCTCCCTCTA ATGGCTGTTT TGTTCATGAA TGTCTCATTG TTAAGCCTAA
ACCATCTTCA GCTCTATTAC AATTCCACCG GATTCATCTT CGTCATTACT CTCGCCATTG TCGGATCCAT
TGTCTTCTTC ATGTCTCGAC CTAGATCCAT CTACCTTCTA GATTACTCTT GCTACCTCCC GCCTTCGAGT
CAAAAAGTTA GCTACCAGAA ATTCATGAAC AACTCTAGTT TGATTCAAGA TTTCAGCGAA ACTTCTCTTG
AGTTCCAGAG GAAGATCTTG ATTCGCTCTG GTCTCGGTGA AGAGACTTAT TTACCGGATT CTATTCACTC
TATCCCTCCG CGTCCTACTA TGGCTGCAGC GCGTGAAGAA GCGGAGCAGG TAATCTTCGG TGCACTCGAC
AATCTTTTCG AGAATACAAA AATCAATCCT AGGGAGATTG GTGTTCTTGT TGTGAATTGT AGTTTGTTTA
ACCCTACGCC TTCTTTATCC GCCATGATTG TTAACAAGTA TAAGCTTAGA GGAAACATTA AGAGCTTTAA
CCTTGGAGGA ATGGGATGTA GTGCTGGTGT TATCGCGGTA GATCTAGCTA GTGATATGTT ACAAATCCAT
AGGAACACTT TTGCTCTTGT GGTTAGTACT GAGAACATCA CTCAGAATTG GTATTTTGGT AACAAGAAAG
CAATGTTGAT CCCTAATTGC TTGTTTAGAG TTGGTGGTTC CGCGGTTCTG CTTTCGAACA AGCCTTTGGA
TCGAAAACGA TCCAAGTATA AGCTTGTTCA TACGGTCAGG ACTCATAAAG GATCTGATGA GAACGCATTC
AATTGTGTGT ATCAAGAACA AGATGAGTGT TTGAAAACCG GAGTTTCTTT GTCTAAAGAT CTTATGGCTA
TAGCTGGAGA AGCTTTAAAG ACGAATATCA CTTCTTTGGG TCCTCTGGTT CTTCCTATAA GCGAGCAGAT
TCTGTTCTTT GCGACTTTTG TTGCTAAGAG ATTGTTCAAT GACAAGAAGA AGAAGCCTTA CATACCGGAT
TTCAAGCTTG CTTTAGATCA TTTCTGTATT CACGCGGGAG GTAGAGCCGT GATTGATGAG CTAGAGAAGA
GTTTAAAGCT TTCTCCAAAA CATGTTGAGG CGTCTAGAAT GACTTTGCAT AGATTTGGAA ACACTTCCTC
TAGCTCTATA TGGTATGAAT TGGCTTACAC GGAAGCTAAA GGAAGAATGA GGAAAGGAAA CAGAGTTTGG
CAGATTGCTT TTGGTAGCGG GTTTAAGTGT AACAGCGCGG TTTGGGTGGC TCTTCGCAAT GTCGAGCCCT
CGGTTAACAA TCCTTGGGAA CATTGCATCC ATAGATATCC GGTTAAGATC GATCTTTGAA CTCATAAAAA
                                                                                  # 82
```

Figure 5. Analysis of the sequence of the *KCS2* gene.
The putative stop codon corresponding to the end of the ORF directly upstream of *KCS2* and the *KCS2* ATG start codon are indicated with black boxes. Primers #123 and #124 used to amplify the 5' upstream region and primers #81 and # 82, used to amplify the ORF are underlined. For the promoter, putative TATA and CAAT boxes, as well as the ACGT element are highlighted in gray.

1.2. Analysis of the KCS2 protein

The *KCS2* open reading frame encodes a 487 amino acid protein. Alignment of the KCS2 protein sequence with previously characterized condensing enzymes shows that it shares a high percentage of amino acid identity with FAE1 (65% identity and 73.8% similarity), CUT1 (61.2% identity and 71.9% similarity) and KCS1 (61.2% identity and 72.3% similarity). The four proteins also show a very conserved region around the proposed active site (Figure 6).

The condensing enzymes for the elongation of VLCFAs studied so far, have shown to be located in the microsomal fraction of the cells (Fehling *et. al.*, 1992). Both CUT1 and FAE1 condensing enzymes have been shown to possess putative membrane spanning domains (Millar *et. al.*, 2000) when analyzed using the TMpred algorithm (Hofmann and Stoffel, 1993). To determine whether KCS2 protein had any membrane spanning domains, the sequence was also analyzed using the TMpred algorithm. The hydropathy plot obtained is highly similar to the ones previously obtained for FAE1 and CUT1 (Figure 7), showing two N-terminal hydrophobic regions, which are likely to be transmembrane domains. This suggests that KCS2, as well as FAE1 and CUT1, is an integral membrane protein.

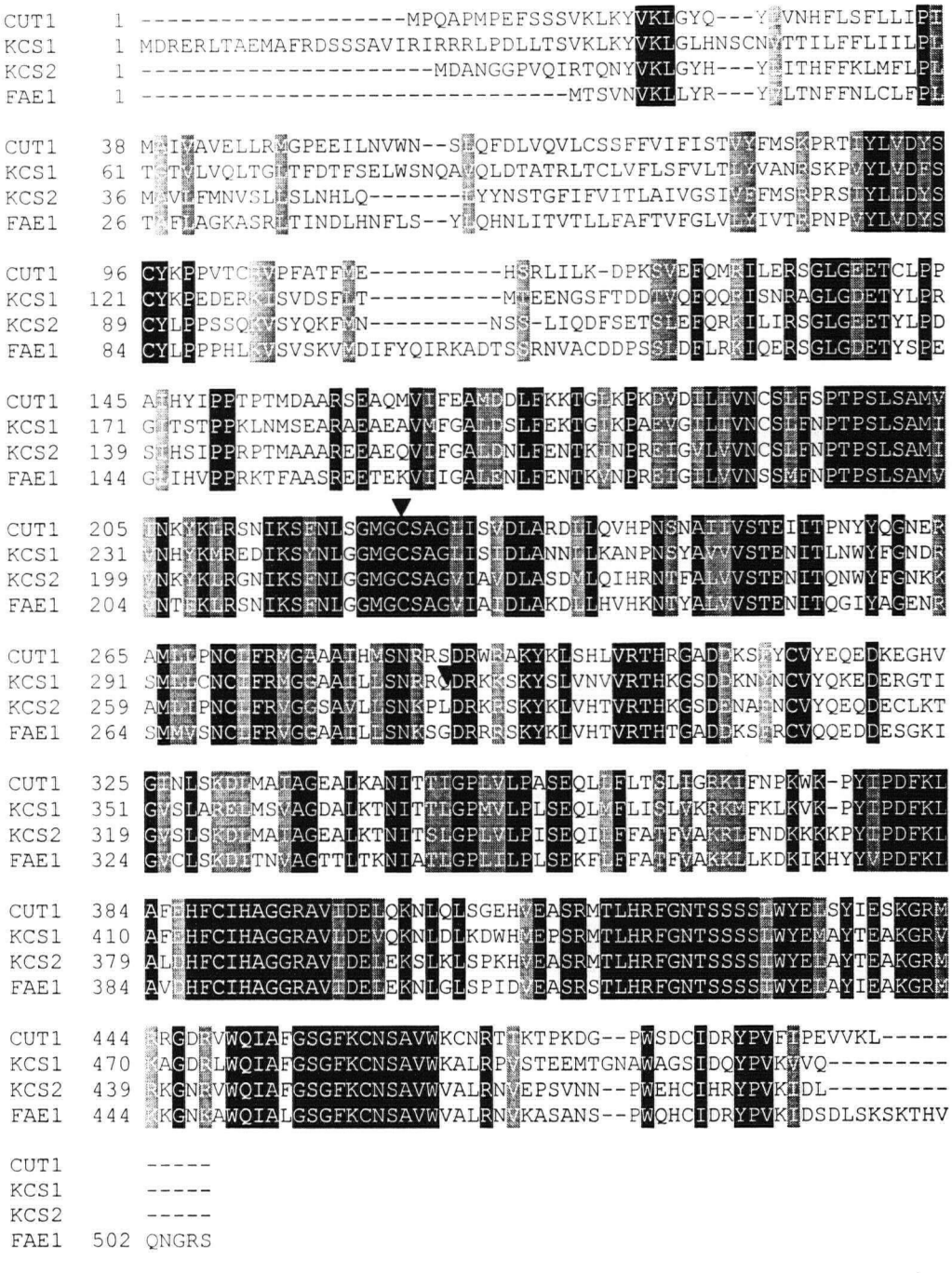

Figure 6. Amino acid sequence alignment of the microsomal condensing enzymes.
Sequences were aligned using the CLUSTAL W program. Identical aminoacids are highlighted on a black background and similar amino acids, on a grey background. Gaps introduced for the alignment are indicated with dots. The arrowhead indicates the predicted active site cysteine. *CUT1* sequence was obtained from Millar and Kunst (1999); *FAE1* sequence was obtained from James *et. al.* (1995); *KCS1* sequences was obtained from Todd *et. al.* (1999).

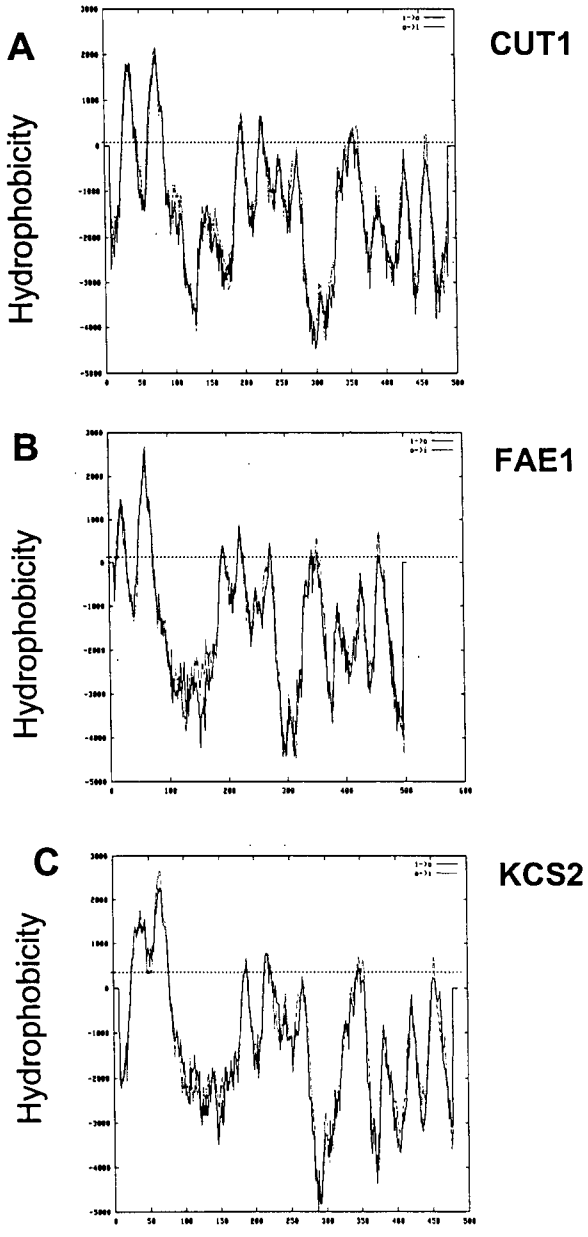

Figure 7. Comparison of the hydropathy plots of the *Arabidopsis* condensing enzymes CUT1, FAE1 and KCS2. The hydropathy plots were obtained using the TMpred algorithm (Hoffmann and Stoffel, 1993). **A**. CUT1, **B**. FAE1, **C**. KCS2

2. *KCS2* expression pattern

2.1. GUS assays

A construct containing the GUS gene under the control of the 1 Kb 5' region directly upstream of the putative translation initiation site of the *KCS2* gene was made (Figure 4-A). Transgenic plants were generated by the floral dip method. The primary transformants (T_0) were grown to maturity and seeds were bulk harvested from all the plants grown in individual pots. Transgenic seeds (T_1) were selected based on their ability to grow on medium containing kanamycin.

Approximately 150 transgenic seedlings (T_1 plant population) were transferred to soil and grown to maturity. T_1 plants were harvested individually and 100 T_2 seeds from 20 T_1 plants were screened on medium containing kanamycin. Three of the lines showed a pattern of kanamycin resistant to sensitive of 3:1 (71:29, 68:32 and 65:35 respectively) indicating that there was only one copy of the construct, or at least of the NPTII gene which confers kanamycin resistance, present in that line. To increase the proportion of transgenic plants homozygous for the GUS gene, two more generations were grown, allowed to self-fertilise, harvested individually and their seeds selected on kanamycin. In parallel, two other T_1 lines were selected which T_2 seeds were all kanamycin resistant, indicating homozigocity. In this way, 10 T_4 lines, originated from five independent transformation events were generated and used for the studies of GUS expression.

GUS histochemical assays using buffer containing X-GLUC were carried out on whole seedlings, leaves at different stages of development, stems, flowers, siliques and roots. Wild type plants treated with GUS buffer under the same conditions were used as controls. In all transgenic lines tested, the pattern of GUS activity was consistent, showing blue staining only in very young leaves and several floral organs (Figure 8). No blue staining was observed for any of the other organs or the wild type controls.

Developing siliques were also dissected and incubated in GUS buffer. The zone where they had been cut shows faint blue staining, but the seeds inside show no GUS expression. In order to compare the activity of *KCS2* with that of *FAE1*, which is known to be seed specific, developing siliques of transgenic plants expressing the GUS gene under the control of the *FAE1* 5' upstream region (p*FAE1*-GUS lines previously generated in the lab), were incubated in GUS buffer. For these plants, a seed specific expression pattern of the GUS gene could be seen (Figure 8-G and H).

For the seedlings, dark blue colour was observed as soon as the leaf became visible (1-2 mm long) and it seemed to gradually disappear when the next leaf started to come out (Figure 8-B). In cotyledons the expression was not consistent, some plants showed a faint blue stain, whereas others did not show expression of the GUS gene. The pattern of colour diffusion in leaves was very consistent, showing a tendency to move towards the tip of the leaves. Strong blue staining was only seen in very small leaves, and never in older ones.

In flowers, GUS stain could be seen only in anthers (Figure 9-B) if the flowers were left in the GUS buffer for approximately one hour. However, if the flowers were left in the buffer for longer periods of time, faint staining was observed in the petals, a part of the stamen filament and the stigma (Figure 8- C and 9-B). No expression was ever observed in sepals.

To examine the pattern of blue staining in flowers more precisely, young flowers were dissected and each of the organs was separately stained for GUS activity. As can be seen in Figure 9-E,G,H and I, when the organs were separately incubated in GUS buffer, only the anthers showed intense blue colour, regardless of the length of time that the organs were left in the buffer. This suggests that in the flowers, the expression is anther specific.

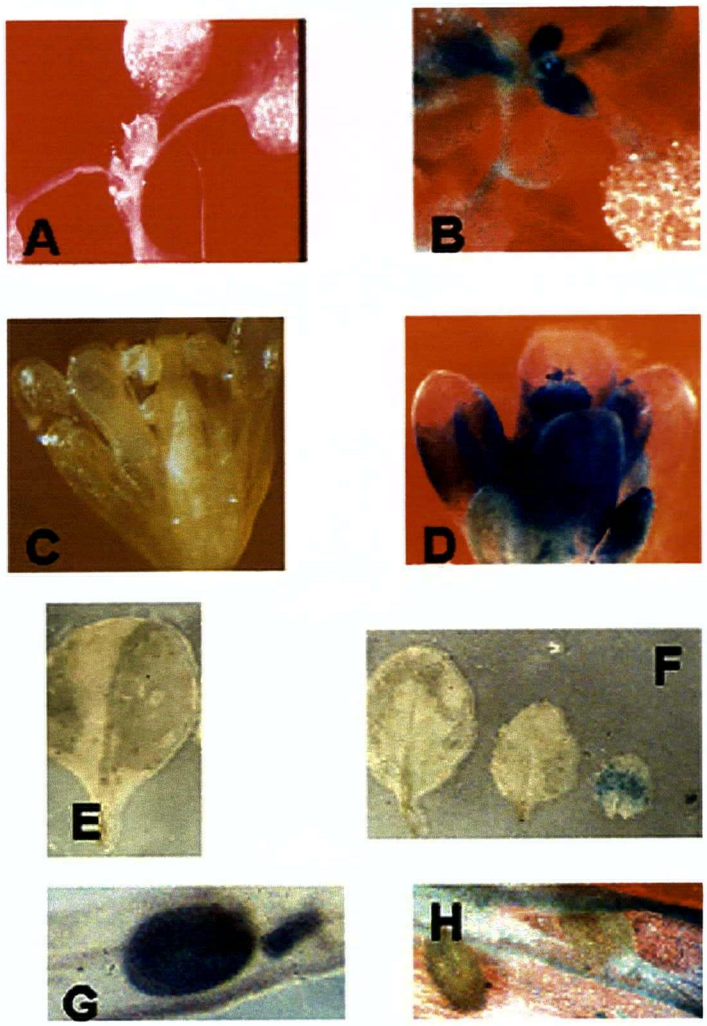

Figure 8. GUS expression in different tissues.
Wild type and *pKCS2*-GUS transgenic tissues were harvested and treated with GUS buffer for 24 hours. The blue staining demonstrates GUS activity in the presence of X-GLUC.
A. Wild type seedling. **B**. *p-KCS2*-GUS seedling, showing GUS activity in very young leaves. **C**. Wild type flower. **D**. *pKCS2*-GUS flower showing GUS activity in anthers, pistil and petals. **E**. Wild type leaf. **F**. Series of leaves of *pKCS2*-GUS plants, expressing the GUS gene. Expression can be seen in the first two leaves but is lost as the leaves get older. **G**. Open developing silique of a plant expressing the GUS gene under the control of the *FAE1* promoter. Expression can be clearly seen in seeds. **H**. Open silique of *pKCS2*-GUS plant. Some blue staining can be seen in the zone of the cut, but the seeds do not show any staining.

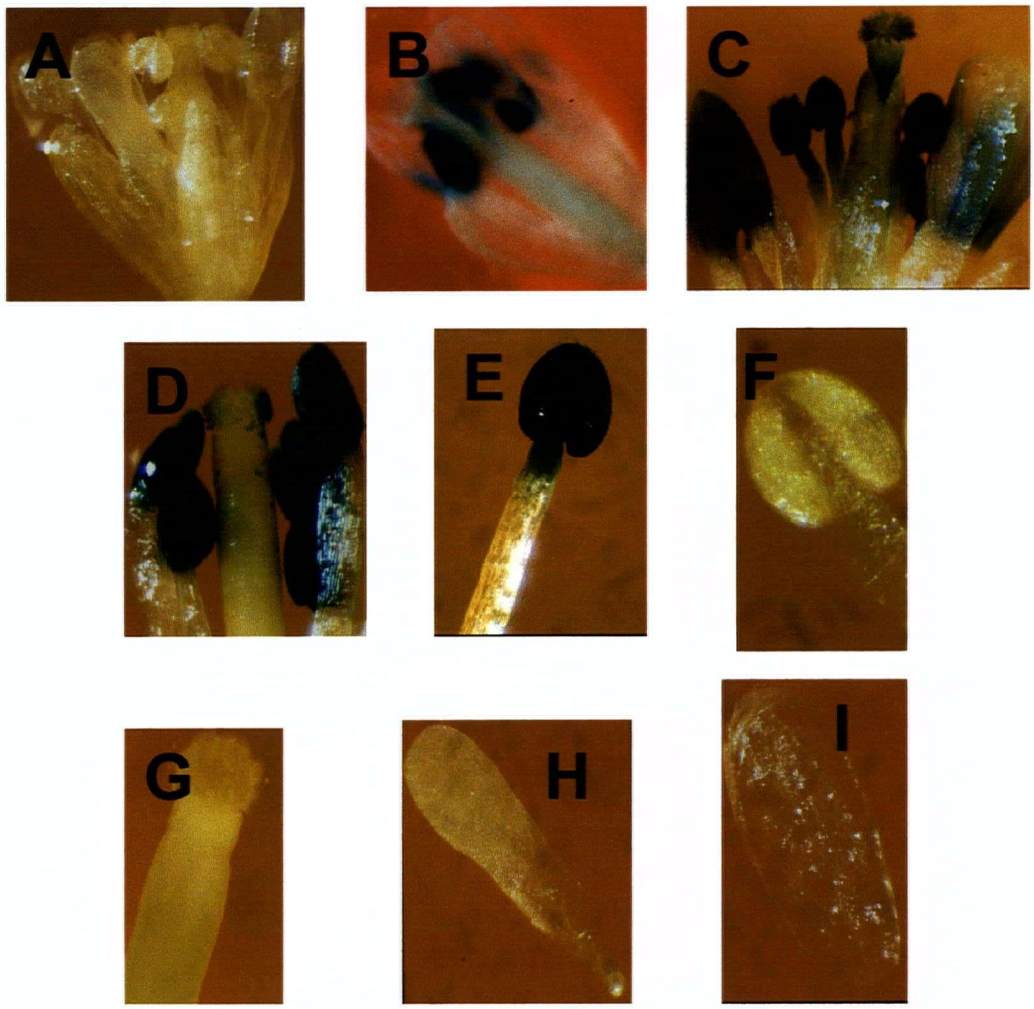

Figure 9. GUS expression in *Arabidopsis* flowers. Wild type and *pKCS2*-GUS transgenic tissues were harvested and incubated in GUS buffer. The blue staining represents the GUS activity in the presence of X-GLUC.
A. Wild type flower. **B**. Flower expressing *pKCS2*-GUS after one hour of incubation in GUS buffer. GUS activity is detected in anthers. **C**. Open flower expressing *pKCS2*-GUS transgene after 24 hours of exposure to the GUS buffer. GUS activity can be seen in anthers, pistil and petals. **D**. Anthers and pistil of transgenic flower expressing GUS. **E**. Anthers of *pKCS2*-GUS plants incubated in isolation. **F**. Wild type anthers incubated in GUS buffer. **G**. Pistil of a transgenic *pKCS2*-GUS plant incubated in isolation. No GUS activity can be seen. **H**. Petal of a transgenic flower incubated in isolation, showing no GUS activity. **I**. Sepal of *pKCS2*-GUS transgenic flower incubated in isolation. Again, no GUS activity can be seen.

2.2. Analysis of *KCS2* expression by RNA blot analysis

To confirm the expression pattern of the *KCS2* gene established by *pKCS2*-GUS fusion experiments, total RNA was isolated from all tissues and used to perform RNA blot analysis, with the full *KCS2* ORF as a probe. The specificity of the probe was tested by DNA blot analysis (Figure 10). As shown in Figure 10, when *KCS2* ORF was used as a probe single bands were observed for all the enzymes used, except for EcoRV, which has two restriction sites within the *KCS2* sequence, generating two visible fragments and a third one, two small (50 bp) to be detected on the blot.

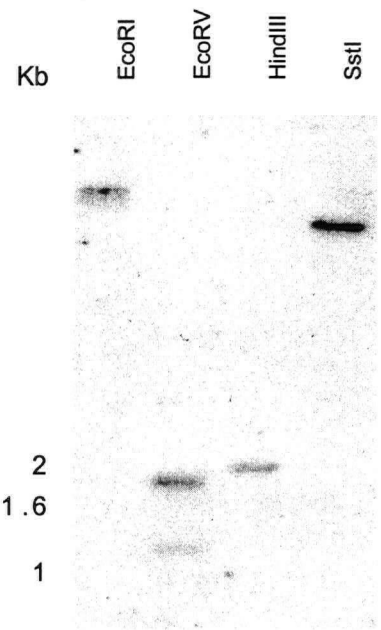

Figure 10. DNA blot analysis of *KCS2* gene.
10 μg per lane of *Arabidopsis* genomic DNA were digested to completion using the indicated restriction enzymes, separated on an agarose gel and blotted on a nylon filter. The blot was hybridized using *KCS2* full coding sequence, washed and exposed overnight to X-ray film at -80°C.

The level of expression of the *KCS2* gene is below the detection levels for total RNA blot analysis for all tissues examined, except for flower buds, where a weak signal was detected after the blot was exposed for several weeks. The expression of *KCS2* in buds is consistent with what had been observed using the GUS assay. An additional analysis using mRNA was performed in order to ensure that the lack of signal in the rest of the plant was not due to poor sensitivity of the assay. Again, hybridization was only detected in flower buds (Figure 11). Unfortunately, it was not possible to obtain mRNA from developing siliques.

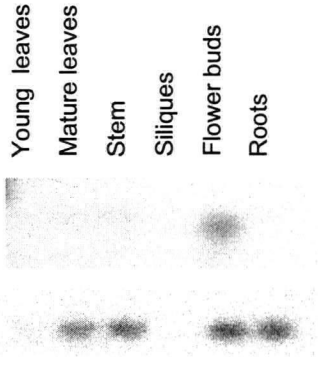

Figure 11. RNA blot analysis of *KCS2* expression in different tissues. 10 μg of mRNA was isolated from total RNA and blotted onto a nylon membrane. The hybridisation was carried out using the full-length *KCS2* ORF as a probe. After washing, the membrane was exposed to X-ray film for one week at -80°C. The upper band shows the expression of *KCS2*, which is only seen in flower buds. The lower bands show the constitutive expression of the *Arabidopsis* cytosolic cyclophilin (*ROC1)* gene (loading control).

3. Analysis of the specificity of the *KCS2* gene product

3.1. Expression of *KCS2* in yeast cells

KCS2 coding sequence exhibits high sequence similarity to previously characterized condensing enzymes. To assess the ability of *KCS2* to elongate fatty acids and determine its substrate specificity, *KCS2* ORF was transformed into yeast cells under the control of GAL10, a galactose-inducible promoter. Yeast (*Saccharomyces cerevisiae*, strain YPH499) cells were used to express *KCS2*. Ten colonies containing *KCS2*, as shown by PCR analysis using the primers #210 and #211, which amplify the full *KCS2* ORF, were grown in galactose. Their fatty acid profile was determined by gas chromatography. Yeast cells transformed with an empty vector and also grown in galactose were used as a control. The identity of the peaks was deduced by comparison to known standards.

Figure 12 shows the chromatograms obtained for the control yeast transformed with an empty vector, and yeast cells expressing *pESC-KCS2*. Yeast cells expressing *KCS2* show at least four extra peaks that do not appear to be present in the control. When these peaks were compared to the standards, they seemed to correspond to the saturated VLCFAs 20:0, 22:0 and 24:0. An additional peak was observed at a position that could correspond to 26:0. Unfortunately, we did not have fatty acid standards longer than C24.

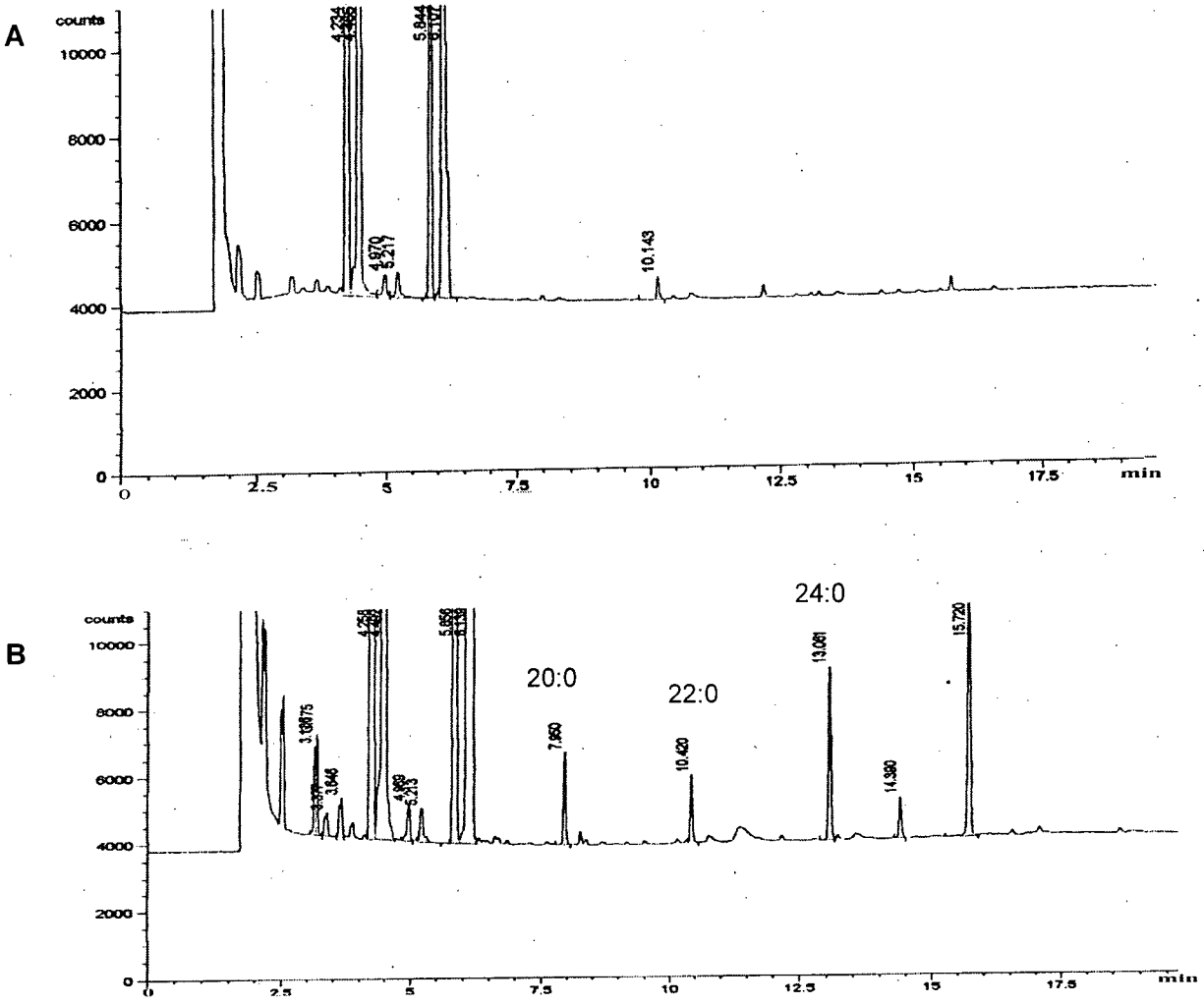

Figure 12. Gas chromatography analysis of yeast fatty acids. **A** shows the gas chromatogram of control yeast cells, which were transformed with an empty pESC-T vector. **B** shows the chromatogram of yeast cells expressing *KCS2* condensing enzyme. The identity of the peaks found only in yeast expressing *KCS2*, was deducted by comparison to known fatty acid standards. The predicted identity is indicated above the peaks.

3.2. Expression of *KCS2* in *Arabidopsis* seeds under the control of the *FAE1* promoter

KCS2 expression in yeast seemed to indicate that the KCS2 gene product was capable of elongating an acyl chain from C18:0 to C26:0. Apparently, the enzyme had a preference for saturated fatty acids, as was shown by the appearance of peaks corresponding to C20:0, C22:0, C24:0 and C26:0. To test the ability of *KCS2* to elongate an acyl chain *in planta*, the full coding sequence was placed behind the *FAE1* seed specific promoter. The recipient plant used for the experiment was the mutant CB25, which contains a lesion in the *FAE1* gene, resulting in a truncated FAE1 protein. Thus, CB25 plants do not make VLCFAs in the seeds, and all the VLCFAs observed would be the product of the KCS2 condensing enzyme.

Gas chromatography analysis was performed on seeds of 50 transgenic lines and compared to CB25 seeds. Ten lines showed a fatty acid profile different from the one observed for CB25 seeds (Table 1). The most dramatic difference was observed in the levels of mono-unsaturated C20:1 fatty acid, which in some lines shows an increase of almost 100 fold when compared to the control (Figure 13). Line 5-8 was used as an example to illustrate the difference in fatty acid profiles between wild type, CB25 and the transformants (Figure 14). The same line was analyzed by PCR to confirm the presence of the *pFAE1-KCS2* insert. Primers #56 (forward primer for *FAE1* promoter) and #211 (reverse primer for *KCS2* ORF) were used in a PCR reaction, using 100 ng of genomic DNA from three transgenic lines, including 5-8, and CB25 as a control. As shown in Figure 15, all the transgenic lines show a band corresponding to the product of the expected size resulting from the amplification of *pFAE1-KCS2* DNA. CB25 shows no such band.

Table 1. Relative % of fatty acids in the seeds of CB25 and transgenic *pFAE1-KCS2* lines.

FA	Samples	CB25	2-17	5-6	5-15	5-8	1-13	6-8	5-10	5-16	7-8	6-7
	RT* (min)	% area	% area	% area	% area	% area	% area	% area	% area	% area	% area	% area
16:0	4.155	12.18	9.60	8.93	9.58	8.89	9.44	11.70	8.65	9.22	13.19	9.44
16:1	4.38	0.67	0.46	0.41	0.53	0.46	0.44	0.77	0.39	0.43	0.75	0.61
18:0	5.750	2.92	3.12	3.69	4.85	3.93	3.58	2.92	3.01	3.49	5.82	3.49
18:1	5.9+6.1	27.59	27.55	20.86	19.95	14.07	28.45	15.16	20.09	25.37	25.99	12.52
18:2	6.499	33.47	32.26	29.90	28.98	27.62	31.63	28.83	30.72	30.97	26.66	29.06
18:3	7.163	22.02	25.91	23.12	19.15	21.60	25.14	22.12	22.18	23.32	21.45	20.67
20:0	7.854	0.63	0.53	1.13	1.84	2.10	0.72	1.35	1.11	0.87	1.29	1.82
20:1	8.1+8.2	0.24	0.269	11.16	14.02	19.49	0.27	15.73	12.89	5.74	4.32	20.45
22:0	10.318	0.16	0.14	0.19	0.25	0.29	0.18	0.23	0.19	0.18	0.26	0.25
22:1	10.659	0.00	0.00	0.45	0.66	1.32	0.00	0.10	0.63	0.26	0.11	1.55
24:1	12.928	0.10	0.13	0.14	0.17	0.21	0.12	0.17	0.14	0.14	0.21	0.14

* RT= Retention times. The identity of the peaks was estimated by comparing their retention times to known fatty acid standards.

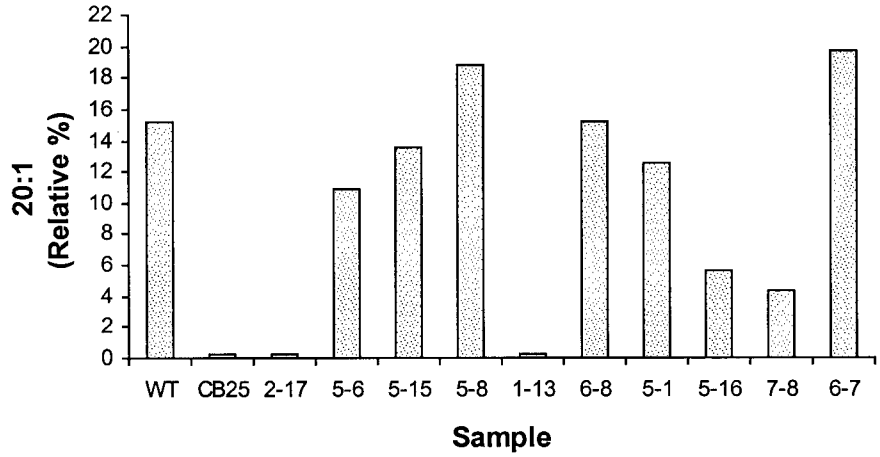

Figure 13. Relative % of 20:1 fatty acid in wild type, CB25 and 10 *pFAE1-KCS2* transgenic lines. The values were obtained by gas chromatography analysis and the identity of the fatty acid was determined by comparison to known fatty acid standards.

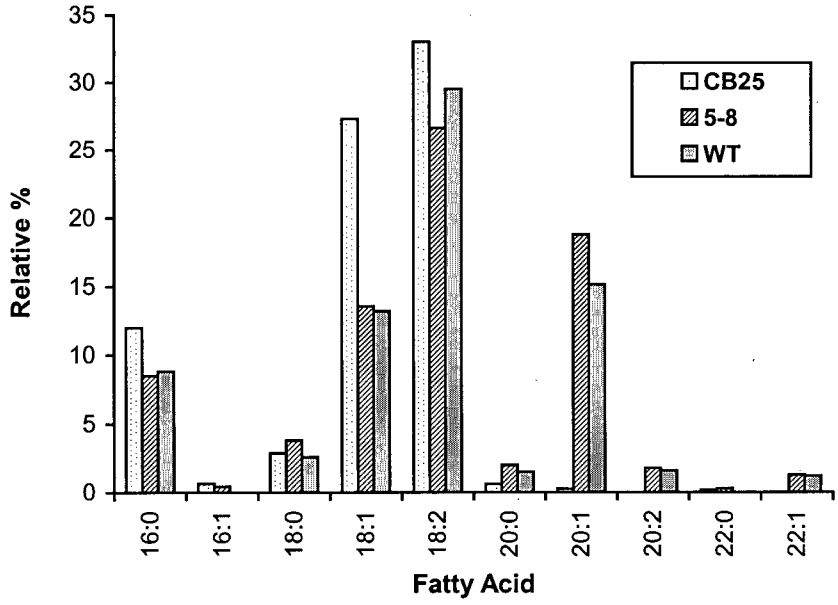

Figure 14. Relative % of major seed fatty acids in wild type, CB25 and *pFAE1-KCS2* transgenic line 5-8. Fatty acid profiles were obtained by analyzing seeds by gas chromatography. The identity of the fatty acids was determined by comparing the retention times with known fatty acid standards.

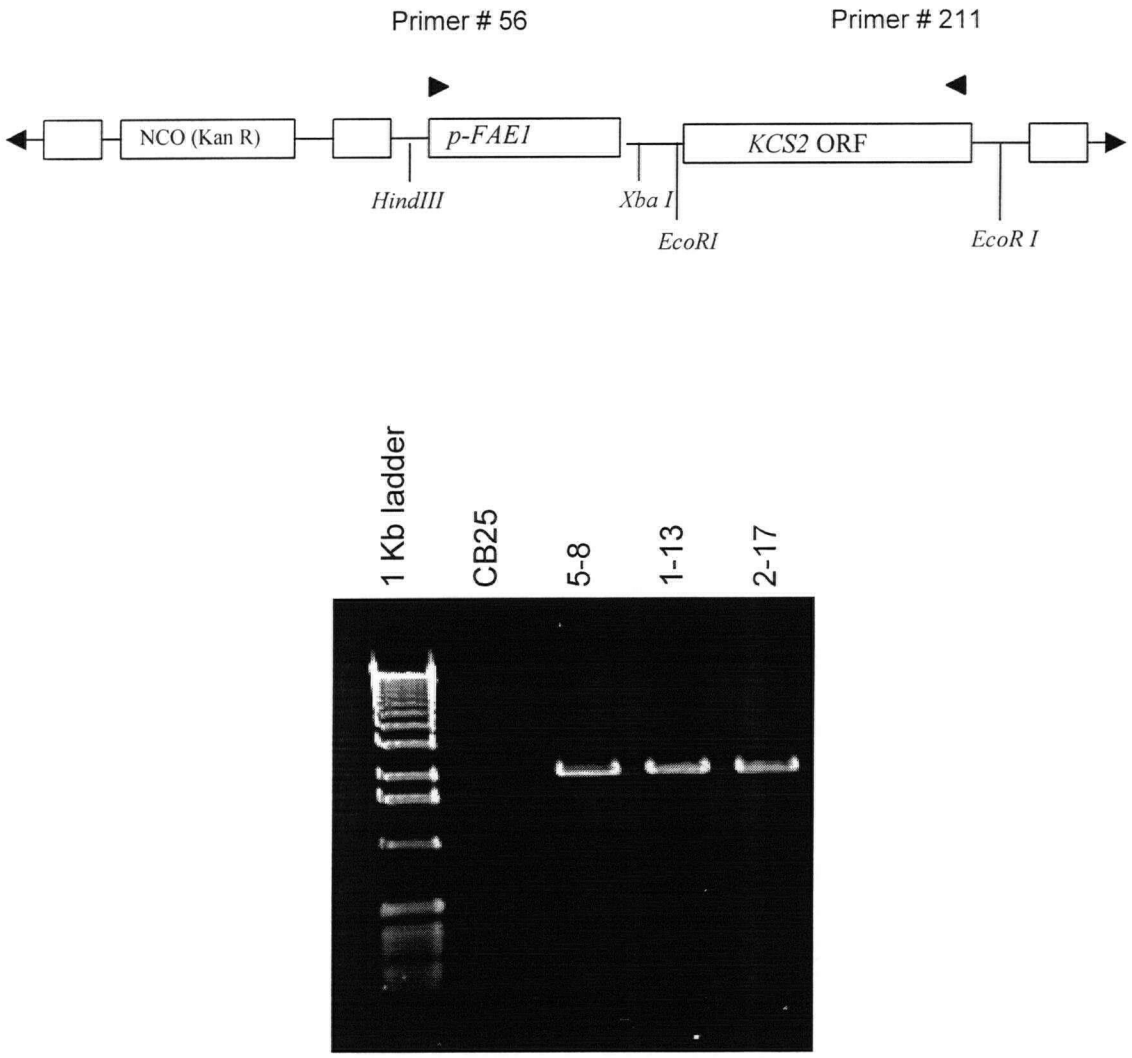

Figure 15. PCR amplification of the *pFAE1-KCS2* transgene using *Arabidopsis* genomic DNA as a template. 100 ng of genomic DNA were used for a PCR reaction, using primers #56 (*pFAE1* forward primer) and # 211(*KCS2* reverse primer) in order to determine the presence of the transgene. The first lane represents 1 Kb ladder. The control, CB25 shows no amplification product, whereas the three transgenic lines show the presence of the transgene. A schematic of the construct used is shown above. The primers used are indicated by arrowheads.

4. Study of the function of *KCS2* in *Arabidopsis*

4.1. Expression of *KCS2* under the control of the CaMV 35S promoter

Results of the experiments described above show the ability of KCS2 to elongate VLCFAs in *Arabidopsis* seeds, whereas the analysis of the *KCS2* expression pattern show that the gene is primarily expressed in flower buds. However, the exact function of *KCS2* remains to be determined. In order to do this, a reverse genetic approach was taken, using the strong CaMV 35S promoter to constitutively express *KCS2* in all plant tissues. The objective of this experiment was to attempt to co-suppress *KCS2* in order to obtain a loss-of-function phenotype, as well as a gain-of-function, over-expression phenotype.

The construct 35S-*KCS2* (Figure 4-B) was used to generate more than 400 transgenic T_1 plants. These plants were carefully observed throughout their development to maturity for differences with wild type. On the basis of wild type *KCS2* expression pattern, a loss-of-function phenotype was expected to be associated with the seedlings (young leaves) or flowers. However, none of the T_1 plants generated showed any differences in comparison with the wild type. Approximately 10 plants died before developing their third leaf, suggesting that the transgene may have been inserted in an essential gene, blocking its function.

4.2. Analysis of the expression of *KCS2* in 35S-*KCS2* transgenic lines

Since none of the 35S-*KCS2* transgenic plants generated showed any visible changes in phenotype, an RNA blot was carried out to assess the level of expression of the transgene in different lines. 10 T_1 lines were randomly selected and 100 seeds per transgenic line were grown on medium containing kanamycin. Approximately 2-3 kanamycin resistant plants per line were chosen and grown in pots. Total RNA was extracted from stems of 8 plants and hybridized to the full length *KCS2* ORF. As seen in Figure 16-A, some

KCS2 gene, whereas the level of expression in wild type stem is below the level of detection.

4.3. Analysis of stem wax load and seed VLCFA levels of 35S-*KCS2* transgenic lines

Once it was determined that there were transgenic lines overexpressing *KCS2*, it became interesting to examine whether the higher level of expression of the gene was correlated with higher levels of VLCFAs in the seed and a greater accumulation of surface wax. In order to do this, gas chromatography analysis was performed to measure the level of seed VLCFAs and total wax load on stems of transgenic 35S-*KCS2* plants.

4.3.1. Analysis of seed VLCFA accumulation

T_2 seeds from the lines previously shown to overexpress the transgene were grown on kanamycin and the resistant plants were transferred to pots and grown to maturity. Approximately 100 seeds per plant were used for gas chromatography to determine the levels of seed VLCFAs. Table 2 shows a comparison of the levels of the major fatty acids found in wild type and transgenic 35S-*KCS2* lines. Fatty acid levels in all the transgenic lines tested differ from the levels observed in wild type, but are very similar among them. The main difference was observed in the levels of 18:1 and 20:1 fatty acids. In the wild type, 18:1 fatty acid comprises close to 36 % of total fatty acids, whereas in all transgenic lines the level of 18:1 fluctuates between 12 and 14 %. This seems to be partly due to the significantly higher levels of 20:1 VLCFAs, found in all transgenic lines (approximately 18% vs. 12% in wild type). Apparently, the expression of KCS2 in seeds increases the rate of conversion of 18:1 to 20:1 fatty acids.

Total VLCFAs were calculated in μg/seed, using 20 μg of 17:1 methylester as an internal standard. As shown in Table 2, all the transgenic 35S-*KCS2* lines show significantly more total VLCFAs than the wild type seeds. However, the amount of VLCFAs does not

seem to be correlated with the level of expression of the transgene. For example, line 11-2-2, which does not over-express *KCS2* (Figure 16-A), has more total VLCFAs (186.46 μg/seed) than line 11-2-3 (96.161 μg/seed) in which *KCS2* is clearly over-expressed.

A calculation of the proportion of VLCFAs with respect to total fatty acids showed that for all the transgenic lines tested, VLCFAs increased in proportion when compared to the wild type. In the wild type, VLCFAs account for 16.9% of total fatty acids, whereas all transgenic lines tested have over 20% of VLCFAs (Table 2). As with total VLCFAs, however, the proportion of VLCFAs with respect to total fatty acids does not seem to correlate with the level of expression of the transgene.

4.3.2. Analysis of stem waxes

To examine whether the over-expression of *KCS2* translated into a thicker wax-load as a result of the increased VLCFA production, lower parts of the stems (5 cm) of 35S-*KCS2* plants were used for surface wax extraction and analysis by gas chromatography. Total wax load was calculated for each plant and compared to the wild type control. As shown in Figure 16-B, the total wax load does not seem to be affected by the over-expression of *KCS2*. Most of the transgenic lines tested show similar accumulation of stem wax when compared to the wild type, and the differences observed do not seem to correlate with the differences in expression of the *KCS2* gene.

Table 2. Relative % of fatty acids in the seeds of wild type and transgenic 35S-*KCS2* lines.

Fatty Acid	Samples	WT	3-6-4	5-10-1	5-10-2	22-6-4	11-2-2	11-2-3	22-6-2
	RT* (min)	% area	% area	% area	% area	% area	% area	% area	% area
16:0	4.203	8.43	9.47	8.67	4.92	8.97	8.50	9.16	9.23
18:0	5.783	3.45	3.02	3.13	2.88	2.90	3.06	3.04	3.15
18:1	6.05+6.10	37.73	14.81	14.83	14.37	13.88	14.74	15.16	11.94
18:2	6.544	21.62	30.45	29.39	30.00	29.57	29.47	29.97	29.61
18:3	7.200	13.51	20.83	22.00	23.82	22.33	21.78	21.87	23.14
20:0	7.877	1.87	1.51	1.48	1.56	1.53	1.51	1.47	1.60
20:1	8.19+8.28	12.34	18.64	19.23	20.87	19.43	19.59	18.16	19.88
22:1	10.684	1.043	1.26	1.26	1.57	1.37	1.34	1.18	1.44
Seed total VLCFAs** (μg/seed)		61.80	101.20	169.03	162.50	204.62	186.46	95.16	N.D
VLCFAs (% of total)		16.9	21.1	23.2	25.0	25.6	24.6	24.0	N.D

* RT= Retention times. The identity of the different fatty acids was determined by comparing their retention times to known fatty acid standards.

** Total VLCFAs were calculated using 20 μg of 17:1 methylester as an internal standard.

N.D. = Not determined.

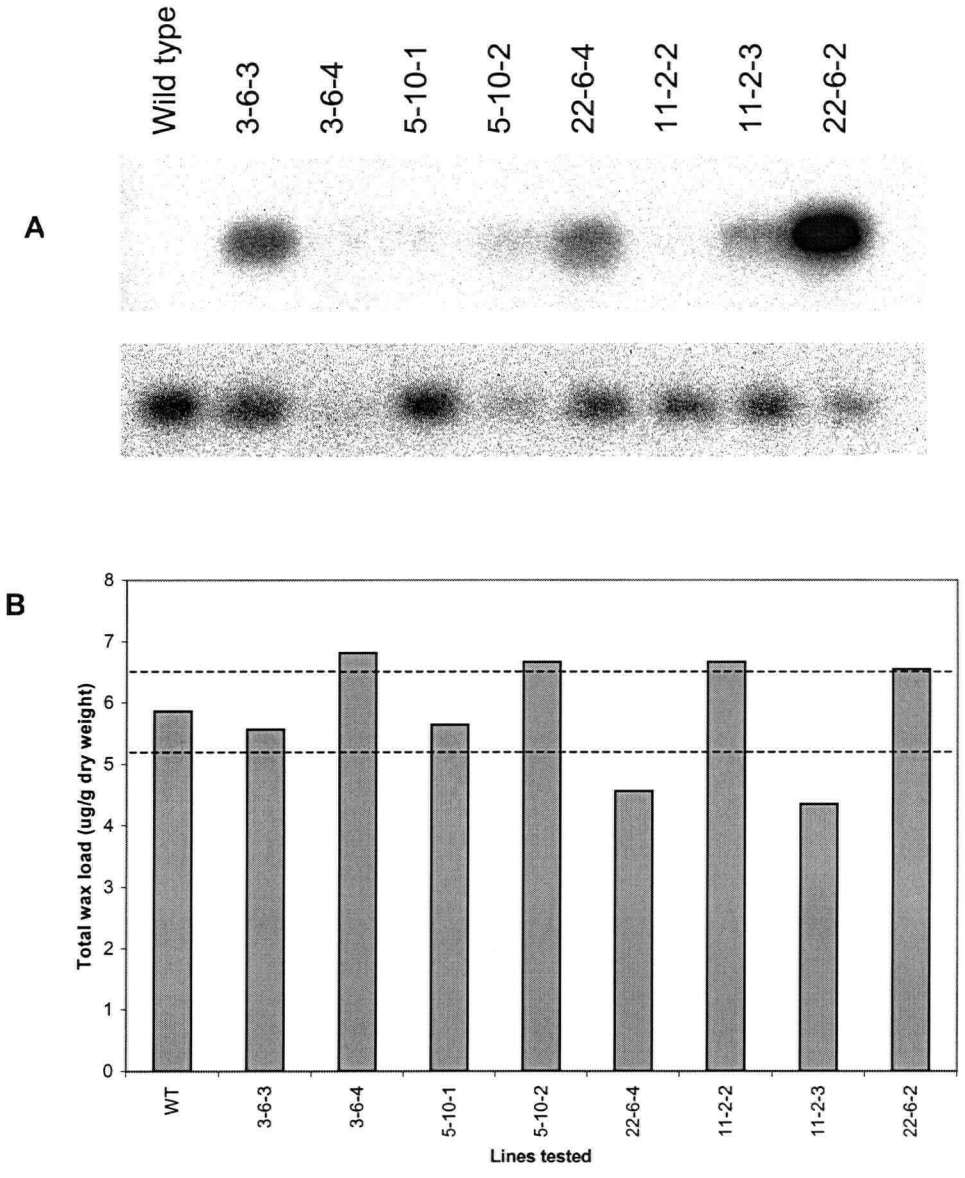

Figure 16. Analysis of *35S-KSC2* transgenic lines.
A. Total stem RNA blot analysis of wild type and eight transgenic lines. The first lane represents the wild type control. The upper bands show the hybridization obtained using *KCS2* ORF as a probe. The lower bands represent the hybridization pattern of the constitutively expressed *Arabidosis* cytosolic cyclophilin (*ROC1*) gene (loading control).
B. Total wax load analysis of wild type and eight transgenic lines. The order of the lines on the graph corresponds to the order shown on the RNA blot showed in **A**. Wild type total wax load value is an average obtained from seven plants. The dotted lines represent the standard error of the average of seven wild type plants.

CHAPTER IV

Discussion

KCS2 is located directly upstream of *FAE1* on chromosome IV

KCS2 gene is located in a tandem array with the *FAE1* gene on chromosome IV (Figure 3), suggesting that *FAE1* and *KCS2* arose by duplication from a common ancestral gene.

There have been several reports on genes of related functions that are present as gene clusters. For example, self-incompatibility genes in the *Brassicaceae* are a part of the *S*-multigene family. In *Brassica campestris*, three of those genes (*BcRK1*, *BcRL1* and *BcSL1*) have been found in a tandem array within a 26.5 kb region in the genome (Suzuki *et. al.*, 1997). In *Arabidopsis*, two genes related to the same S-multigene family (*ARK1* and *ARK2*) have also been found to be arranged in a tandem (Dwyer *et. al.*, 1994). In fatty acid biosynthesis, two isozymes of acetyl-CoA carboxylase (ACCase) -ACC1 and ACC2- are found in a tandem array within an approximately 25 kb region of chromosome I of *Arabidopsis.* Even though they are 93% identical, they show completely different expression patterns (Yanai *et. al.*, 1995). Another example of genes found in clusters includes the *nit1/nit2/nit3* gene cluster encoding the enzymes that participate in the last step of auxin synthesis in *Arabidopsis.* Even though there are four isoforms of this gene, the three previously mentioned genes are grouped together on chromosome III. *nit4* is located on chromosome V and, interestingly, it is the more distantly related isoform. Even though the coding sequences of those genes are very similar, their promoters differ significantly, which suggests differential regulation, maybe in response to developmental or environmental factors (Hillebrand *et. al.*, 1998). Finally, vegetative storage proteins genes (Vsp1 and Vsp2)

of *Arabidopsis* have been shown to be clustered and also be differentially expressed in both vegetative and reproductive organs (Utsugi *et. al.*, 1998).

The above mentioned examples represent a wide variety of physiologically significant gene families of which some members are located on the same chromosome, generally in a tandem configuration. Commonly, these genes show differential expression and differ significantly in their promoter sequences (Hillebrand *et. al.*, 1998). This seems similar to what is observed with *FAE1* and *KCS2*. Both genes have a high degree of sequence similarity within their coding region and they are both transcriptionally active. Their structures are also very similar, in that they do not contain introns. *KCS2* expression seems to be located primarily in flower buds, specifically in anthers, and young emerging leaves. In contrast, *FAE1* is expressed exclusively in developing seeds (James *et. al.*, 1995). This suggests that *FAE1* and *KCS2* evolved from a common ancestor and that their roles may have differentiated during evolution.

KCS2 is expressed in flowers and very young leaves

As previously mentioned, RNA blot and *KCS2* promoter-GUS studies of *KCS2* expression indicate that it differs from that of *FAE1*, supporting the theory that if these genes come from a common ancestor, their promoters evolved to form two differentially expressed condensing enzymes. However, the present study is not definitive in the assessment of the exact expression pattern of *KCS2*.

Results of both *pKCS2*-GUS fusion and mRNA blot analyses are in agreement showing that *KCS2* is primarily expressed in flower buds, whereas no KCS2 expression was detected in stems, older leaves or siliques. More precisely, GUS assays of whole flowers revealed that *KCS2* is expressed mainly in anthers but also in petals and pistil. Wild type flowers did not exhibit any staining when treated with GUS buffer under the same conditions (Figure 8).

However, the results of GUS histochemical assays differ somewhat from those obtained by RNA blot analysis in that a clear blue staining is observed in very young leaves of *pKCS2*-GUS plants incubated in GUS buffer. In contrast, no hybridization was observed in either total RNA or mRNA blot analysis for young leaves. When analyzing the expression pattern of seedlings treated with GUS, the highest level of GUS expression was seen in emerging leaves 1 or 2 mm long (Figure 8-B). The GUS staining in these leaves became less intense by the time the second leaf started to emerge. When harvesting young leaves for RNA blot analyses, the first and the second leaves were selected. It could be that since the first leaf was extremely small, and the expression seemed to decrease in the second leaf, the level of *KCS2* transcript could not be detected by an RNA blot.

Diffusion of the GUS stain is another problem that initially led to a misinterpretation of the results. When assaying whole flowers, GUS expression could be seen in anthers after approximately one hour, but when the flowers were left in the assay buffer for an extended period of time (overnight), the pistil and petals also showed faint blue staining. In order to determine if the expression in pistil and petals was real, flowers of Arabidopsis plants containing the GUS transgene were dissected and each organ was tested separately in GUS buffer. The results of this test showed that only the anthers possessed GUS activity, whereas the remaining floral organs (petals, pistil and sepals) did not show any GUS staining (Figure 9).

Based on these results, it seems that *KCS2* expression in flowers is anther-specific, suggesting the role of KCS2 in the elongation of VLCFAs for the synthesis of pollen grain lipids. The outer layers of the pollen grain, sporopollenin and pollen coat or tryphine layer, contain VLCFAs (Piffanelli *et. al.*, 1997). It has been shown in maize, that the levels of VLCFAs decreased considerably in the sporopollenin when a thiocarbamate herbicide (EPTC), known to inhibit the synthesis of VLCFAs was used (Wilmesmeier and Wiermann, 1995). Pollen coat analyses, on the other hand, have detected very long chain wax esters,

which seem to have an essential role in pollen hydration upon landing on the stigma surface (Preuss *et. al.*, 1993). In fact, some mutants of Arabidopsis defective in VLCFA synthesis are also male sterile, due to the lack of long chain wax esters in the tryphine layer. These mutants can be rescued by growing the plants in high humidity conditions (Preuss *et. al.*, 1993).

All the lipid components of the sporopollenin and pollen coat are synthesized in the tapetum cells that surround the developing microspores. When the microspores develop into mature pollen grains, the tapetum undergoes programmed cell death and releases its contents to the pollen grains (Piffanelli *et. al.*, 1998). The time at which lipids are synthesized in the tapetum appears to be the later stages of pollen development. In *Brassica napus*, it has been shown that lipid biosynthesis was undetectable at the meiosis/tetrad stages and did not peak until the first pollen mitosis (Evans *et. al.*, 1992; Piffanelli *et. al.*, 1997). *KCS2* expression in the anther using GUS reporter gene was detectable throughout flower development. However, when analyzing *KCS2* transcript accumulation by RNA blotting, *KCS2* expression could only be detected in flower buds. Since GUS staining in anthers is very strong, it is possible that even low levels of KCS2 expression are able to trigger the β-glucoronidase reaction. Thus it may be that the GUS assay does not allow accurate detection of the *KCS2* activity in the anther. It would be very important to do a time-course experiment, since this would help determine the exact timing of *KCS2* expression. In addition, *in situ* hybridization would help to determine the spatial pattern of *KCS2* activity in the anther. These two assays may give clues as to the possible function of the KCS2 condensing enzyme.

KCS2 is expressed at very low levels in wild type *Arabidopsis*

Experiments discussed above showed that *KCS2* was expressed at very low levels in wild type plants. In fact, *KCS2* mRNA was only found in flower buds and was barely detectable. Based on the results of the GUS assays, it is possible that *KCS2* has a specific function in the synthesis of VLCFAs in the tapetum at a distinctive stage of pollen development. The level of expression of *KCS2* appears to be below the detection level of an mRNA blot in the rest of the plant. In order to determine this, it may be useful to perform a more sensitive assay such as RT-PCR.

On the other hand, since the tissue was harvested from healthy plants growing in optimal conditions, the possibility that *KCS2* expression is environmentally regulated cannot be ruled out. Some plant responses that involve the participation of VLCFAs are triggered by several environmental factors. For example, suberin deposition in aerial tissues can be observed when the tissue is wounded, as a way to avoid water loss and fungal or bacterial attack (Kolattukudy, 1980). *KCS1* is thought to be regulated in response to water stress, as shown by the lack of ability of the mutant to grow in low humidity conditions (Todd *et. al.*, 1999).

Another possibility would be that *KCS2* is regulated by developmental signals and is active during a narrow window of time that did not coincide with the time that the tissue was harvested. It would be very useful to perform additional experiments using different developmental stages and environmental conditions to further examine *KCS2* expression in different tissues.

KCS2 elongates VLCFAs from C20:0 to C26:0 in yeast

The activity of *KCS2* in yeast was assayed by gas chromatography of yeast fatty acids. Ten colonies that contained the *KCS2* gene, as shown by PCR analysis, all conatined three prominent GC peaks that were typically not seen (or at very low levels) in the

chromatograms of the control yeast. A comparison of these peaks to known fatty acid standards showed that they aligned with 20:0, 22:0, 24:0 and maybe 26:0 fatty acids. The last peak, presumably corresponding to 26:0, has been observed before in wild type yeast chromatograms. In fact, it has been documented that *Saccharomyces cerevisieae* has a minor amount of sphingolipids in its membranes and that the VLCFA comprising the ceramide is almost exclusively 26:0 (Oh *et. al.*, 1997). Therefore, it is possible that the last peak observed was not a novel VLCFA. However, the relative area % of the peak compared to the control, suggests that 26:0 VLCFA was being produced at higher levels in cells expressing *KCS2*. In the control, no major peaks were detected beyond C18:1, whereas in pESC-*KCS2* expressing yeast, the values of relative percentage of C20:0, 22:0, 24:0 and 26:0 were 0.4%, 0.3%, 0.2% and 1.7%, respectively.

The peaks observed in yeast expressing *KCS2* are very clear in the chromatograms (Figure 12) and seem to be equally spaced, suggesting increments in two carbons. However, these results have been difficult to reproduce. In addition, the same fatty acids are present in the wild type yeast but only occasionally and at much lower levels. Thus, at the present time it is not clear whether this result is real, or should be considered an artifact.

Expression of *KCS2* can restore wild type levels of VLCFAs in seeds of the CB25 mutant deficient in C18 fatty acid elongation

Expression of the *KCS2* in yeast cells suggested that the KCS2 condensing enzyme was capable of elongating fatty acids from 18 to 26 carbon atoms with a distinct preference for saturated VLCFAs. In order to test the substrate specificity of *KCS2* in plant tissues, the *KCS2* ORF was expressed in seeds under the control of the *FAE1* seed-specific promoter. The results showed that some transgenic lines were capable of producing 20:1 fatty acid at levels up to almost 100 fold the levels seen in the control CB25 seeds (from 0.2% of total fatty acids in CB25 to almost 20% in some of the lines). The levels of 20:1 VLCFAs

corresponded to the ones documented for wild type plants (Lemieux *et. al.*, 1990; Kunst *et. al.*, 1992), indicating that the expression of *KCS2* in the seed could restore VLCFAs to wild type levels (Figure 14). In CB25 there is also no detectable 22:1, whereas some transgenic lines have almost 1.5% of this VLCFA. Similarly, 20:0 VLCFA showed an increase from 0.6% in the control to approximately 1.7% of total fatty acids in some transgenic lines.

Even though saturated VLCFAs increase with the expression of *KCS2*, they do not seem to do it to the same extent as mono-unsaturated VLCFAs. This contradicts the results obtained for yeast, in which saturated VLCFAs seem to be the ones primarily elongated by KCS2. In addition, in yeast, the acyl chain length specificity of KCS2 appears to be C18 to C26, suggesting that the condensing enzyme is capable of participating in at least 4 elongation cycles. In seeds of *Arabidopsis*, however, the results suggest that KCS2 is capable of participating in only two elongation cycles, from C18 to C22, similar to the FAE1 condensing enzyme.

In both yeast and seeds of the CB25 mutant, unsaturated fatty acids are more abundant than saturated fatty acids as substrates for elongation. In the case of yeast, the available substrate seems to be 18:1 fatty acid (34.8%, as opposed to 5.4% for 18:0) (Oh *et. al.*, 1997), and in seeds of CB25 mutant, three C18 fatty acids are available for elongation: 18:1 (15.4%), 18:2 (32.7%) and 18:3 (20.3%). However, the accumulation of products does not always coincide with the availability of substrates, as shown by the higher accumulation of saturated VLCFAs in yeast and of mono-unsaturated VLCFAs in seeds, even though poly-unsaturated C18 fatty acids are more abundant. This might have to do with the sinks for the VLCFAs produced. Yeast cells produce sphingolipids, comprising approximately 10% of the total membrane lipids. The most abundant species in wild type are 26:0 and hydroxy 26:0, which comprise approximately 2.2 and 0.9% of the total fatty acid mass respectively (Oh *et. al.*, 1997). Since yeast needs C26:0 for its sphingolipids, it accumulates them,

whereas other VLCFAs might get degraded if they are not incorporated into a lipid. Similarly, in seeds of *Arabidopsis*, the major product of fatty acid elongation for the production of TAGs in the wild type is 20:1 (Lemieux *et. al.*, 1990) and 22:1 in much lower amounts (up to 3.0%- Lemieux *et.al.*, 1990). Thus, it is possible that, even if the KCS2 condensing enzyme was capable of elongating saturated VLCFAs or VLCFAs longer than C22, they would get degraded if they do not accumulate as part of the seed TAGs.

The results of the expression of *KCS2* in yeast cells and seeds of *Arabidopsis* showed that the gene is transcriptionally active and that the condensing enzyme has the capacity for elongating fatty acids. However, experiments to determine the exact acyl chain length specificity of *KCS2* are inconclusive. More accurate data concerning the substrate specificity of *KCS2* will be obtained by *in vitro* enzymatic assays, in which radioactively labeled C18:CoA is provided to yeast and seed microsomes.

Altered expression of *KCS2* did not result in a visible phenotype

Expression studies suggest the *KCS2* gene to be preferentially expressed in the anthers of *Arabidopsis*. By using reverse genetics, we were hoping to generate a phenotype that could give an idea about the function of the gene. For example, considering the importance of long chain lipids in pollen grains, one could expect that a loss of function of *KCS2* might result in a male sterility phenotype.

It has been shown that highly expressed transgenes introduced into the plant genome can inhibit the expression of the plant native genes by triggering the destruction of similar transcripts (Elmayan and Vaucheret, 1996; Jorgensen *et. al.*, 1998). This phenomenon is known as co-suppression or homology-dependent gene silencing (Wassenegger and Pélissier, 1998). Even though the mechanisms for co-suppression are still not well understood (Jones *et. al.*, 1998), it has been widely used to study gene functions and as a tool in biotechnology (Jones *et. al.*, 1998; Flipse *et. al.*, 1996; Vaucheret

et. al., 1995). A good example of a successful co-suppression was the study of the *CUT1* gene. When the *CUT1* cDNA was introduced into *Arabidopsis* under the control of the 35S promoter, plants with a shiny stem phenotype were obtained. This was a key step in figuring out the function of *CUT1* in wax biosynthesis (Millar and Kunst, 1999). With this in mind, *KCS2* cDNA was subcloned in a sense orientation behind the CaMV 35S promoter and more than 400 plants were generated. In contrast to what was observed with the *CUT1* plants, no visible phenotype was observed in any of the transgenic 35S-*KCS2* transformants, suggesting that no loss-of-function had been obtained. However, considering the possibility that the phenotype may be subtler, it may have to be detected using methods other than visual screening. For example, levels of VLCFAs may have to be measured in the floral tissue, and compared to the wild type VLCFA composition.

In order to examine the level of accumulation of *KCS2* transcript in the plants transformed with 35S-*KCS2*, RNA blot analysis of total stem RNA was performed for several transgenic lines and compared to the wild type. This analysis showed that in at least three lines, the *KCS2* gene was being over-expressed (Figure 16-A). Since the level of *KCS2* transcript in wild type stem is undetectable, only the over-expressors could be identified. It then became interesting to analyze these lines in order to estimate the effect of a gain-of function of the *KCS2* gene. Thus, 35-*KCS2* transgenic lines were analyzed for their content of VLCFAs in seeds and stem wax accumulation.

Transgenic lines over-expressing *KCS2* accumulate less 18:1 and more 20:1 fatty acids

When seeds of some of the over-expressor lines were studied for their content of VLCFAs it was found that they accumulated higher levels of C20:1 (19% in 35S-*KCS2* versus 13% in wild type) and considerably less 18:1 (14% in 35S-*KCS2* versus 37% in wild type). The levels of 22:1 were also slightly higher in 35S-*KCS2* plants. This could be

explained by a higher elongation of the C18 substrate, due to the availability of an additional condensing enzyme. As has been shown previously, the condensing enzyme is the rate limiting activity of the fatty acid elongation pathway (Millar and Kunst, 1997). In addition, it has been shown that FAE1 is the only condensing enzyme present in seeds of *Arabidopsis* (James *et. al.*, 1995). It is not surprising then, that when an additional condensing enzyme is supplied, a greater conversion of 18:1 fatty acids to VLCFAs occurs.

Measurements of the proportion of VLCFAs with respect to the total fatty acids revealed that this proportion increased from 16% in the wild type to above 20% in all trasngenic lines tested. This is in accordance to what was observed when introducing additional copies of the *FAE1* gene into *Arabidopsis* under the control of the pNapin seed specific promoter. In some of the pNapin-*FAE1* transgenic, the proportion of VLCFAs increased up to 40% of total fatty acid content of the seed.

In addition to the higher VLCFA accumulation, higher levels of 18:2 (29%) and 18:3 (21%) were also observed in the 35S-*KCS2* transgenic seeds, in comparison to wild type (21 and 13% respectively). This result is puzzling, since the introduction of double bonds to the acyl chains is achieved by the action of different enzymes, fatty acid desaturases (Heinz, 1993).

KCS2 over-expression does not affect the total wax load in stems of transgenic plants

Transgenic lines showing over-expression of *KCS2* were also analyzed for their total wax accumulation on stems. We reasoned that, if *KCS2* participated in the elongation of VLCFAs, constitutive expression of the gene could affect the levels of precursors for wax biosynthesis and increase the total wax load (Millar and Kunst, 1999). *KCS2* transcript in stems increased from undetectable levels in the wild type to high levels of expression in some of the transgenic lines (Figure 16-A). However, higher levels of *KCS2* expression did not translate into an increased accumulation of wax in transgenic plants (Figure 16-B). It is

possible that the levels of *KCS2* expression were not high enough to affect wax deposition, because 35S promoter does not have a strong activity in the epidermis. It seems that for some tissues, endogenous tissue-specific promoters work better. For example, by using *CUT1* gene under the control of 35S promoter, it has not been possible to obtain higher wax loads on stems of *Arabidopsis* plants. In contrast, when additional copies of *CUT1* under the control of its native promoter were introduced, total wax accumulation was significantly higher in some of the transgenic lines (Tanya Hooker, personal communication). This has also been observed for lignin biosynthesis. In an attempt to increase syringyl units in *Arabidopsis* stems, the enzyme F5H, specific for the synthesis of syringyl lignin monomers, was expressed constitutively under the control of 35S promoter. Even though the levels of lignin obtained in the transformants were greater than in the wild type, they were still significantly lower (three fold in some lines) than the levels obtained using C4H, a lignin specific promoter (Meyer *et. al.*, 1998). Considering this, it may be important to try to express *KCS2* under the control of the epidermis specific *CUT1* promoter.

Conclusion

The present study has shown that *KCS2* is a transcriptionally active gene, expressed in a different fashion than *FAE1*. Their tandem position on the chromosome and their high degree of similarity suggests that both genes evolved from a common ancestor and that their promoters diverged to play different roles in *Arabidopsis* plants. Expression studies indicate that *KCS2* is preferentially expressed in anthers, which suggests the participation of the gene in the elongation of VLCFAs for the outer layers of the pollen grain. The generation of a *kcs2* mutant is required to confirm these predictions. Finally, it has been demonstrated that the KCS2 condensing enzyme is capable of elongating fatty acids from C18 to C22 in

length when ectopically expressed in seeds. However, its exact specificity requires further study.

Future experiments

The fact that co-suppression of *KCS2* did not result in a visible phenotype, makes it difficult to predict a possible function for the condensing enzyme. If the lack of a phenotype was due to an inefficient co-suppression technique, several other approaches could be taken in order to effectively co-suppress the *KCS2* gene. The utilization of a double 35S promoter has resulted in some cases in more successful co-suppression events (Vaucheret *et. al.*, 1995). Another possibility is the use of a novel technique that combines a simultaneous expression of a sense and anti-sense copy of the gene. The presence of both the sense and the anti-sense copies of the gene in the same construct, results in the formation of an RNA duplex that apparently triggers the degradation of the endogenous transcript (Waterhouse *et. al.*, 1998).

Another possibility for the lack of a phenotype when co-suppressing *KCS2* is that *KCS2* function is redundant and that it could be performed by another condensing enzyme. Support for this possibility stems form the fact that there are at least 15 cDNAs with high sequence similarity to the known condensing enzymes in *Arabidopsis*. So far, the number of condensing enzymes involved in a particular elongation activity and the degree of overlap is not known. It appears, however, that there is some overlap in the substrate specificity of different condensing enzymes. For example, *CUT1* is thought to elongate fatty acids beyond 24 carbon atoms in vegetative tissues. *KCS1* condensing enzyme seems to have a preference for C18 to C24 acyl groups, and its activity seems to be related to both wax and suberin synthesis. Similarly, when *KCS2* was expressed in yeast, it elongated VLCFAs from

C18 to C26 in length. Therefore it could be that *KCS2* specificity overlaps both *CUT1* and *KCS1*. If this is the case and the overlap occurs in the same tissue, the loss-of function of *KCS2* will not result in a visible phenotype. Thus, another approach might be necessary. For example, an attempt to isolate a *kcs2* mutant from a population of T-DNA tagged mutants using a PCR approach could be successful.

Based on our data obtained so far, it seems that *KCS2* is expressed in the anthers and possibly in very young leaves. However, more accurate studies of its expression pattern in the young leaves to confirm the data obtained in promoter-GUS fusion experiments and its expression within the anther are needed. *In situ* hybridization is a very useful technique to determine the expression pattern at a cellular level. This technique could be used in both anthers and emerging leaves to determine the cellular localization of the *KCS2* transcript and also to clarify the discrepancy between the RNA blots and the GUS assays concerning the expression of *KCS2* in young leaves. In addition to that, it would be very important to perform expression studies under different environmental conditions or at different developmental stages, considering the possibility that stress or developmental signals could have a role in regulating *KCS2* expression.

The expression of KCS2 in yeast, showed that the condensing enzyme was capable of elongating VLCFAs from C18 to C26, showing an accumulation of saturated VLCFAs. However, since it has been difficult to reproduce these results, it is not clear yet if they should be considered real. On the other hand, it was shown that in *Arabidopsis* seeds, *KCS2* was capable of elongating fatty acids between C18 and C22 in length, accumulating a higher amount of mono-unsaturated VLCFAs. In order to determine the exact specificity of the KCS2 condensing enzyme, it would be useful to perform *in vitro* enzymatic assays using yeast and seed microsomes and feeding them with radioactively labeled C18-CoA. Both C18:0-CoA and C18:1-CoA could be used to determine the preference of the KCS2 condensing enzyme for different fatty acids as substrates for elongation.

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