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UNIVERSITY OF ZAGREB
FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

GRADUATE THESIS

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Antea Talajić

1032/MB

Agrobacterium-mediated transformation of flax (*Linum usitatissimum* L. cv. Sara) with the *phaC1* gene

Experimental work for this Graduate thesis was done at the Department of Biotechnology and Food Microbiology, Faculty of Biotechnology and Food Science – University of Environmental and Life Sciences, Wrocław. The thesis was made under the guidance of assistant professor Anamarija Štafa, PhD., and with the help of associate professor Magdalena Wróbel-Kwiatkowska, PhD.

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Besides, I would like to thank assistant professor Anamarija Štafa for being such a supportive co-supervisor at my home University, for her helpful advices and invested time.

I na kraju, veliko hvala mojoj obitelji, prijateljima i Vedranu, što su mi pružali potporu i razumijevanje tijekom cijelog studija i bez kojih ovo ne bi bilo moguće.

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Transformacija vrste *Linum usitatissimum* L. cv. Sara genom *phaC1* pomoću bakterije *Agrobacterium tumefaciens*

Antea Talajić, 1032/MB

Sažetak: Polihidroksialkanoati srednje duljine lanaca (*mcl*-PHA) pripadaju skupini prirodnih polimernih materijala koje sintetiziraju mikroorganizmi, a privlače sve veću pozornost zbog svoje biorazgradivosti. Pogodni su za toplinsku obradu što ih čini dobrom zamjenom za sintetsku plastiku te se vjeruje kako bi smanjili potrebu za njezinom proizvodnjom i upotrebom, a samim time i negativan utjecaj na okoliš. Osnovni problem su visoki troškovi mikrobne proizvodnje ovih polimera, koji su često veći nego prilikom kemijske proizvodnje plastičnih materijala. Zbog toga se sve više istražuje upotreba genetički modificiranih biljaka s ciljem ekonomski prihvatiljive proizvodnje PHA polimera u velikim količinama. U ovom je radu lan (*Linum usitatissimum* L. cv. Sara) transformiran metodama genetičkog inženjerstva *phaC1* genom koji kodira za PHA sintazu. Glavni cilj sinteze PHA polimera u peroksisomima stanica lana je poboljšanje mehaničkih svojstava lanenih vlakana te povećanje učinkovitosti njihove primjene. Dobivenim rezultatima utvrđeno je kako je lan pogodan za transformaciju pomoću *Agrobacterium tumefaciens* konstruktom koji sadrži *phaC1* gen. Ekspresija gena na razini transkripcije potvrđena je u svim genetički modificiranim biljkama.

Ključne riječ: *mcl*-PHA, prirodni polimeri, *phaC1* gen, lan, transformacija pomoću *A. tumefaciens*

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Agrobacterium-mediated transformation of flax (*Linum usitatissimum* L. cv. Sara) with the *phaC1* gene

Antea Talajić, 1032/MB

Abstract: Medium-chain-length polyhydroxyalkanoates (*mcl*-PHAs) belong to the group of polymeric materials naturally synthesized by many species of microorganisms. These biopolymers attract increasing attention due to their biodegradability and great thermo-mechanical properties, making them an excellent alternative for petroleum-based plastics and subsequently reducing the consumption and demand for plastic-goods. Therefore, such application of *mcl*-PHAs would, without a doubt, make a great positive impact on the environment. However, higher production costs of microbial production of these biopolymers compared to chemical production of conventional plastics, pose a great challenge. Thus, genetically modified plants are emerging as a great platform for large-scale PHA synthesis. In this thesis, flax plants (*Linum usitatissimum* L. cv. Sara) were genetically transformed with the *phaC1* gene, fused to the isocitrate lyase peroxisomal targeting sequence (ICL). The main objective of such genetic modification is improving mechanical properties of flax fibers for their further applications. The results indicate that flax is suitable for transformation via *A. tumefaciens* with the *phaC1* gene construct. It was determined that transgene was successfully integrated and expressed in transformed plant lines.

Keywords: *mcl*-PHA, biopolymers, *phaC1* gene, flax, *Agrobacterium*-mediated transformation

Thesis contains: 46 pages, 16 figures, 6 tables, 58 references, 1 appendix
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1. INTRODUCTION

Synthetic plastics have become an integral part of our daily lives, due to their various chemical and mechanical properties that allow them to replace traditional materials like wood and metals. They are widely used for several applications, such as computer equipment, packaging materials, components in automobiles, home appliances, etc. Plastics have been widely and necessarily used even for medical applications (Zinn et al., 2001). The two main problems of using synthetic plastics are durability of disposal materials and unsustainable production starting from petrochemicals. These non-biodegradable materials accumulate in the environment as pollutants and interfere with the normal functioning of ecosystems, negatively affecting numbers of species, especially in oceans. The need for plastics is rapidly increasing every year with the continuously growing world population. Therefore, it is necessary to switch from petroleum-based plastics to bio-alternatives produced from renewable resources, which can be subjected to biodegradation and composting, in order to establish a more sustainable society and to solve global environmental and waste management problems (Iwata, 2015; Koller, 2017).

Polyhydroxyalkanoates (PHAs), macromolecule-polyesters naturally produced by many species of microorganisms, are being considered as a replacement for conventional plastics (Suriyamongkol et al., 2007). Unlike petroleum-based plastics, products made of these materials are natural, non-toxic, biocompatible, and completely degradable by a variety of microorganisms in nature (Dobrogojski et al., 2018). Because of the ability to use different renewable carbon sources and precursors for microbial biosynthesis of PHA polymers, it is possible to produce materials with a wide range of physical properties (Kunasundari and Sudesh, 2011). Despite the numerous advantages of using biodegradable polymers, commercialization of PHAs has been outgoing with limited success, primarily because of high production costs in bioreactors. Considering the low price of most commodity plastics derived from petroleum, such as polyethylene and polypropylene, PHAs cannot currently compete with bulk production of petrochemical plastics (Chee et al., 2010).

The ability to produce PHAs in photosynthetic organisms, such as transgenic plants, offers the opportunity to produce these bioproducts from CO_2, thus eliminating the substrate input cost. Plants use photosynthetically fixed CO_2 and water to generate bioplastic, which is degraded back to CO_2 and water after its disposal (Lu et al, 2009; Suriyamongkol et al., 2007).

Production of PHAs in plants is a promising alternative for large-scale and low-cost production of this polymer. The highest level of PHAs accumulation was achieved in transgenic *Arabidopsis thaliana* (up to 40% of the dry weight of the leaf) , and among crop plants in *Cannabis sativa* (up to 20% of the dry weight of the seed) (Dobrogojski et al., 2018).

Flax (*Linum usitatissimum* L.) is an annual plant species widely cultivated for either fiber or oil, depending on the cultivar. In case of fibrous plants, the main product is fiber derived from phloem tissue (Wróbel-Kwiatkowska et al., 2018). Recently, due to research development, a renewed interest in flax fibers production has been observed. Flax fibers are becoming increasingly widely used in automobile and construction industry as a recyclable composite material. It was also suggested that fibers enriched with PHA by genetic engineering might serve as components of biodegradable packing materials or as scaffolds for tissue engineering (Kulma et al., 2015). Plant cells of a fibrous flax cultivar (*Linum usitatissimum* L. cv. Sara) were genetically modified in order to improve properties of its fibers. The impact of introduced modifications on flax fibers, as well as possibilities for industrial applications should be tested in future experiments. For species that are suitable for transformation using *Agrobacterium*, including flax (because of its totipotency, small genome, and availability of haploids), T-DNA-mediated gene transfer is preferred approach due to its simplicity and efficiency (Gelvin and Liu, 1994).

The main aim of this thesis was to transform flax plants with the *phaC1* gene encoding PHA synthase from *Pseudomonas aeruginosa* via *Agrobacterium*-mediated technique and to generate flax plants overexpressing *phaC1* gene. The plasmid used for genetic transformation contains the *phaC1* gene under control of the cauliflower mosaic virus (CaMV) 35S promoter, fused to the isocitrate lyase peroxisomal targeting sequence from *Brassica napus*. Contrary to the enzyme derived from *Cupriavidus necator*, which synthesizes short chain length (*scl*) PHAs, the enzyme from *P. aeruginosa* can produce medium chain length (*mcl*) PHAs with great elastomeric properties. Previous studies showed that peroxisomes have a high potential for PHA biosynthesis in transgenic plants, primarily due to the high reductive strength of the nicotinamide adenine dinucleotide (NADH) involved in this process and presence of β-oxidation intermediates, the substrates for *mcl*-PHA production (Dobrogojski et al., 2018; Wróbel-Kwiatkowska et al., 2018).

2. LITERATURE REVIEW

2.1. BIOPOLYMERS

Natural polymers are macromolecular compounds, molecular weights of several thousand to several hundred thousand, found in nature as parts of plant or animal tissues. They are also called biopolymers. Such materials originate from renewable resources and are very diverse, complex compounds. (Andričić, 2009). Biopolymers present a possible alternative to common plastics because most can be degraded by various microorganisms (bacteria, fungi, algae) or composted.

Biopolymers can be divided into three categories (Fig. 1), depending on their source:

1. Polymers extracted directly from different types of biomass, which include proteins (high functionality and excellent properties), lipids and polysaccharides (e.g. starch and cellulose-based biopolymers);
2. Polymers produced by natural or genetically modified microorganisms, e.g. polyhydroxyalkanoates (PHAs), biogenic polyesters that accumulate in microbial cells as storage materials, commonly during stress conditions;
3. Polymers obtained from bio-derived monomers, such as polylactic acid (PLA) or bio-polyethylene (PE) (Burgincourt et al., 2014; Peelman et al., 2015).

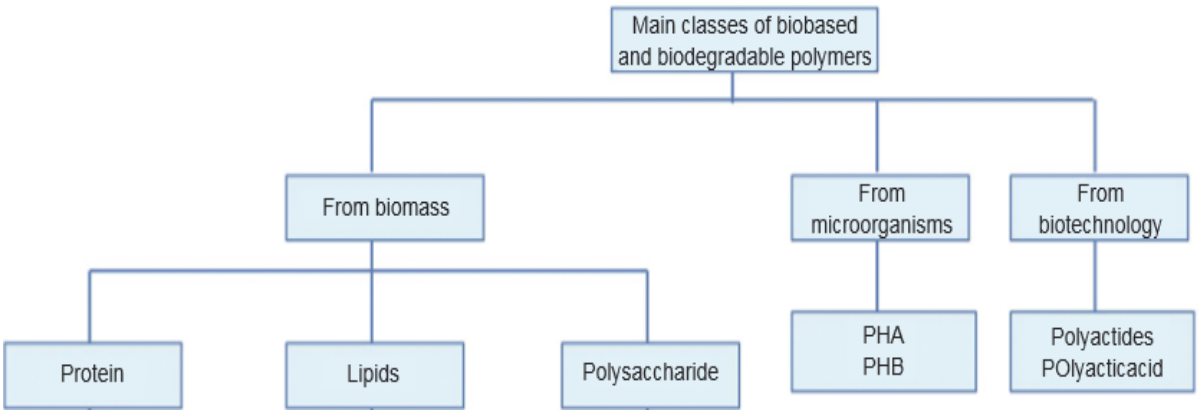


Figure 1. Different classes of polymers that are bio-based and biodegradable (therefore not including biodegradable plastics from petrochemical resources and non-biodegradable bio-sourced plastics) (Bugincourt et al., 2014).

Biodegradable polymers provide dual advantages over petroleum-based plastics, the conservation of fossil resources and reduction in CO_2 emissions (greenhouse gas), which contribute to a sustainable economy. Since biobased materials are produced from biomass, they provide a unique potential for CO_2-neutrality. In theory, the amount of CO_2 released in the environment from disposal of bioplastics is no greater than the amount fixed by the plant during its life cycle. This is in great contrast to conventional plastics derived from fossil raw materials that have a high carbon footprint. However, most currently used biobased plastics are not yet CO_2-neutral, mainly because the energy used during their production chain is petroleum-based (Bugincourt et al., 2014; Peelman et al., 2015).

Nowadays, a variety of manufacturers are introducing the term "green plastics" for polymeric materials they commercialize on the market. These products must be classified in one of the following categories: biodegradable, compostable, biocompatible, or biobased, to be considered "green plastics". Such attributes only apply to plastics if they meet strict requirements defined by standardized norms and certificates (CEN/TR 15932, 2010). Material (plastic) can be defined as 'biodegradable' if 90% of its carbon is metabolized within 180 days. Likewise, the material is 'compostable' if no more than 10% of material remains in a 2 mm sieve after 180 days of composting. The classification 'biocompatible' refers to materials exerting no negative impact on living organisms or environment involved, when using standardized ecotoxicity assessment methods. Also, a polymer is considered 'biobased' if the production of its building blocks, i.e. its monomeric units, is based on renewable resources; afterward, polymerization of the monomers may occur chemically (e.g. polymerization of biosynthesized lactic acid to PLA) or biologically (e.g. *in vivo* polymerization of hydroxyacyl-CoAs by PHA synthases towards PHAs) (Koller et al., 2012).

Among biopolymers, PHAs attract increasing attention as biobased and biodegradable "green plastics" due to the integration of their life cycle into nature's closed carbon balance (Muhr et al., 2013). Moreover, PHAs offer good thermomechanical and barrier properties, as well as great structural variety. The development of copolymer production or blending PHAs with other monomers has extended their applications. Thus, PHAs and their derivatives find use in many applications, from food packaging to biomedical materials (Bugincourt et al., 2014; Du et al., 2012).

2.2. PHAs - STRUCTURE AND PROPERTIES OF POLYHYDROXYALKANOATES

Polyhydroxyalkanoates (PHAs) are biologically produced polyesters which can consist of a diverse set of repeating unit structures (Lu et al., 2009). More than 80 hydroxyalkanoates (HAs) have been detected as constituents of PHAs, which allows these thermoplastic materials to have various mechanical properties depending on the incorporated monomer units. (Lee, 1996). Among all known classes of biobased polymers with plastic-like properties, PHAs are the only ones that are entirely produced and degraded by living cells.

PHAs mainly consist of 3-hydroxyalkanoates (3HAs) as monomeric building blocks. 3HAs commonly are enantiomerically pure, R-configurated chiral compounds. The general chemical structure is provided in Fig. 2 (Koller et al., 2012).

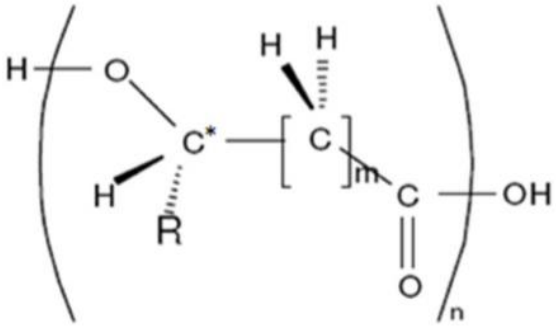


Figure 2. General chemical structure of polyhydroxyalkanoates (PHAs). The chiral center is indicated by an asterisk (*) (Koller et al., 2012).

For the producing microbial cells, PHAs act as intracellular carbon and energy storage compounds. Accumulation of PHAs occurs usually if a nutrient, for example nitrogen or phosphorus, is limited while carbon is in excess (Koller et al., 2012; Kunasundari and Sudesh, 2011). When the supply of limiting nutrient is restored, PHAs can be degraded by intracellular depolymerase and subsequently metabolized as carbon and energy source (Winnacker, 2019). PHAs fulfill important biological functions as they protect cells against environmental stress conditions like osmotic shock, UV irradiation, desiccation, and thermal or oxidative stress. Further, they are involved in special metabolic processes in different microbial species, for instance, sporulation, cyst formation, germination, control of exopolysaccharide excretion and, considering diazotrophic species, the energy flow during nitrogen fixation (Koller et al., 2012).

PHAs accumulate in the cytoplasm of bacterial cells as discrete granules, the number per cell and size of which can vary among different species. The average size is approximately 0.2 - 0.5 μm. Fig. 3 shows the morphology of PHA granules when observed using various microscopy techniques. These granules appear as highly refractile and bright inclusions under a phase-contrast microscopic observation (Lee, 1996). When thin sections of PHA-containing bacteria are observed by transmission electron microscopy, the PHA inclusions appear as electron-dense bodies (Khanna and Srivastava, 2005). Atomic force microscopy analysis shows the presence of a protein monolayer on the surface of PHA granules (Kunasundari and Sudesh, 2011).

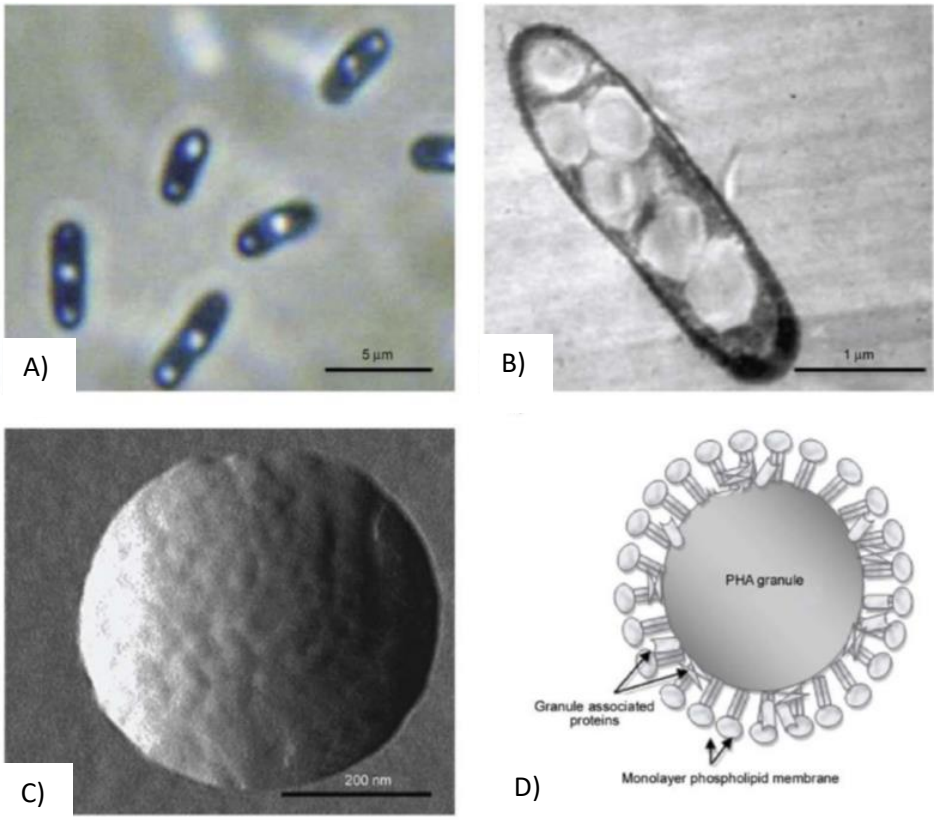


Figure 3. Morphology of PHA granules in the bacterial cells. PHA granules observed under (A) Phase contrast and (B) Transmission Electron Microscope. C) Atomic force microscope deflection image showing the presence of globular particles on the granule surface. D) A model representing the native PHA granule with a protein monolayer on the surface (Not drawn according to actual scale) (Kunasundari and Sudesh, 2011).

The first PHA, poly(3-hydroxybutyrate) (PHB), was discovered in bacterium *Bacillus megaterium* by the French scientist Lemoigne in 1926. He reported that this bacterium can accumulate intracellularly a homopolymer consisted of 3-hydroxybutyric acids that were linked through ester bonds between the 3-hydroxyl group of the first and the carboxylic group of the

next monomer. Since then, PHB and related PHAs were shown to occur in many different genera of bacteria, encompassing both Gram-positive and Gram-negative species, in aerobic and anaerobic habitats (Poirier et al., 1995; Zinn et al., 2001). However, the occurrence of PHA is not limited to the intracellular collection in granules and bacteria. Further research revealed that PHB is found not only in eubacteria but also in eukaryotic cells (yeast, peanut, spinach, sheep, cat muscles and many others). Thus, it is not surprising that PHB has excellent biocompatibility in animals (Zinn et al., 2001).

In general, PHAs may be classified by the number of carbon atoms in their monomer units into three main classes:

a. short-chain-length (*scl*) PHAs, which consist of monomers with chain lengths of 3–5 carbon units;
b. medium chain-length (*mcl*) PHAs, which consist of monomers with chain lengths between 6 and 14 carbon units;
c. long-chain-length (*lcl*) PHAs, which are composed of monomers with carbon chain lengths greater than 14 units (Lu et al., 2009).

The length of the side chain and its functional group considerably influence the properties of bioplastics, e.g., melting point, glass transition temperature and crystallinity (stiffness/flexibility) (Zinn et al., 2001). Polymers composed wholly of *scl*-monomers are thermoplastics with a high degree of crystallinity, while polymers composed of *mcl*-subunits generally have elastomeric properties, for instance, a low degree of crystallinity and low melting temperature. *Mcl*-PHAs have a high elongation at break, which makes them much less rigid than *scl*-PHAs, and therefore easier to shape (Kim 2007; Lu et al., 2009).

2.2.1. PHA biosynthesis

Table 1. lists the genera of bacteria that have PHA biosynthesis. Besides bacteria, genetically modified yeasts and plants are also being developed for PHA production (Novak et al., 2015).

Table 1. Genera of bacteria shown to produce PHAs (Lu et al., 2009).

Gram-positive bacteria		
Actinomycetes	*Bacillus*	*Caryophanon*
Corynebacterium	*Clostridium*	*Micrococcus*
Microcystis	*Nocardia*	*Rhodococcus*
Staphylococcus	*Streptomyces*	
Gram-negative bacteria		
Acinetobacter	*Alcaligenes*	*Aphanocapsa*
Aphanothece	*Aquaspirillum*	*Asticcaulus*
Azomonas	*Azospirillum*	*Azotobacter*
Beggiatoa	*Beijerinckia*	*Beneckea*
Caulobacter	*Chloroflexus*	*Chlorogloea*
Chromatium	*Chromobacterium*	*Derxia*
Ectothiorhodospira	*Escherichia*	*Ferrobacillus*
Gloeothece	*Haemophilus*	*Halobacterium*
Haloferax	*Hyphomicrobium*	*Lamprocystis*
Lampropaedia	*Leptothrix*	*Methanomonas*
Methylobacterium	*Methylocystis*	*Methylomicrobium*
Methylomonas	*Methylosinus*	*Methylovibrio*
Microcoleus	*Moraxella*	*Mycoplana*
Nitrobacter	*Nitrococcus*	*Oceanospirillum*
Paracoccus	*Photobacterium*	*Protomonas*
Pseudomonas	*Rhizobium*	*Rhodobacter*
Rhodopseudomonas	*Rhodospirillum*	*Sphaerotilus*
Spirillum	*Spirulina*	*Stella*
Syntrophomonas	*Tetrahymena*	*Thiobacillus*
Thiocapsa	*Thiocystis*	*Thiodicotyon*
Thiopedia	*Thiosphaera*	*Vibrio*
Xanthobacter	*Zoogloea*	

PHA polymers are produced by a series of enzymatic reactions in both native and recombinant organisms. The properties of PHA polymers are dependent on a starting carbon feedstock, metabolic pathways for the conversion of those feedstocks into PHA precursors, and

specific activities and substrate specificities of enzymes involved in the process (Lu et al., 2009).

Cupriavidus necator (formerly known as *Ralstonia eutropha*) is the most extensively studied microbial *scl*-PHA producing microorganism, due to its ability to accumulate a large amount of PHB from simple carbon sources (Khanna and Srivastava, 2005; Kunasundari and Sudesh, 2011). In this bacterium, PHB is synthesized in a three-step pathway. The biosynthesis of PHB is initiated by the condensation of two acetyl-CoA molecules by β-ketothiolase (PhbA) to form acetoacetyl-CoA. Subsequently, NADPH-dependent acetoacetyl-CoA reductase (PhbB) catalyzes the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. In the final step, PHB is synthesized by polymerization of (R)-3-hydroxybutyryl-CoA molecules by PHB synthase (PhbC). PHB biosynthetic pathway is schematically depicted in Fig. 4 and is common in a wide range of bacteria (Shrivastav et al., 2013).

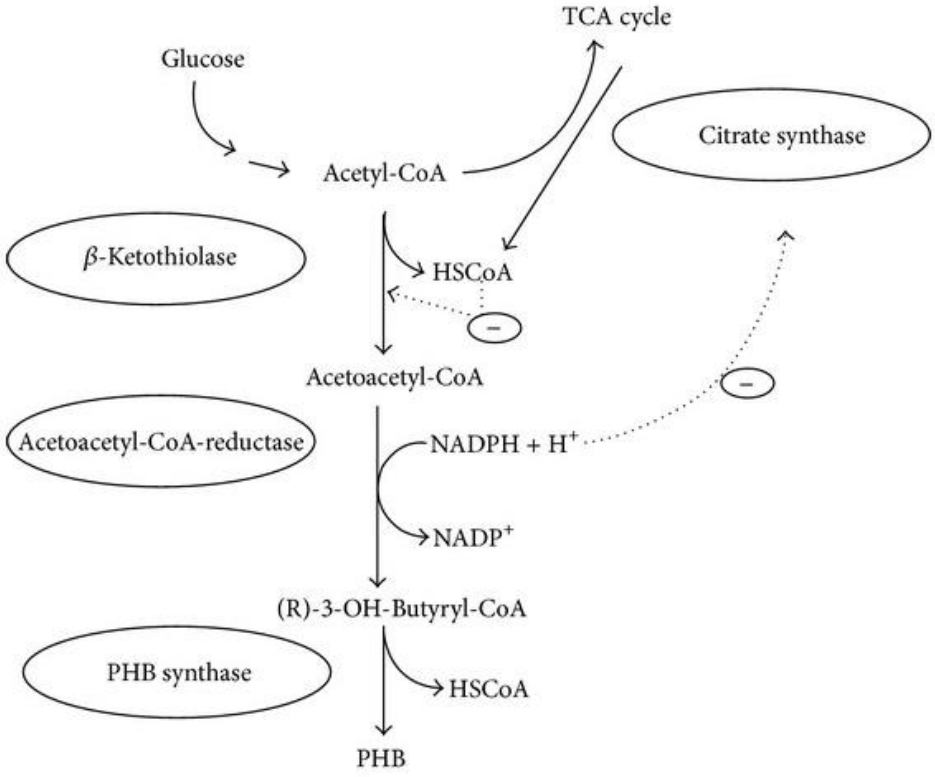


Figure 4. PHB synthesis pathway of *C. necator* and regulation of enzymes activity. The first enzyme of PHB synthesis, β-ketothiolase, is negatively regulated by the coenzyme A (HSCoA), which is also a product when acetyl-CoA enters the TCA cycle under nonlimited conditions. The high concentration of NADPH and NADH inhibits the citrate synthase of the TCA cycle, which ensures the availability of acetyl-CoA for β-ketothiolase. (Shrivastav et al., 2013).

Mcl-PHA biosynthesis in bacteria is closely linked to three different metabolic routes that generate *mcl*-PHA precursor molecules (Fig. 5):

a. *de novo* fatty acid biosynthesis pathway, which produces (R)-3-hydroxyacyl-CoA precursors from non-related carbon sources such as glucose and gluconate;
b. β-oxidation pathway, which produces *mcl*-PHA precursor molecules from fatty acids;
c. chain elongation pathway, in which acyl-CoA is extended with acetyl-CoA.

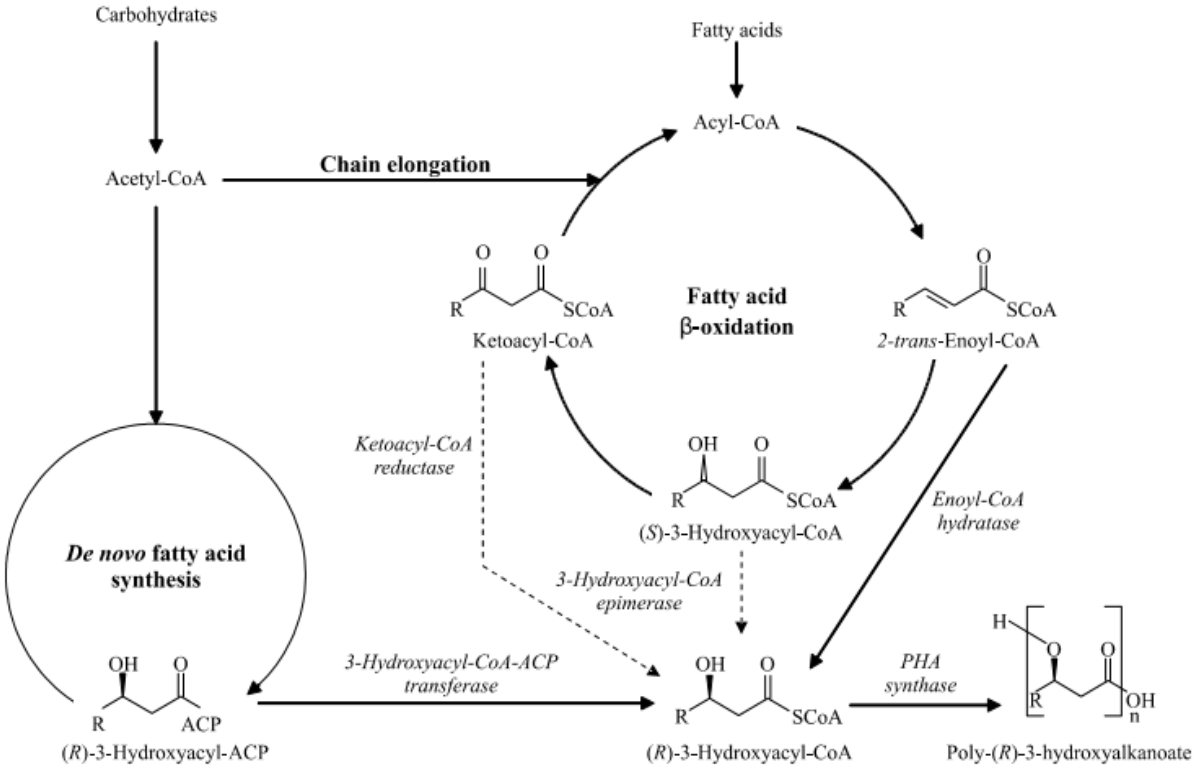


Figure 5. Metabolic pathways for *mcl*-PHA biosynthesis (Kim et al., 2007).

These metabolic pathways produce various intermediate precursors of *mcl*-PHA, such as (R)-3-hydroxyacyl-acyl carrier protein (ACP), 2-trans-enoyl- CoA, (S)-3-hydroxyacyl-CoA, and 3-ketoacyl-CoA. In the final step of *mcl*-PHA biosynthesis, *mcl*-PHA synthases (PhaC) catalyze the conversion of (R)-3-hydroxyacyl-CoA molecules into *mcl*-PHAs with the concomitant release of CoA. To date, two *mcl*-PHA synthase genes, *phaC1* and *phaC2*, have been identified and characterized in *Pseudomonas spp*. Studies have demonstrated that PhaC1 and PhaC2 from the same *Pseudomonas* strain have obvious differences in substrate specificity, although they both have very similar properties (Kim et al., 2007).

2.2.2. Biodegradation of PHA

The most attractive feature of PHAs is their biodegradability. Both aerobic and anaerobic microorganisms containing PHA depolymerases are widespread in our environment, including bacteria and fungi from soil, activated and anaerobic sludge, and sea or lake water (Lee, 1996; Winnacker, 2019). These microorganisms secrete extracellular PHA depolymerases to break down the polymer into its molecular building blocks, called hydroxy acids, which are then utilized by microorganisms as a carbon source for their growth (Reddy et al., 2003). The end products under aerobic conditions are carbon dioxide and water, whereas methane is produced under anaerobic conditions, and corresponding intermediates are not harmful (Lee, 1996).

Degradation of PHAs is determined by two main factors, environmental conditions and properties of plastic material to be degraded (Fig. 6). Humidity (moisture level), presence or absence of oxygen and availability of nutrients have an enormous influence on the growth of microorganisms, while temperature and pH affect the activity of PHA degrading enzymes (Ong et al., 2017). PHAs fit perfectly well in the ecosystem due to their complete biodegradation. This process has been reported to be the fastest in anaerobic sewage sludge and the slowest in seawater. Lee (1996) has reported the time taken for complete degradation of P(3HB-*co*-3HV) in anaerobic sewage, soil, and the seawater to be 6, 75, and 350 weeks, respectively (Nehra et al., 2017).

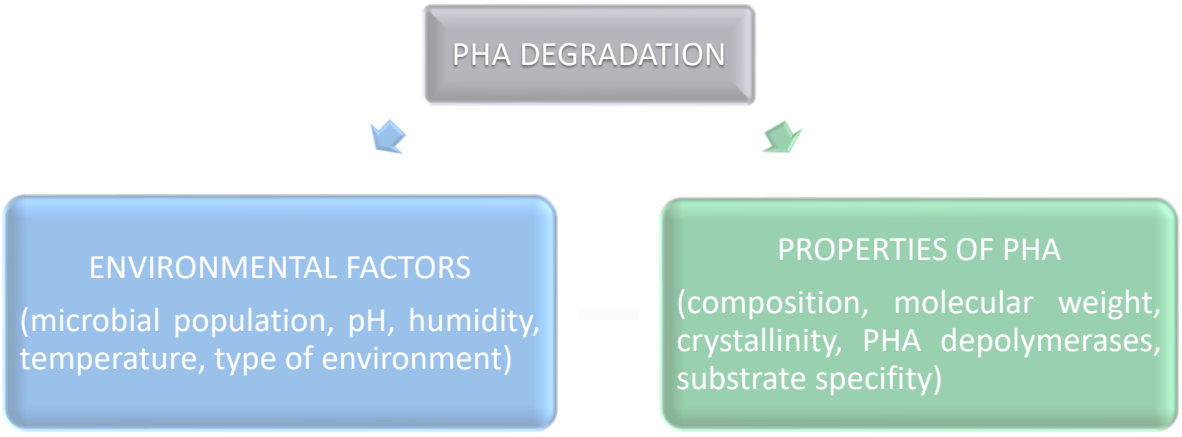


Figure 6. Factors affecting degradation of PHA (Ong et al., 2017).

2.2.3. Applications of PHAs

Comparable to all bioplastics, PHAs can be used for certain applications when their physical and chemical properties offer benefits. It is particularly significant that PHAs are biodegradable, which makes them suitable for food producing industry, especially in such areas where a high oxygen barrier is required (for packaging of easily perishable foods). Further, they are important as flexible films used as compostable waste bags for organic waste, where they contribute to reduce landfill and make composting process more effective (Koller et al., 2012; Winnacker, 2019).

Depolymerization of PHA to the monomers generally results in a rich source of chiral synthons. They may serve as starting materials for production of fine chemicals and marketable products such as:

- pheromones,
- aromatics,
- vitamins,
- antibiotics

or can even be used as pharmaceutically active compounds (Koller et al., 2012). The ester bond can be easily split either chemically with acid or base, enzymatically or trough *in vivo* degradation. Obtained monomers promote cell proliferation and prevent necrotic cell death. Some of these chiral acids also display biological activity against pathogenic bacteria or viruses (Koller et al., 2012; Zinn et al., 2001).

Degradable product of PHB, R-3-hydroxybutyric acid, is a normal constituent of human blood plasma. Therefore, PHAs are compatible with the blood and tissues of mammals and non-cytotoxic (Bugnicourt et al., 2014; Lee, 1996). Due to their biocompatibility, PHAs find many applications in the medical field. They can be used for tissue engineering, for *in vivo* application as implants, surgical pins, screws, meshes and sutures, or as carrier matrices for controlled drug release (Koller et al., 2012; Winnacker, 2019). Elastomeric *mcl*-PHAs are better suited for vascular applications and controlled drug delivery then *scl*-PHAs, which are too rigid and brittle. However, these medical applications have been limited by the low physical properties and hydrophobic character of *mcl*-PHAs. For this reason, chemical and physical modifications of functional groups (the addition of polar groups, block-copolymerization, or graft copolymerization) have been successfully applied (Kim et al., 2007; Winnacker, 2019).

2.3. PRODUCTION OF PHA BY GENETICALLY ENGINEERED PLANTS

There is a growing interest in making use of high productivity and metabolic flexibility of plants to produce variety of organic materials, including proteins and various other polymers (Poirier et al., 1992). PHA production in plant systems is simply based on water, mineral salts, CO_2 and light, which makes it considerably less expensive than microbial production in a costly fermentation process. Besides, plants are not capable of degrading PHA polymers as bacteria do (Dobrogojski et al., 2018; Suriyamongkol et al., 2007). The possibility of PHA production in genetically modified plants could allow synthesis of biodegradable plastics in the million-tonne scale compared to fermentation, which produces material in the thousand-tonne scale. For this purpose, PHAs would have to be synthesized in plants to a level of 20-40% of dry weight and thus competitive to petroleum-based plastics. In addition to high production yields, commercialization of plant derived polymers requires transgenic crop plants with normal plant phenotypes and transgenes that are stable over several generations (Reddy et al., 2003).

The biosynthesis of PHA in transgenic plant cells is possible considering general availability of acetyl-CoA, the primary substrate in PHA biosynthesis. Acetyl-CoA is a major metabolite of both catabolic and anabolic processes, including cycle of tricarboxylic acids and biosynthesis and degradation of fatty acids. Unlike the bacterial cell, plant cells have different subcellular compartments in which PHA synthesis can be metabolically localized since they are particularly rich in acetyl-CoA. Those compartments are cytoplasm, plastids, peroxisomes and mitochondria. They were all taken under consideration to synthesize and accumulate various PHAs and mitochondria were found to be the least effective structure for this purpose, mainly due to the rapid use of acetyl-CoA in cellular respiration (Dobrogojski et al., 2018; Suriyamongkol et al., 2007).

Because of the relative simplicity of its biosynthesis, PHB has received most attention as a target molecule for production in plants. The entire pathway of PHB biosynthesis in the cytoplasm of plant cell is based on the well-known bacterial process (Dobrogojski et al., 2018; Somleva et al., 2013). The first genetically modified plant producing PHB was *Arabidopsis thaliana.* For this purpose, *A. thaliana* was engineered to express genes encoding reductase (*phbB*) and PHA synthase (*phbC*) from *Cupriavidus necator*. The *phb* genes were under transcriptional regulation of the 35S CaMV promoter. A β-ketothiolase (*phbA*) was not included as thiolase activity is known to be present in the cytoplasm of plant cells (Poirier et al., 1992). Low levels of PHB polymer were obtained, and transmission electron microscopy (TEM)

analysis demonstrated that granules were located not only in the cytoplasm but also in the nucleus and vacuole (Somleva et al., 2013). Although of no agricultural importance, *A. thaliana* was chosen because of its extensive use as a model system for genetic and molecular studies of plants (Poirier et al., 1995). Since the first report describing engineering of *A. thaliana* to produce PHB (Poirier et al., 1992), numerous articles have been published describing PHA synthesis in different plant species, tissues and organelles (Somleva et al., 2013).

2.3.1. Biotechnology of fibrous flax

Flax (*Linum usitatissimum* L.) is an annual plant cultivated in temperate climates and commonly used for its fibers or linseed oil production (Yunus et al., 2008). Fibrous flax grows up to 140 cm tall, with grayish leaves and flowers with usually five petals. The glossy brown or yellow seeds, color depending on plant variety, are 4-7 mm long and sticky when wet. Plant has one short, branched taproot, which may extend to a depth of 1 m, with lateral roots (Kulma et al. 2015). The nuclear genome of flax is small (373 bp based on the flow cytometry, Wang et al., 2012), with a chromosome number of 2n=30 (Basiran et al., 1987).

There is a wide range of possible applications of flax, for example, long linen fibers are used in textile industry, while short fibers find application in paper production or as a part of isolation materials. Flax seeds are rich in antioxidants and have high nutritional value, which is significant in food, pharmaceutical and healthcare industries. A unique feature of flax is the possibility of whole plant exploitation with almost no waste products. For this reason, flax has important potential for biotechnological applications (Czemplik et al., 2011).

The use of flax fibers by textile industry is limited because they are of lower quality than those of cotton origin (Yunus et al., 2008). To improve at least thermoplastic properties of the fibers, transgenic plants synthesizing PHAs in their tissues have been developed. Under natural conditions, flax fiber consists of several polysaccharides such as cellulose, hemicellulose, pectin and phenolic lignin polymers. The slight accumulation of PHA in flax stem cells improves the elasticity and strength of flax fibers (Wróbel-Kwiatkowska et al., 2018). For this purpose, flax plants (cv. Nike) were transformed using construct containing *phaC1* gene derived from *P. aeruginosa* for *mcl*-PHA biosynthesis. Results showed that the introduced modification significantly improved mechanical properties of whole flax stems, which corresponded to changes in biochemical composition of the cell wall. Also, cellulose

crystallinity was increased in generated flax plants over-expressing *phaC1* gene when compared to wild-type plants (Wróbel-Kwiatkowska et al., 2018). Since *mcl*-PHAs are known as elastomers, manipulation of *phaC1* gene gave better results than previous researches concerning expression of genes necessary for PHB synthesis (*phbA*, *phbB* and *phbC* from *C. necator*) in transgenic flax plants (Wróbel et al. 2004; Wróbel-Kwiatkowska et al., 2009).

2.3.2. Plant transformation

Genetic transformation is an important technique for the study of plant functional genomics, i.e., gene discovery, new insights into gene function, and investigation of genetically controlled characteristics. It is also a method for introducing foreign genes into crop plants to create new genetically modified plants having desirable traits (Narusaka et al., 2012). The methods for introducing DNA into plant cells can be divided into two types:

1. direct, where physical and chemical processes are responsible for DNA introduction, and
2. indirect, where exogenous DNA is introduced by a biological vector.

Direct delivery of DNA is achieved using biolistic particle bombardment, electroporation, microinjection, silicone fibers, etc. In general, many of these techniques are highly effective, but can also result with insertion of unwanted DNA, multiple and fragment copies at high numbers and expression instability. Indirect *Agrobacterium* or virus-mediated techniques are based on the use of vectors to transfer DNA into target plant cells.

For successful plant transformation, the structure and copy number of transgenes along with stability must be established. By far the most successful approaches have been based on *Agrobacterium*-mediated transformation and particle DNA bombardment, due to high transformation frequencies. Those methods have been optimized to deliver genes of interest to the nuclear genomes of a range of plant species and are responsible for almost all existing transgenic plants. The process of *A. tumefaciens* mediated transformation is, at the technical level, simpler than direct gene transfer methods and has minimal equipment costs. Contrary to direct gene transfer, experiments showed that *Agrobacterium*-mediated technique usually produces plants with single insertions of low copy numbers (Alves et al., 1999; Puddephat, 2003).

2.3.2. Agrobacterium-mediated plant transformation

Agrobacterium tumefaciens is a Gram-negative soil phytopathogen that naturally infects plants and causes crown gall disease (the formation of tumors). In nature, wounded plant tissues secrete a wide range of chemical compounds that can function as chemotactic agents to attract *Agrobacterium* to wounded sites for infection (Hwang et al., 2017). The virulence is based on delivery of transferred DNA (T-DNA) segment, a part of large bacterial tumor-inducing plasmid (Ti plasmid), from bacterial cells into host plant cells.

A. tumefaciens Ti plasmid consists of five components:

a. the T-region, which codes for sequences that are transferred to the plant host;
b. the *vir* region, which directs the processing and transfer of the T-DNA;
c. the *rep* region, which is required for replication of the Ti plasmid;
d. the *tra* and *trb* loci, which direct the conjugal transfer of the Ti plasmid; and
e. genes that direct uptake and catabolism of opines.

T-region consists of T-DNA delimited by left (T_L-DNA) and right (T_R-DNA) border sequences, which are crucial for transfer of T-DNA into the host cell. Collectively, T_L-DNA and T_R-DNA encode 13 proteins. One group of transferred genes direct production of plant growth hormones that are responsible for proliferation of transformed plant cells (Zhu et al., 2000). Oncogenes in the T-DNA indirectly influence auxin and cytokinin biosynthesis and escalate plant sensitivity to hormone uptake. The upregulation of auxin and cytokinin levels can lead to uncontrolled cell division and tumorigenicity (Chandrasekaran et al., 2019; Lee and Gelvin, 2007). The second set of T-DNA genes direct production of opines, on which bacterium feeds (Zhu et al., 2000).

To date, *A. tumefaciens* has become the most common "tool" to deliver genes of interest into a plant, due to a more comprehensive knowledge of how this bacterium interacts with host plant cells. The identification of bacterial and plant genes involved in tumorigenesis made it possible to replace oncogenes in the T-DNA region with any gene of interest and thus transform plant using the *Agrobacterium*-mediated technique (Hwang et al., 2017).

However, Ti plasmids are very large and T-DNA regions do not generally contain unique restriction endonuclease sites that are not found elsewhere on Ti plasmid. Because of that scientists developed a binary system in which the T-DNA region and virulence (*vir*) genes

required for T-DNA processing and transfer are split into two replicons (T-DNA binary vector and *vir* helper, Fig.7). Binary vectors are small and easy to manipulate in both *E. coli* and *A. tumefaciens*. T-DNA regions of binary vectors generally contain multiple unique restriction endonuclease sites into which genes of interest can be cloned. Also, many disarmed A*grobacterium* strains containing *vir* helper plasmids have been developed, including LBA4404, GV3101 MP90, AGL0, EHA101 and its derivative strain EHA105, and NT1 (pKPSF2). This binary system permits easy manipulation of *A. tumefaciens* to create transgenic plants (Gelvin, 2003). Inside the plant nucleus, the transferred DNA (containing gene of interest) integrates into the plant genome for an inheritance to the next generation (i.e. stable transformation). Alternatively, the foreign DNA can transiently remain in the nucleus without integrating into the genome but still be transcribed to produce desirable gene products (i.e. transient transformation) (Hwang et al., 2017).

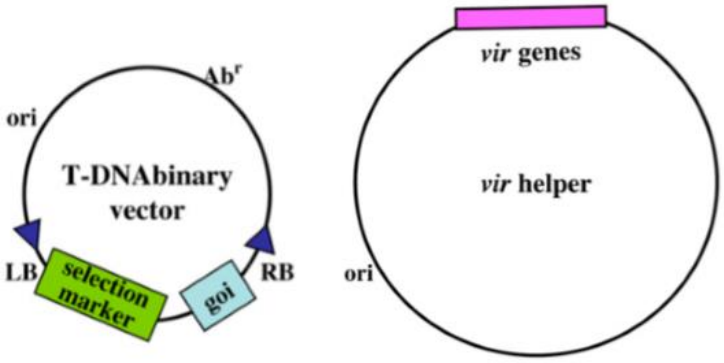


Figure 7. T-DNA binary vector system. Genes of interest are maintained within the T-DNA region of a binary vector. Vir proteins encoded by genes on a separate replicon (*vir* helper) mediate T-DNA processing from binary vector and T-DNA transfer from the bacterium to the host cell. The selection marker is used to indicate a successful plant transformation. ori, the origin of replication; Ab^r, an antibiotic-resistance gene used to select presence of the T-DNA binary vector in *E. coli* (during the initial stages of gene cassette construction) or *Agrobacterium* (Lee and Gelvin, 2007).

The *Agrobacterium*-mediated transformation method has several advantages over other transformation methods; it is easy to use, relatively inexpensive, and commonly results in low copy numbers and well-defined DNA insertions into the host cell chromosome (Hwang et al., 2017). Still, this method has some limitations, most importantly, the host restriction. Thus, gene transfer is very difficult for *Gramineae*, leguminous plants and tree species, in which it occurs at extremely low frequencies (Beyaz et al., 2017).

3. MATERIALS AND METHODS

3.1. MATERIALS

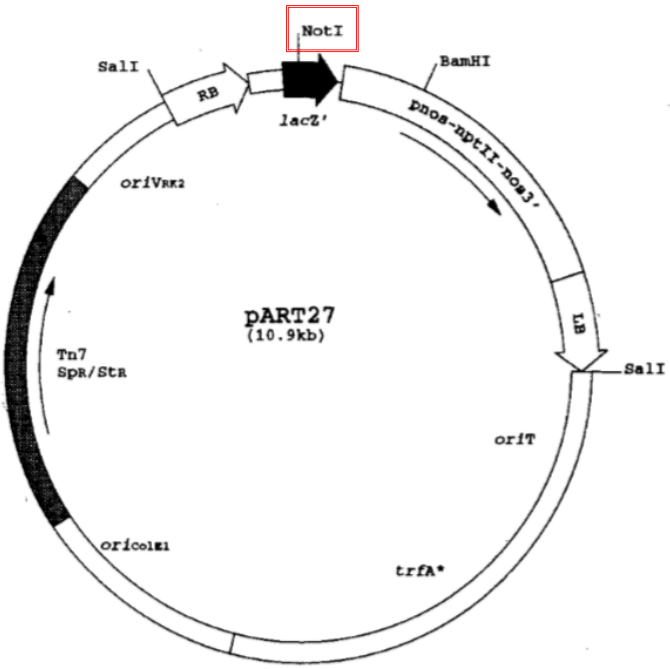
3.1.1. Plasmid

For the transformation of *Linum usitatissimum* L. and biosynthesis of *mcl*-PHAs in plant cells the plasmid p27.C1.A1+, which is 14940 bp in length, was used (Fig. 8). This plasmid can be replicated in bacteria *E coli.* and *A. tumefaciens* and was kindly provided by Prof. Poirier, University of Lausanne, Switzerland (Mittendorf et al., 1998).

p27.C1.A1+ contains the gene encoding PHA synthase (*phaC1*) from *Pseudomonas aeruginosa*, modified for peroxisome targeting by addition of the last 34 amino acids of *Brassica napus* isocitrate lyase gene, as previously described (Mittendorf et al., 1998). The modified *phaC1* gene was subcloned into the EcoRI–XbaI sites on the expression cassette of the primary cloning vector pART7 (Gleave, 1992), followed by cloning into the binary vector pART27.

The expression cassette of the primary cloning vector, pART7, comprises 35S promoter of a cauliflower mosaic virus (CaMV), multiple cloning site and transcriptional termination region of the octopine synthase gene (ocs 3'). The entire cassette can be removed from pART7 as a NotI fragment and introduced directly into the binary vector pART27. Recombinants are selected by blue-white screening technique. The binary vector pART27 consists of RK2 minimal replicon for replication in both *Escherichia coli* and *Agrobacterium tumefaciens*, ColEI replicon for a high-copy number in *E. coli* and Tn7 spectinomycin/streptomycin resistance gene for bacterial selection. The T-DNA carries lacZ' region (encoding the lac α peptide) and chimeric kanamycin resistance gene (nptII gene), allowing kanamycin to be a selectable marker in plants. Modified lacZ' region has a unique NotI site for cloning of the pART7 expression cassette (Gleave, 1992).

A)



B)

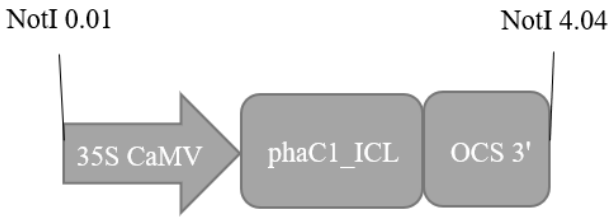


Figure 8. Vectors and expression cassettes used in this work. A) Binary vector, pART27. Right border (RB) and left border (LB) are indicated by arrowed boxes; T-DNA consists of the lacZ' region (encoding the lac α peptide) represented by the dark arrowed box and the chimeric kanamycin resistance gene (nopaline synthase promoter-neomycin phosphotransferase II-nopaline synthase terminator) (Gleave, 1992). B) Schematic illustration of the *phaC1* cassette used for flax transformation, that provides expression cassette of a primary cloning vector pART7 (35S CaMV and ocs 3') with the gene of interest *phaC1*, modified for peroxisome targeting. The entire expression cassette with the *phaC1* gene (*35-mcs-ocs 3'*) was introduced into the binary vector pART27, as a NotI fragment.

3.1.2. Primers

Table 2. List of primers used for PCR reactions. Orientation of primers can be forward (fw) or reverse (rev).

Primers	**Sequence**
PhaC1_fw	5'-GCTGAACCTGAATCCGGTGA-3'
PhaC1_rev	5'-ACTACCTCGATGGCCTCCTT-3'
35S CaMV promoter_fw	5'-GAAAAGGAAGGTGGCTCCTA-3'
35S CaMV promoter_rev	5'-GGTCTTGCGAAGGATAGTGG-3'
ACT_fw	5'-TTGCTGACCGTATGAGCAAG-3'
ACT_rev	5'-TTCGAGATCCACATCTGCTG-3'

3.1.3. Microorganism

In this thesis *Agrobacterium tumefaciens* LBA4404 strain was used for genetic transformation of the flax plant. This strain is derived from *A. tumefaciens* Ach5, i.e. isolated from the crown gall on yarrow (collected in Contra Costa County, CA, USA), by T-DNA deletions. The disarmed LBA4404 strain has only *vir* and *ori* region of the Ti plasmid and is widely used for plant transformation (Huang et al., 2015).

A binary vector (p27.C1.A1+) was introduced into bacteria by electroporation and they were kept in glycerol on -80°C before use.

3.1.4. Plant material

A fibrous cultivar of flax (*Linum usitatissimum* L. cv. Sara) was chosen for the experiments. In tissue culture, plants were grown on MS medium (Murashige and Skoog, 1962) containing 1% sucrose and 0.8% agar.

3.1.5. Growth media

Solid media were prepared by adding agar to liquid media before sterilization (20 g l^{-1} agar for bacteria or 8 g l^{-1} agar for plant tissue). Solutions of NaOH and HCl were used for pH adjustment.

3.1.5.1. Growth media for Agrobacterium tumefaciens

YEM medium (Yeast Extract-Mannitol medium)

Yeast extract	0.4 g l^{-1}
Mannitol	10.0 g l^{-1}
NaCl	0.1 g l^{-1}
$MgSO_4x7H_2O$	0.2 g l^{-1}
$K_2HPO_4x3H_2O$	0.5 g l^{-1}
H_2O	to 1000 ml
pH was adjusted to 6.8-7.0	

Medium with antibiotics: Streptomycin and rifampicin were added to the sterile medium cooled to room temperature to final concentrations of 25 mg l^{-1} and 20 mg l^{-1}, respectively.

3.1.5.2. Growth media for Linum usitatissimum

MS medium (Murashige and Skoog Basal Medium)

MS	4.4 g l^{-1}
Sucrose	10 g l^{-1}
H_2O	to 1000 ml
pH was adjusted to 5.8	
agar 8 g l^{-1}	

PPM™ Plant Preservative Mixture (Plant Cell Technology) was added to the medium cooled to room temperature to the final concentration of 750 μg l^{-1}.

2MS medium

Composition is equal to the MS medium, only with 2% sucrose (20 g l^{-1}).

Callus-induction medium (CIM)

MS	4.4 g l^{-1}
Sucrose	25 g l^{-1}
Glucose	25 g l^{-1}
H_2O	to 1000 ml

pH was adjusted to 5.8
agar 8 g l^{-1}

Phytohormones: After autoclaving, 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) were added to the medium cooled to room temperature to final concentrations of 1 mg l^{-1} and 0.05 mg l^{-1}, respectively.

Shoot-induction medium (SIM)

MS	4.4 g l^{-1}
Sucrose	25 g l^{-1}
H_2O	to 1000 ml

pH was adjusted to 5.8
agar 8 g l^{-1}

After autoclaving, antibiotics and phytohormones were added to the medium cooled to room temperature: kanamycin, cefotaxime, BAP and NAA to final concentrations of 50 mg l^{-1}, 0.4 mg l^{-1}, 0.02 mg l^{-1} and 0.001 mg l^{-1}, respectively.

3.1.6. Chemicals

All chemicals used during this research were purchased from common manufacturers and were of analytical grade or the highest purity available.

All solutions and media were prepared with distilled water (dH_2O) and sterilized in an autoclave at 121°C for 20 minutes.

3.1.7. Buffers and solutions

- Solutions for plasmid extraction

BibidoI

Tris-Hcl (pH 8)	25 mM
EDTA (pH 8)	10 mM
Glucose	50 mM
Lysozyme	50 mg ml^{-1}

BibidoII

NaOH	0.2 N
SDS	1 %

BibidoIII

Potassium Acetate (pH 4.8)	3 M
Acetic Acid, glacial	11.5 %

❖ Buffers for RNA isolation (RNeasy Plant Mini Kit, Qiagen)

Buffer RLT	lysis buffer (high concentrations of guanidine isothiocyanate)
Buffer RW1	buffer for washing membrane-bound RNA
Buffer RPE	concentrated buffer for washing membrane-bound RNA
RNase-Free Water	30-50 µl, for elution of RNA

The RLT Buffer was mixed with β-mercaptoethanol (10 µl of β-ME was added to 1 ml of RLT Buffer) before use. The RPE Buffer was diluted by adding 4 volumes of 100% ethanol for a working solution.

❖ Solutions for PCR reactions

Thermo Scientific DreamTaq Green PCR Master Mix (2x) composition:

DreamTaq Green PCR Master Mix contains DreamTaq DNA polymerase, 2x DreamTaq Green Buffer, dNTPs (0.4 mM each), 4 mM MgCl2, and nuclease-free water. DreamTaq Green Buffer is optimized for robust performance in PCR for longer PCR products (up to 6 kb from genomic DNA and up to 20 kb from viral DNA). It contains a density reagent and two dyes for monitoring electrophoresis progress (the blue dye migrates with 3-5 kb DNA fragments and the yellow dye migrates faster than 10 bp DNA fragments in a 1% agarose gel).

Thermo Scientific 2x Phire Plant Direct PCR Master Mix composition:

Thermo Scientific 2x Phire Plant Direct PCR Master Mix contains Phire Hot Start II DNA Polymerase, Dilution Buffer, dNTPs, 1.5 mM $MgCl_2$, and nuclease-free water. It also includes a density reagent and two tracking dyes for direct loading of PCR products on the gel. The Dilution Buffer has been optimized to release DNA from a wide variety of different sample materials such as plant leaves and seeds.

❖ Solutions for RT-qPCR reaction

Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR composition:

Thermo Scientific Maxima First Strand cDNA Synthesis Kit contains double-strand specific DNase (dsDNase) with 10x dsDNase Buffer. The kit uses Maxima Enzyme Mix containing

Maxima Reverse Transcriptase (RT) and Thermo Scientific RiboLock RNase Inhibitor. 5x Reaction Mix contains the remaining reaction components: reaction buffer, dNTPs, oligo$(dT)_{18}$ and random hexamer primers.

- Solutions for gel electrophoresis

TAE buffer (50x)

Tris Base (2 M)	121.1 g l^{-1}
Acetic Acid, glacial (1 M)	60.5 ml l^{-1}
EDTA Sodium salt dehydrate (50 mM, pH 8)	372.24 g l^{-1}

Agarose gel (1%)

Agarose	0.3 g
TAE buffer (1x), prepared by diluting TAE buffer (50x)	30 ml
Midori Green DNA Stain	1.5 µl

DNA Gel Loading Dye (6x)

Tris-HCl (pH 7.6)	10 mM
Bromophenol Blue	0.03 %
Xylene Cyanol FF	0.03 %
Glycerol	60 %
EDTA	60 mM

DNA Loading Dye (6x) is mixed with DNA sample in proportion 1:6.

3.2. METHODS

3.2.1. Bacterial plasmid DNA isolation

Prior to the isolation of plasmids from *A. tumefaciens* cells, pure bacterial cultures were prepared via streak plate method. The culture was grown for two days at 28°C. *Agrobacterium* single cell-derived colonies were used to inoculate liquid medium (YEM + AB). Bacterial plasmid was extracted to verify the presence of binary vector following the DNA Miniprep from *Agrobacterium* liquid cultures protocol. For this purpose, 15 ml of liquid culture was grown to saturation at 28°C. In the first step, bacterial cells were collected by centrifugation (10 000 rpm, 7 min) of the saturated culture (15 ml). The collected cell pellet was resuspended in 200 µl of BibidoI solution and incubated for 30 minutes at 37°C by gentle shaking. After incubation, 400 µl of BibidoII and 300 µl of BibidoIII solution were added to the tube, its contents gently mixed each time and incubated at room temperature for 5 minutes. The suspension was centrifuged (13 000 rpm, 10 min) and supernatant was transferred to a new microfuge tube, mixed with 750 µl of isopropanol afterward. After 15 minutes, plasmid DNA was collected by centrifugation under the same conditions. Isolated pellet was washed twice in 70% ethanol, dried at 56°C, and resuspended in 30 µl of water with RNase.

3.2.2. Plant genomic DNA extraction

Isolation of DNA from *Linum usitatissimum* L. was performed using Thermo Scientific Phire Plant Direct PCR Master Mix kit, as recommended by the manufacturer. Small samples of flax leaves were taken in sterile conditions and placed directly into Dilution Buffer. After mixing with a micropipette, solution was greenish in color. Supernatant was used for further PCR reactions.

3.2.3. Polymerase chain reaction

The polymerase chain reaction (PCR) is a technique used to amplify a region of DNA, thus allowing it to be detected with high sensitivity (Mosier, 2010). The basic steps of PCR are:

(1) denaturation, in which double-stranded DNA templates are separated by raising the temperature; (2) annealing, when the temperature is lowered to enable primers to attach to single stranded DNA molecules; and (3) extension, in which the heat-stable DNA polymerase begins adding nucleotides onto the ends of annealed primers. These steps are repeated 25–35 times to exponentially produce exact copies of the target DNA. (Garibyan and Avashia, 2013). In experimental part of this thesis, PCR was used to detect presence of introduced gene in bacterial cells and plant genome. Gel electrophoresis (1% agarose) was carried out to separate amplified products.

Plasmid DNA isolated from *A. tumefaciens* LBA4404 strain was a template for the first PCR reaction. 12.5 µl of DreamTaq Green PCR Master Mix (2x) solution was mixed with 0.5 µl of each primer solution and 0.5 µl of template DNA isolated from bacterial culture. Nuclease-free water was added to the final volume of 25 µl. Conditions for PCR reaction were as recommended in the protocol of Thermo Scientific DreamTaq Green PCR Master Mix (2x), shown in Table 3.

When conducting PCR directly from fresh plant leaves, Phire Plant Direct Master Mix (Thermo Fischer) was used. Genomic DNA isolated from flax plants regenerated on root induction medium was screened with primers specific for the *phaC1* gene and 35S CaMV promoter. DNA (0.5 µl) was added to 2x Phire Plant Direct PCR Master Mix (10 µl) previously mixed with 0.5 µl of each primer solution and 8.5 µl of water. Conditions of the reaction were as recommended in the protocol (Table 4).

Table 3. PCR program used for the amplification of the *phaC1* gene from bacterial binary plasmid

Step	**Temperature [°C]**	**Time**	**Number of cycles**
Initial denaturation	95	3 min	1
Denaturation	95	30 s	25-40
Annealing	63	30 s	
Extension	72	1 min	
Final extension	72	5-15 min	1

Table 4. PCR program used for the amplification of the *phaC1* gene from flax genomic DNA

Step	Temperature [°C]	Time	Number of cycles
Initial denaturation	98	5 min	1
Denaturation	98	5 s	40
Annealing	63	5 s	
Extension	72	20 s	
Final extension	72	1 min	1

A device used for conducting PCR reactions is TPersonal Thermocycler, Biometra, shown in Fig. 9.

Figure 9. TPersonal Thermocycler, Biometra.

3.2.4. Gel electrophoresis

Gel electrophoresis is a method used to identify, quantify, and purify nucleic acids or proteins. Samples loaded into wells of an agarose gel are subjected to an electric field, causing negatively charged nucleic acid to move toward positive electrode. Shorter DNA fragments migrate more rapidly through the gel matrix than longer fragments, resulting in separation based on size (Lee at al., 2012).

For adequate separation of DNA fragments 1% agarose gels were prepared. Midori green DNA stain was added to gels for visualizing DNA fragments. Before loading, DNA samples were mixed with DNA loading dye in proportion 6:1. GeneRuler 1kb DNA ladder (Thermo Fischer) was used as a molecular weight standard. The electrophoresis was performed in 1x TAE buffer at 100 V for 40 minutes. Finally, Midori green-DNA complexes were visualized under UV transilluminator and photo-documented using a basic system for gel documentation (DOC-PRINT VX2, Vilbert Lourmat).

3.2.5. *Agrobacterium*-mediated transformation of flax

The procedure of genetic transformation and regeneration applied in this thesis was previously described by Dietze et al. (1995), but for potato (*Solanum tuberosum*). Firstly, seeds of Sara cultivar were treated with PPM^{TM} solution for disinfection (vortex, 10 min.). The seeds were sterile transferred onto MS medium and kept in the dark for germination at 21°C, for 7-14 days. After cotyledons and hypocotyls of flax plants appeared, they were transformed with chosen *Agrobacterium* culture (#5). The first step of transformation was to immerse plant explants in Petri dishes containing 10 ml of 2MS liquid medium and 50 µl of *Agrobacterium* culture (grown to late logarithmic phase), for T-DNA transfer. Plates were gently shaken (to evenly spread the bacteria) and kept in the dark at 22-24°C for two days. After two days, transformed plant leaves were transferred onto callus induction medium to obtain callus, and after 14 days onto shoot induction medium. Once the first shoots on callus tissue appeared, they were transferred and rooted on root induction medium (MS with 1% sucrose). The remaining callus was cultivated further to obtain more plants. Flax plants regenerated on root induction medium were screened by the PCR method for amplification of the *phaC1* gene.

3.2.6. Selection of flax plants at the transcriptional level

3.2.6.1. Isolation and purification of flax RNA

Total RNA was isolated from plant material according to RNeasy Plant Mini Kit protocol (Qiagen). 100 mg of plant tissue was homogenized by grinding under liquid nitrogen and directly transferred into 450 µl of RLT Buffer, which maintains the integrity of RNA during lysis. After centrifugation of homogenized sample (14 000 rpm, 2 min), 225 µl of 100% ethanol was added to the supernatant. Suspension was mixed using a micropipette and centrifuged (8

000 rpm, 15 sec). After discarding the flow-through, Buffer RW1 (700 µl) and Buffer RPE (500 µl) were added to the RNeasy spin column, followed each time by centrifugation under the same conditions. Buffer RPE (500 µl) was once again added to the tube and suspension was centrifuged (8000 rpm, 2 min). Collected pellet was dissolved in RNase-free water and centrifuged at 8000 rpm for 1 min. Isolated RNA was stored at -80°C.

Total RNA was purified from traces of DNA by digestion with DNase. For this purpose, 1 µl of DNase and 2 µl of 10x dsDNase Buffer were mixed with 17 µl of isolated RNA dissolved in RNase-free water. The mixture was incubated for 30 min at 37°C. Enzymatic reaction was stopped with EDTA (2 µl) and suspension was incubated at 65°C for 10 min. Purity of RNA was checked by dilution of samples and absorbance measurements using WPA Biowave II UV-Vis Spectrophotometer (Biochrom, Fig. 10).

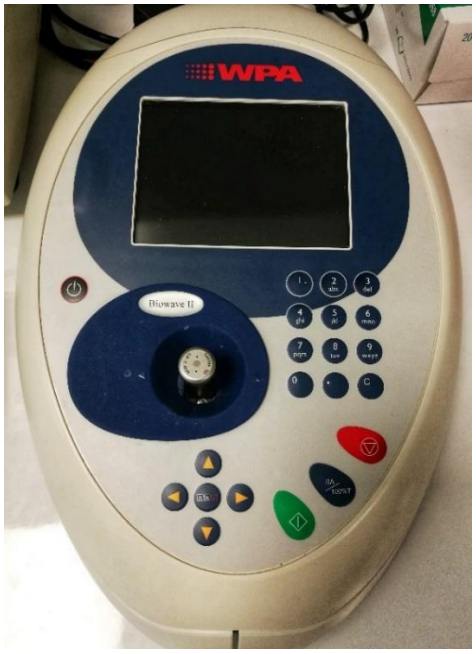


Figure 10. WPA Biowave II UV-Vis Spectrophotometer, Biochrom.

3.2.6.2. cDNA synthesis in quantitative RT-PCR

Isolated RNA was a template for cDNA synthesis (Maxima First Strand cDNA Synthesis Kit, Thermo Fisher). When conducting RT-qPCR, purified RNA isolated from plant material (14 µl) was mixed with 4 µl of 5x Reaction Mix and 2 µl of Maxima Enzyme Mix. Conditions for RT-qPCR reaction were as recommended in the protocol (Table 5). Plant RNA was reverse transcribed by Maxima Reverse Transcriptase (RT), an enzyme developed through

in vitro evolution of Moloney Murine Leukemia Virus RT. The Maxima Enzyme Mix also contains recombinant RiboLock RNase Inhibitor which effectively protects RNA templates from degradation.

Table 5. RT-qPCR program used for cDNA synthesis

Step	Temperature [°C]	Time	Number of cycles
Incubation	25	10 min	1
Reaction temperature	60	15 s	1
Termination	85	5 min	1
Final extension	4	pause	1

3.2.6.3. PCR amplification of synthesized cDNA

cDNA synthesized in reverse transcription reaction (0.5 µl) was a template for PCR amplification with primers specific for *phaC1* gene (792 bp) and housekeeping actin gene (100 bp). The reaction volume was 25 µl. Protocol and conditions for PCR reaction using Thermo Scientific DreamTaq Green PCR Master Mix (2x) were as previously described. PCR products were detected on agarose gel (0.8%) and quantified by densitometry analysis (BIO-GENE 11.9 software, Vilber Lourmat).

4. RESULTS AND DISCUSSION

This thesis analyses the transformation frequency of industrially important plant, *Linum usitatissimum* L. cv. Sara, transformed with the *phaC1* gene encoding PHA synthase using *Agrobacterium*-mediated technique. The study aimed to obtain flax plants that exhibited transgene integrated into their genomic DNA and to select flax plants with the expression of introduced gene at transcriptional level. Flax plants with overexpression of *phaC1* gene are of interest for further characterization in terms of their biochemical and mechanical properties, together with their potential industrial applications.

4.1. *Agrobacterium tumefaciens* plasmid isolation and *phaC1* gene amplification

Pure cultures of *Agrobacterium* were isolated via a streak plate method (Fig. 11) and used to inoculate liquid media to control for the presence of binary vector p27.C1.A1+. Plasmid DNA isolated from bacterial cultures was screened by PCR method with primers specific for 35S CaMV promoter (220 bp) and *phaC1* gene (792 bp). The template for positive control reaction was pART27-phaC1 plasmid from *E. coli*. Gel electrophoresis was conducted to confirm the presence of binary vector p27.C1.A1+ containing *phaC1* gene (Fig. 12).

Figure 11. The results of streak plate method. It was performed to isolate pure cultures of *Agrobacterium tumefaciens* LBA 4404.

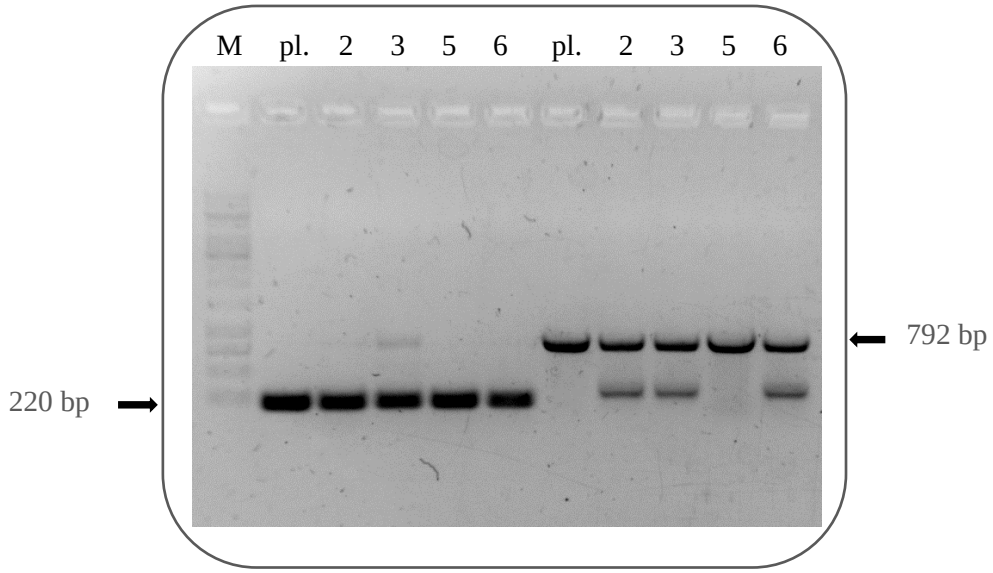


Figure 12. Selection of *Agrobacterium* cultures by PCR method. M, marker, represents the 1Kb ladder; pl, plasmid used for transformation (pART27-phaC1); and lanes 2, 3, 5, 6 correspond to plasmids isolated from bacterial pure cultures. Fragment of 35S CaMV promoter (220 bp) and fragment of *phaC1* gene (792 bp) were amplified with the use of specific primers (Table 2).

PCR results showed amplification of sequence of interest (corresponding to 35S CaMV promoter and *phaC1* gene) and confirmed the presence of binary plasmid in all bacterial cultures. Colony #5 was chosen for further experiments due to the presence of one clear band when screening for *phaC1* gene, the same as in positive control. All other samples showed two bands on gel obtained after PCR reaction, the band corresponding to transgene (792 bp), and nonspecific band around 250 bp.

4.2. The frequency of *Agrobacterium*-mediated flax transformation

Young flax seedlings were transformed with *phaC1* gene, as previously described in the transformation protocol (Fig. 13, Materials and metods, 3.2.5.). Hypocotyls of flax (7-day old seedlings) were used as explants for *Agrobacterium*-mediated transformation since they are the most suitable material for this type of manipulation (Shysha et al., 2013).

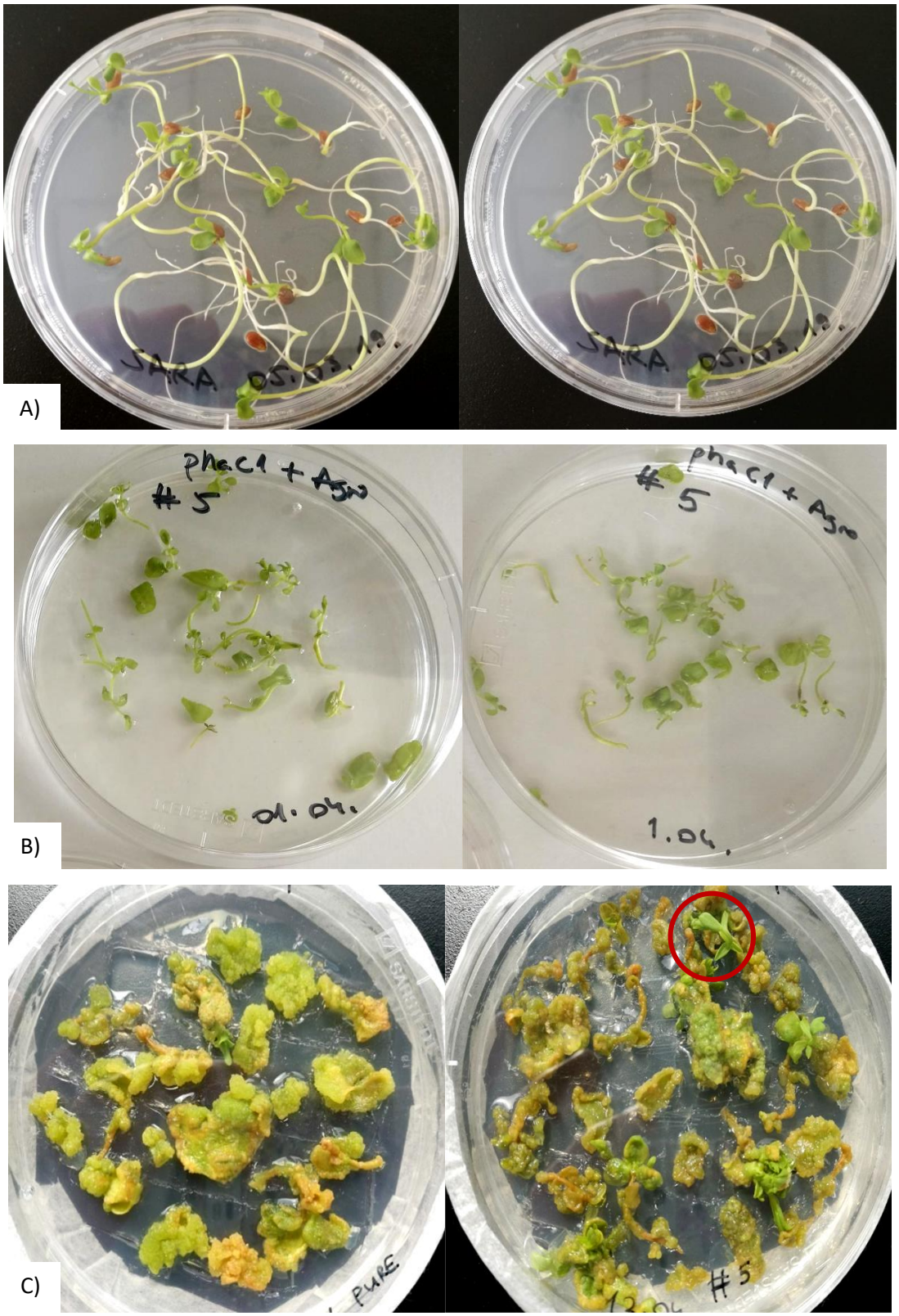


Figure 13. Plant regeneration of *Linum usitatissimum* L. at different stages of the transformation procedure. A) Germination of young flax seedlings used for the genetic transformation; B) flax explants immersed in 2MS liquid medium with medium with transformed *Agrobacterium tumefaciens* cultures; C) left- flax explants on callus induction medium forming callus; right-flax shoots obtained from callus on shoot induction medium.

A total of 137 flax explants were used for *Agrobacterium*-mediated genetic transformation. All of them formed callus tissue and 29 shoots were obtained on shoot induction medium, with kanamycin selection. The transformation frequency was determined as a percentage ratio of explants with regenerated shoots on selective media to the total number of explants taken for the experiment. Hence, the shoot regeneration frequency reached 21.2%, which is similar to results achieved for fibrous flax cv. Nike (18.9%) using the same transformation construct (Wróbel-Kwiatkowska et al., 2018). The highest transformation efficiency when transforming flax plants via *Agrobacterium*-mediated technique was found to be 93.54% (Beyaz et al., 2017). In that study, *A. tumefaciens* strain GV2260 harboring p35S GUS-INT plasmid (containing nptII gene and GUS reporter gene) was used for inoculation of 7-day old sterile flax seedlings.

Obtained shoots were transferred onto root induction medium for regeneration, some of them showing stunted growth (Fig. 14).

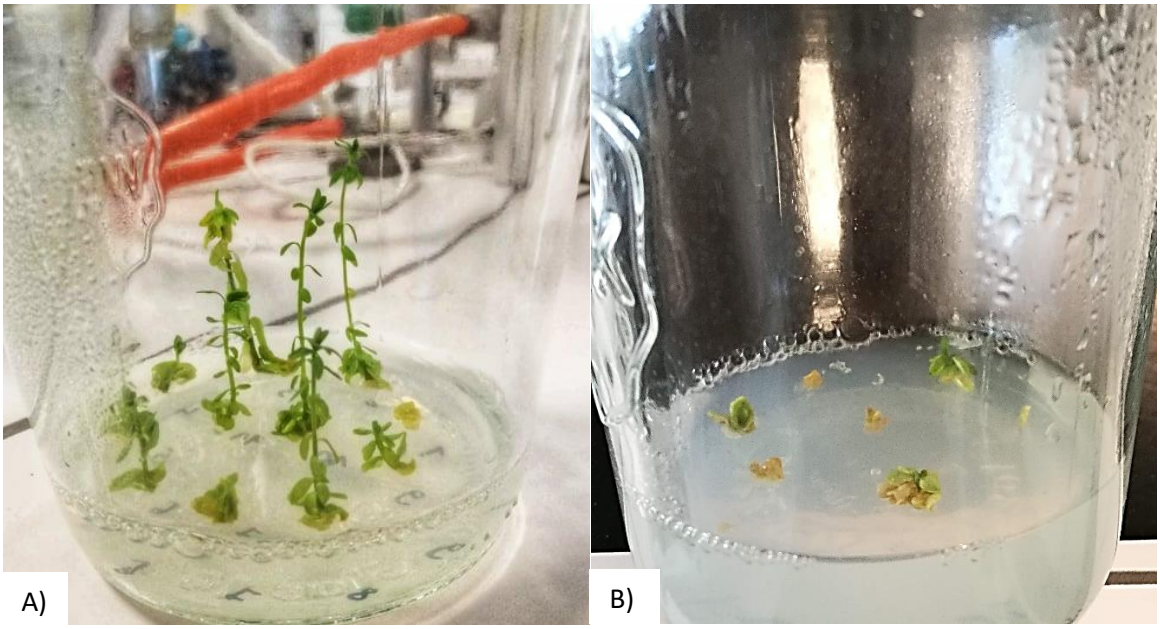


Figure 14. Flax plants on root induction medium that show (A) normal and (B) stunted growth.

Among 29 regenerated plants, 16 were fast growing and they were analyzed for transgene integration in their genomic DNA by PCR reaction (Materials and methods, 3.2.3.). Therefore, fragment of the introduced *phaC1* gene (amplicon 792 bp) and fragment of 35S CaMV promoter (amplicon 220 bp) were analyzed (Fig. 15). Template for positive control reaction was pART27-phaC1 plasmid from *E. coli*, and in negative control there was no DNA template.

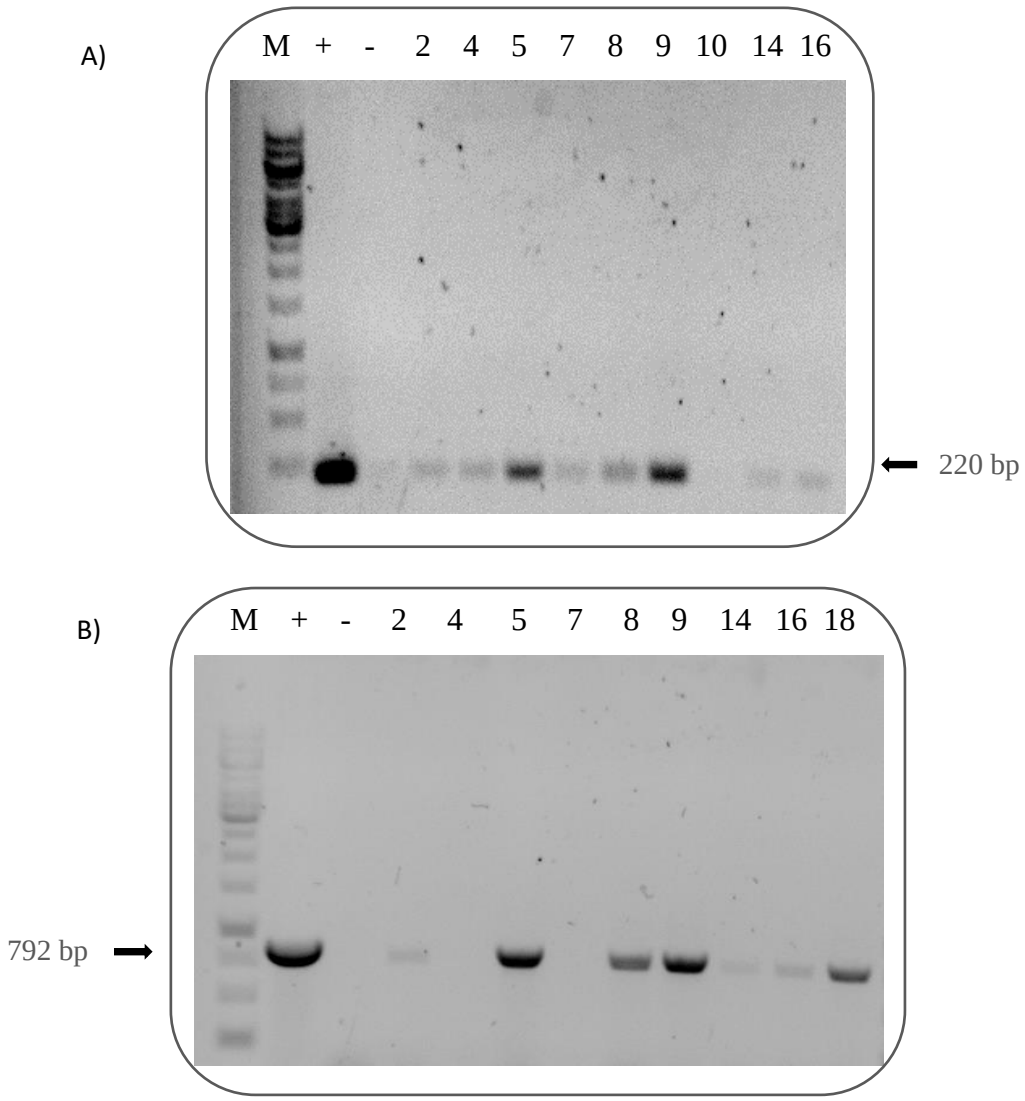


Figure 15. Selection of generated plants by PCR method. M, marker, represents the 1 kb ladder; +, positive control (pART27-phaC1); -, negative control; and lanes 2-18 correspond to DNA isolated from different plant material. A) DNA primed with oligonucleotides specific to 35S CaMV promoter (220 bp). B) DNA primed with oligonucleotides specific to *phaC1* gene (792 bp).

PCR analysis showed amplification of 220 bp corresponding to the 35S CaMV promoter and 792 bp corresponding to the *phaC1* gene, indicating presence of transgene in 7 out of 16 recovered plants. Thus, this thesis demonstrated a successful *Agrobacterium*-mediated transformation of fibrous flax cv. Sara with the modified *phaC1* gene in combination with the selective nptII marker gene. The negative results could be due to non-transformed shoots surviving in the selection medium (Hess et al. 1990; Langridge et al. 1992). Also, some plants were tested positive for promoter and negative for *phaC1* gene. The reason might be incorporation of incomplete cassette.

4.3. Quality assessment of total RNA isolated from transgenic flax plants

Purity of isolated RNA was determined by measuring UV absorbances at 260 and 280 nm (Materials and methods, *3.2.6.1.*). When using a spectrophotometer, ratio of absorbances at 260 and 280 nm (A_{260}/A_{280}) greater than 1.8 is usually considered an acceptable indicator of RNA purity (Manchester, 1996). Nucleic acids can be quantified at 260 nm because it is well established that a solution of DNA in a 10 mm pathlength cell with an optical density of 1.0 has a concentration of 50, or 40 µg/ml in the case of RNA (Koetsier and Cantor, 2019). Concentration of RNA can be calculated using following equation:

$$\text{Concentration (RNA)} = A_{260} \text{ x dilution factor x } 40 \text{ µg/ml}$$

Table 6. Purity and quantity of total RNA isolated from transgenic plants.

RNA sample	**A_{260}/A_{280}**	**c [µg/ml]**
#2	1.962	41.6
#5	2.098	51.2
#8	2.094	44.4
#9	2.046	53.2
#14	2.000	38.4
#16	1.967	47.2
#18	2.081	30.8

All RNA samples were considered pure due to A_{260}/A_{280} ratio between 1.9 and 2.1 and taken for further experiments (Table 6).

4.4. mRNA transcript analysis

Prior to mRNA transcript analysis, seven flax plants were found transformed with *phaC1* gene and propagated. Total RNA was isolated from transgenic lines and purified to control for the expression of introduced *phaC1* gene. Afterward, quantitative RT-PCR was conducted for cDNA synthesis in reverse transcription reaction, using isolated RNA as a template (Materials and methods, *3.2.6.2.*). Expression of investigated *phaC1* gene on

transcriptional level was determined by PCR reaction from synthesized cDNA with specific primer pairs. The housekeeping actin gene served as a reference gene to measure the transcription rate of *phaC1* gene (Fig. 16). Template for negative control reaction was cDNA prepared from total RNA isolated from plant tissue of wild-type plant.

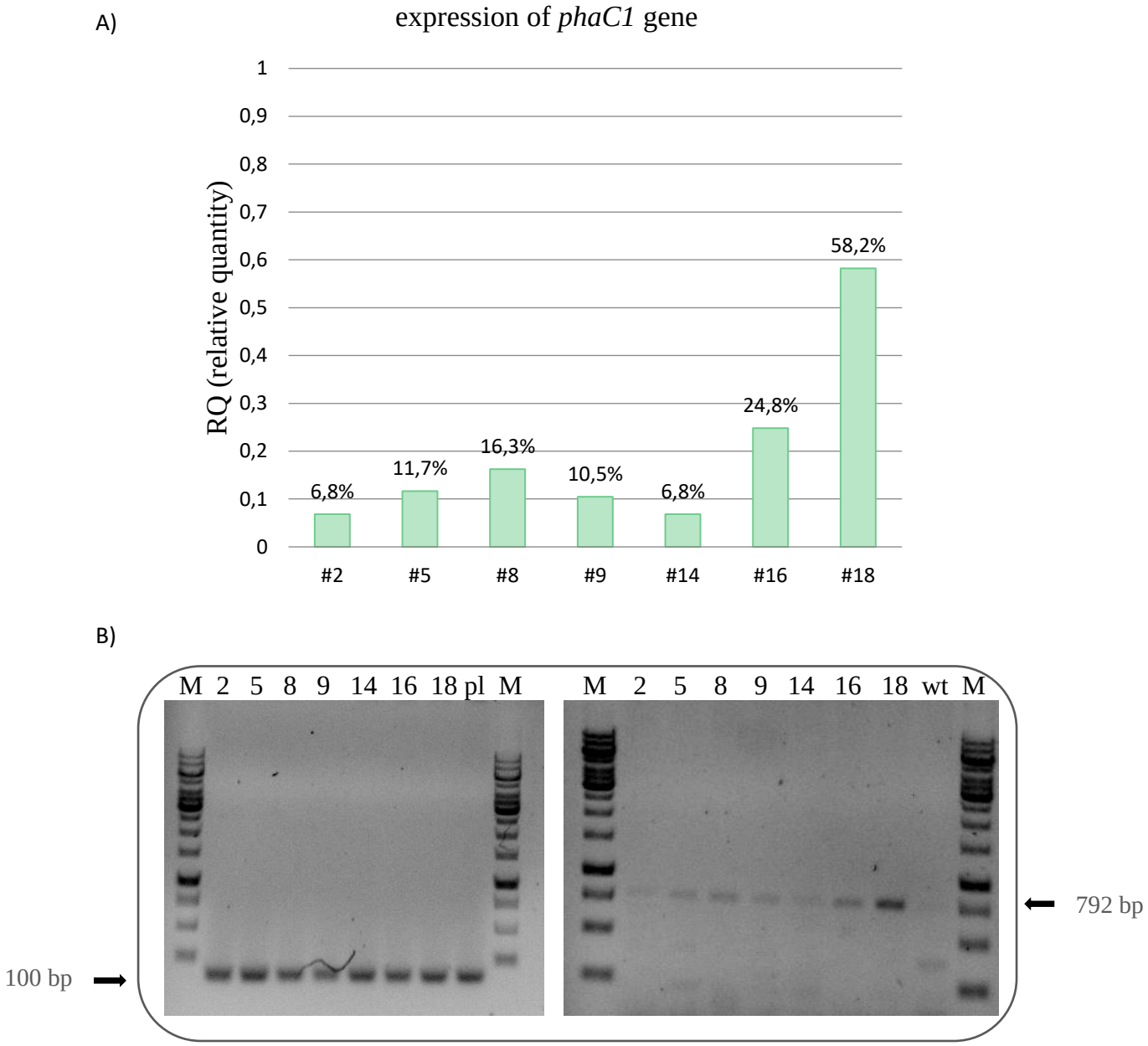


Figure 16. Analysis of selected transgenic plants. A) Expression of *phaC1* gene in transgenic plants. Quantitative data were normalized with expression of actin gene in each sample. B) PCR amplification of cDNA; M, marker, represents the 1Kb ladder; wt, cDNA prepared from wild type plant; and lines 2-18 correspond to cDNA samples prepared from transgenic plants. Left gel: cDNA screened with primers specific for actin gene (100 bp) Right gel: cDNA screened with primers specific for *phaC1* gene (792 bp).

The cDNA synthesis was verified for all transgenic plant lines by amplification of the actin gene using specific primers. Transcription of introduced *phaC1* gene was confirmed for all 7 lines (amplicon 792 bp) by PCR analysis (Fig. 16.b). In order to quantify gene expression, BIO-GENE 11.9 software (Vilber Lourmat) was used. Obtained data was normalized against a reference actin gene. When compared with expression rate of housekeeping actin gene, most of transformed plants showed low transcriptional level of *phaC1* gene (6-25%). Only one line (#18) was detected with transcriptional rate at high level of 58.2%. The produced lines can be used in the future for biochemical, mechanical, and structural analyses of whole plants and fibers derived from them.

5. CONCLUSIONS

Based on the results presented in this thesis, the following conclusions can be made:

1. *Agrobacterium*-mediated transformation has proven to be successful in generating a transgenic fibrous flax cultivar Sara. Transformation frequency of 21.2% was achieved and 55% of obtained shoots regenerated easily *in vitro*.
2. In all newly constructed transgenic plants that contained *phlaC1* gene, as determined by PCR, RT-qPCR confirmed successful transcription.
3. Plant line #18, showing the highest expression of *phaC1* gene at transcriptional level, should be used in further studies in order to analyze the impact of *mcl*-PHA synthesis on mechanical properties of flax stems and fibers.

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APPENDIX

Table of abbreviations

ACP	Acyl carrier protein
BAP	6-Benzylaminopurine
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CIM	Callus-induction medium
CoA	Coenzyme A
EDTA	Ethylenediaminetetraacetic acid
GUS	β-glucuronidase
HA	Hydroxyalkanoate
ICL sequence	Isocitrate lyase peroxisomal targeting sequence
***lcl*-PHA**	long chain length PHA
***mcl*-PHA**	medium chain length PHA
MS	Murashige and Skoog Basal Medium
NAA	1-Naphthaleneacetic acid
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced
nptII	Neomycin hosphotransferase II
PCR	Polymerase chain reaction
PE	Polyethylene
PHA	Polyhydroxyalkanoate
PHB	Poly(3-hydroxybutyrate)
P(3HB-co-3HV)	Poly(β-hydroxybutyrate-β-hydroxyvalerate)
PLA	Polylactic acid
PPM	Plant Preservative Mixture
RT-qPCR	Quantitative reverse transcription PCR
***scl*-PHA**	short chain length PHA
SIM	Shoot-induction medium
TAE	Tris-acetate-EDTA
TCA cycle	Tricarboxylic acid cycle
T-DNA	Transfer DNA
Ti plasmid	Tumor-inducing plasmid
UV-VIS	Ultraviolet–visible spectroscopy
YEM	Yeast Extract-Mannitol medium

STATEMENT OF ORIGINALITY

This is to certify, that the intellectual content of this thesis is the product of my own independent and original work and that all the sources used in preparing this thesis have been duly acknowledged.

Antea Talajić

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