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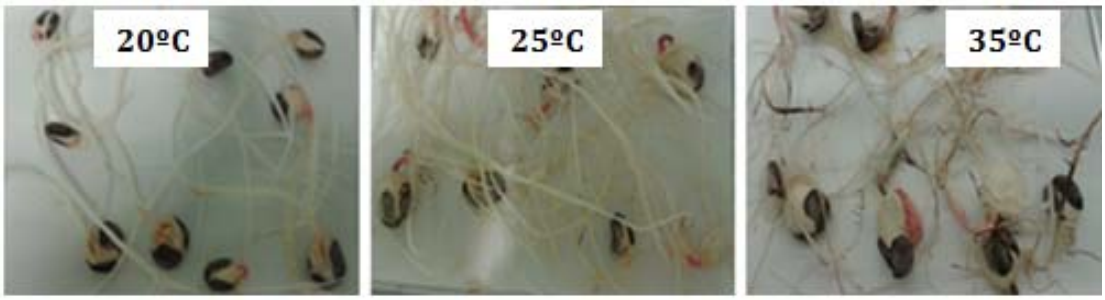

Marie Chantal Mutimawurugo

Wageningen University

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Wageningen Seed Lab

Laboratory of Plant Physiology

Wageningen University

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Marie Chantal Mutimawurugo MSc Student Plant Sciences

Registration number : 761012592080

Code : PPH-80439

Supervisor : **Paulo Roberto Ribeiro de Jesus**

Examiners : **Dr. Ir. Wilco Ligterink (WUR)**
Dr. Henk Hilhorst (WUR)

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Abstract

Background: *Ricinus communis* L. (Castor bean) is an oilseed crop widely grown for vegetable oil and renewable bio-products for pharmaceutical and industrial purposes. However, castor oil production does not meet the increase of its world consumption due to the use of cultivars with low genetic potential to adapt to some environmental conditions like harsh temperatures. Castor bean utilises storage oil for seed germination and breakdown of this reserve is the main source of energy that is required for successful seedling establishment before the plants become autotrophic. Temperature greatly affects germination and seedling performance. However, the mechanisms behind the effect of temperature on the physiology, biochemical and molecular aspects are not fully understood. **Objectives:** To assess temperature effect on germination and post-germination growth and to clone and characterize the function of three temperature responsive genes: glycerol kinase (*GK*), malate synthase (*MLS*) and phosphoenolpyruvate carboxykinase (*PCK*). **Materials and Methods:** Castor seeds were allowed to imbibe and to germinate at 20, 25 and 35°C. Seed germination and seedling growth were followed for two weeks. Then, the number of healthy seedlings at each temperature was recorded. Cotyledons and roots of the healthy seedlings were used to extract RNA. cDNA was synthesized from the isolated RNA, which was used during further experiments. Gene cloning was performed through gateway technology. For functional characterization of the cloned genes we used the *Nicotiana benthamina* transient expression system and transformed leaves were used for metabolite profiling experiments. **Results:** Germination percentage increased with the increasing temperature while seedling performance decreased with the increasing temperature. Thus, seed incubation at 20°C led to lower percentage of germinated seed while both 25 and 35°C are reported to be the optimum temperature for optimum germination rate in castor bean. Moreover, seed germination at 20°C resulted to high number of seedling survival followed by 25°C while at 35 °C all seedlings died. Transient expression of *R. communis GK*, *MLS* and *PCK* genes in *N. benthamiana* leaves led to the reduction of glucose levels with subsequently accumulation of starch. **Discussion:** Castor bean germination and seedling performance are strongly affected by the temperature. In fact, the increasing temperature resulted to high percentage of germinated but also to low seedling survival. This is due to the fact that high temperature results to rapid water uptake required for germination but also leads to low expression of genes that encode the enzymes *GK*, *MLS* and *PCK* which are involved in lipid breakdown to provide energy for seedling growth. **Conclusion:** High temperature during germination led to low seedling performance due to low expression of genes that encode *GK, MLS* and *PCK* enzymes. A reduction in glucose levels which was followed by an increase in starch content in leaves that were infiltrated with *MLS* and *GK* genes indicates that these genes are required for *R. communis* growth at the early of seedling establishment.

Key words: Castor bean, seed germination, seedling establishment, lipid mobilization.

Abbreviations

AGS: Amylo-glucosidase

BLAST: Basic local alignment search tool

cDNA: complementary Deoxyribonucleic acid

$CHCl_3$: Chloroform (synonym: Methylidyne trichloride or Trichloromethane)

DMSO: Dimethy sulfoxide

DTT: DL-dithiolthreitol

EGTA: Ethylene glycerol-bis –tetraacetic acid

GC-MS: Gas chromatography-mass spectrophotometry

GK or GLI: Glycerol kinase

G3P: Glycerol-3-phosphate

HCl: Hydrochloric acid

HPLC: High performance liquid chromatography

ICL: Isocitrate lyase

KOH: Potassium hydroxide

LB: Lysogeny broth

MLS : Malate synthase

MeOH: Methanol anhydrous

MES: 2-(*N*-morpholino)ethanesulfonic acid

Na-DOC: Sodium deoxycholate

NaOH: Sodiumhydroxide

NCBI: National center for biotechnology information

OD: Optical density

PCK: Phosphoeonylpyruvate corboxykinase

PCR: Polymerase chain reaction

PVP: Polyvinlypyrrolidone

SDS: Sodium dodecyl sulfate

TAG: Triacylglycerol

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1. Introduction

1.1. Castor bean production and usages

Castor bean (*Ricinus communis* L) belongs to Euphorbiaceae family. Castor bean is a perennial plant and it originates from Eastern Africa probably in Ethiopia. Castor bean is a plant which prefers semi-tropical and tropical conditions for germination and growth. It can grow between 300-1500m altitude and occasionally up to 3000m (Miller, 2008). Castor bean grows in almost any soil, with the exception of very heavy clay and poorly drained soils. It can be grown in unsuitable soil which is less fertile and therefore less suitable for food crops and this is a considerable advantage in land use efficiency. However, the highest castor bean yield is obtained when it is grown in fertile and appropriate soil. For instance, it was reported that for getting a total yield of 1700kg/ha, plant takes up 50kg N, 20kg P and 16kg K. In addition, the plant can tolerate salinity up to 7.1 dS m^{-1} beyond which seed germination can be affected with losses above 75% (Severino and Dick, 2013).

For optimum germination, a seed must be viable and contain enough reserve material (Miller, 2008). Moreover, the optimum environmental conditions like water to activate enzymes during germination and breakdown of storage material, temperature and air (especially O_2) are also required. Cheema and colleagues (2010) concluded that moisture availability and optimum temperature are important factors which affect seed germination. Soil moisture content of 15-20% normally enables good germination at the optimum temperature (Weiss, 2000). As mentioned above, oxygen is also required and 19% O_2 is the optimum for germination while high CO_2 (above 4%) is harmful (Bewley and Black, 1994). However, Miller (2008) reported that CO_2 concentration higher than 0.03% prevents castor seed germination.

The environmental conditions also affect plant growth and development and consequently the seed yield. For instance, the temperature regulates seed germination by determining the capacity and rate of germination or by removing/ inducing seed dormancy depending on the species (Bewley and Black, 1994). Severino and colleagues (2012) reported that temperature affects seed germination in castor bean and they revealed in one experiment that 14 to 15°C is the minimum temperatures for castor bean germination. Miller (2008) defined the optimum temperature as the one giving the highest percentage of germination in the shortest time. Thus, from a study conducted by Moshkin (1986) (reviewed by Severino and Dick, 2013) for castor bean germination, the optimum temperature was reported to be 31°C and the maximum was 36°C. However, in another experiment Moshkin (1986) revealed 25°C as the optimum (reviewed by Severino and Dick, 2013). Moreover, Cheema and colleagues (2010) also reported that

although seeds can germinate at 10°C, the faster germination occurs at 25°C temperature. Beside seed germination, temperature also affects other developmental stages (Weiss, 2000). For instance, castor bean flowering occurs between 20-26°C. When the temperature rises to 40°C at flowering stage, flower basting occurs and there is a poor seed set. Moreover, too low or high temperatures adversely affect castor seed composition. It was reported that below 15°C or above 35°C, oil and protein content of castor seed are reduced. A temperature of -2°C for 4 hours usually kills the plant at any growth stage (Weiss, 2000).

R. communis is widely grown for different usages. The most important is its production for pharmaceutical purposes for human and veterinary medicines (production of hydroxylated fatty acids such as ricinoleic acid) (Kakakhel, 2008), petro-chemicals and other industrial purposes (oil for lubrication of different equipments, biodiesel production, source of renewable polyurethanes, cosmetics, soap, paints) (Severino *et al*, 2012; Kakakhel, 2008). This also reveals the role of castor oil in environmental protection by using vegetable oils for biofuel and renewable bio-products (Severino *et al*, 2012). Moreover, castor bean contains other compounds such as allergens, the protein 'ricin' and the alkaloid 'ricinine' which are used for different purposes. Allergen extracts are used in insect pest management in agriculture because they have an inhibitory effect on α-amylase activity that prevents the hydrolysis of starch into sugars in the metabolism of insects. Ricinine at high quantity can also be used as organic insecticide while ricin has a negative effect on plant parasitic nematodes by reducing their egg laying rate.

A combination of nematicide and castor meal reduces the growth of nematodes like *Pratylenchus* sp.(Severino *et al*, 2012). In addition, incorporation of castor vegetative tissue into soil reduce the growth and reproduction of *Meloidogyne incognita* in tomato and lentil cultivation and castor bean leaf extract kills *M. exigua* juveniles in coffee. In agriculture, castor bean is also used as organic fertilizer because the husk by-product has high K content (Kakakhel, 2008). Castor by-products also can be used in animal feed application as source of protein after removal of toxic compounds like ricinine and ricin (Severino *et al*, 2012). Castor bean also is used as an ornamental plant due to its attractive colours of leaves and inflorescences (Kakakhel, 2008).

Beside the mentioned roles, castor bean is cultivated because of its high ability to tolerate saline and drought conditions (Graham, 2008). These aspects play an important role in land use systems as most of the farmers tend to grow food crops in good soil and other crops in the rest. Drought tolerance in castor bean is mainly due to its large and well-developed tap-roots with considerable lateral roots both enabling a deep penetration and high soil exploration to take maximum soil moisture (Weiss, 2000).

Another reason of this potential in drought tolerance could be due to its high stomatal control enabling plants to minimize the transpiration rate by keeping also a high level of net CO_2 assimilation under water stress conditions (Severino *et al.*, 2012). These physiological traits enable castor bean to use the soil moisture more efficiently than most food crops (Weiss, 2000). Another important role of castor plant is its use for phytoremediation of soils containing high levels of heavy metals such as Pb, Zn, Cd and Ni. This plant is tolerant to those metals and accumulates them in its tissues. Thus, it can make soils more productive for food crops (Romeiro *et al*., 2006; Liu *et al*., 2008 cited by Severino *et al*., 2012). An additional advantage of growing castor plant in a rotation plan is that it induces the germination of *Striga* spp, a parasitic plant of cereals crops especially in Africa, to which itself is resistant. Therefore, crops following castor bean benefit from lower *Striga* infestation (Weiss, 2000).

However, castor bean production (around 0.15% of vegetable oil produced in the world) and supply do not meet the world demand with a demand increase of 50% during the past 25 years (Severino *et al*., 2012) especially in developed countries where they prefer the usage of natural oils (Weiss, 2000). This is due to the fact that only a few countries in the world such as Brazil, India, China, Russia, Thailand and Mozambique produce a considerable amount of castor bean (Severino and Dick, 2013; Kakakhel, 2008) and the seed oil content also varies for different growing locations (Severino *et al*., 2012). Moreover, other constraints in castor bean production are due to the low technology in production and the use of varieties with low genetic potential. Therefore, breeding for cultivars that combine the potential of high yield and adaptation to different environmental conditions by tolerating temperature, salt, and drought would be a good way of increasing overall castor bean production (Weiss, 2000). This requires a good understanding of the interaction between genetic traits, the environment and crop management (Severino and Dick, 2013).

1.2. Storage material mobilization and seedling establishment

Generally, there are three major food reserves in plants: fats or oils, carbohydrates, and proteins. Fats are the most efficient form for energy storage as they contain more than twice the energy stored in other reserves mentioned above (Quettier and Eastmond, 2009; Huang, 1992). Bewley and Black (1994) reported different reserves in castor bean endosperm which is mainly composed of oil (64%), protein (18%) and negligible carbohydrate. Moreover, Weiss (2000) reported that castor seed is mainly composed of oil (40-60% depending on cultivar and environment), triglycerides (ricinolein) and fatty acid (ricinoleic acid). Castor bean, like other oil crops, accumulates oil reserves in the seeds during seed filling and maturation. In castor bean

seeds, the oil reserves are stored in the form of triacylglycerol (TAG) compounds (Baud and Lepiniec, 2010) which consist of esters of glycerol and fatty acids (Quettier and Eastmond, 2009). These compounds are accumulated within specialised structures called oil bodies localized in cytosol (Theodoulou and Eastmond, 2012). These TAGs serve as reserve for seed germination and its mobilization is essential as source of carbon for successful seedling establishment (Graham, 2008). The transition from seed to seedling is a critical step in the life cycle of plants and in oilseed crops it is determined principally by the breakdown of TAG reserves (Quettier and Eastmond, 2009; Eastmond and Graham, 2001).

The breakdown of TAG reserves into carbohydrates can start before the germination is completed and continues during post-germinative growth with the major mobilization in the growing organs after radicle elongation (Graham, 2008; Bewley and Black, 1994). It is mentioned above that the oil reserve is required during seed germination, but its mobilization is only needed for seedling establishment; a stage in which seedlings require the carbohydrates for energetic resource for rapid growth and development before they become autotrophic organisms via photosynthesis (Kelly *et al.*, 2011). Therefore, the higher the oil reserves and its solubility, the better seedling establishment and vigour of seedlings for ultimate crop establishment and high seed yield (Kornberg and Beevers, 1957; Graham, 2008).

In castor bean like in other oil crops, lipid reserves undergo a complex pathway for the conversion to carbohydrates required for seedling development and supporting photoautotrophic metabolism. This mechanism involves a metabolic pathway which occurs through the following pathways: TAG lipolysis, β-oxidation, glyoxylate cycle and gluconeogenesis. The main enzymes involved in this long pathway are lipase in TAG lipolysis, glycerol kinase (*GK*) in the direct conversion of glycerol into glycerol-3-phosphate (G3P), acyl-CoA synthetases (*LACS*), acyl-CoA oxidase (*ACX)*, multifunctional proteins (*MFPs*) and 3-ketoacyl- CoA thiolase (*KAT*) in *β*-oxidation, isocitrate lyase (*ICL*) and malate synthase (*MLS*) in glyoxylate cycle and finally phosphoenolpyruvate carboxykinase (*PCK*) in gluconeogenesis (Baud and Lepiniec, 2010; Graham, 2008).

1.2.1. TAG lipolysis

The first step of oil breakdown in oilseed crops is TAG lipolysis, a pathway by which lipase enzymes hydrolyse TAGs stored in cytosolic oil bodies into glycerol and free fatty acids (FA) (Penfield *et al.*, 2005, Karim *et al.*, 2005). TAG lipase is encoded by sugar dependent 1 (*SDP1*) locus (Graham, 2008; Eastmond, 2006) (Figure 1).

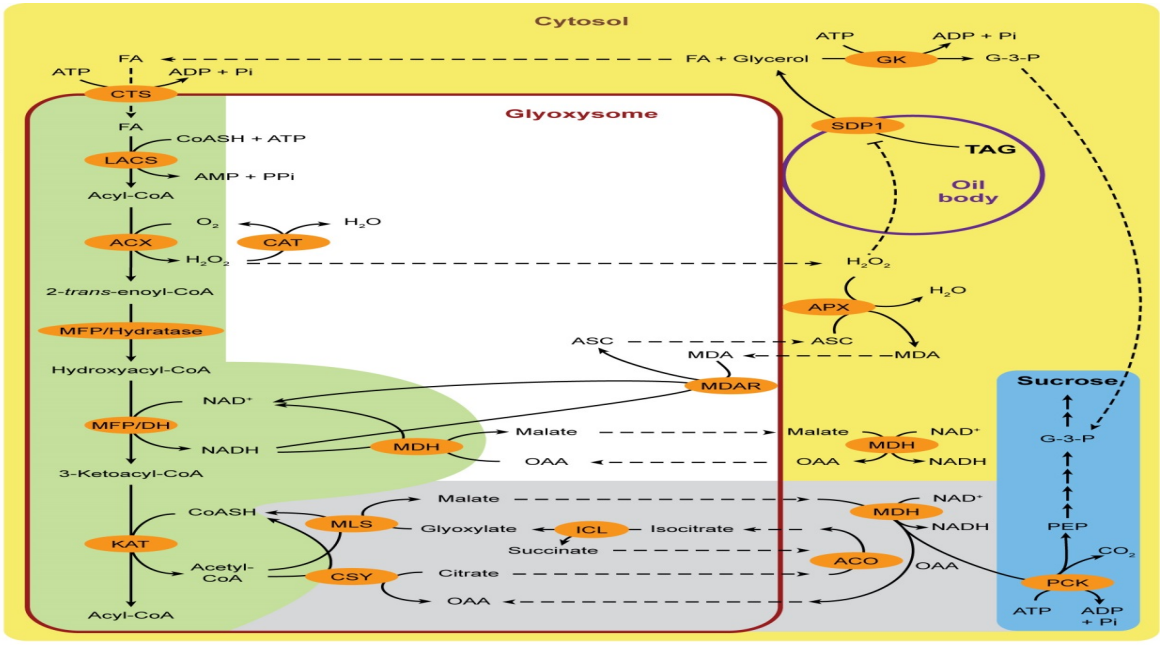


Figure 1. Storage oil breakdown. Yellow color indicates oil body and cytosolic pathway location, green indicates pathway involved in direct glyoxysomal β-oxidation, grey (glyoxylate cycle), and blue (gluconeogenesis). Graham (2008). Annual review. Plant Biology, 59: 115-142.

TAG lipolysis produces FAs and glycerol in a 3:1 ratio in which glycerol represents approximately 5% of the total carbon produced. Glycerol is phosphorylated by glycerol kinase (*GK*) to produce glycerol-3-phosphate (G3P) and the later enters gluconeogenesis to yield carbohydrates (hexose for cell wall synthesis or sucrose for seedling development) (Graham, 2008) (Figure 1) or be used as a respiratory substrate after its conversion to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase (Quettier and Eastmond, 2009; Penfield *et al*., 2005). Although glycerol represents a small carbon reserve its contribution to seedling growth is significant and essential especially in the absence of a functional glyoxylate cycle where it can compensate for the lack of carbons released from this pathway (Penfield *et al.*, 2005).

1.2.2. β-oxidation

β-oxidation in oilseeds occurs within glyoxysomes (Borek *et al.*, 2013). Therefore, fatty acids must be transferred from the oil body to this cell compartment (Theodoulou and Eastmond, 2012) by Transporters such as COMATOSE (*CTS)* ATP-binding cassette (*ABC*) (Graham, 2008; Penfield *et al*., 2005). This pathway results in the production of molecules of two carbon unit (C2) Acetyl-CoA from FA oxidation and additionally hydrogen peroxide (H_2O_2) (Borek *et al.*, 2013; Graham, 2008; Bewley and Black, 1994). The later is ultimately destroyed by peroxisomal

catalases (Penfield *et al.*, 2005). FAs are first esterified into acyl-CoAs by two long chain acyl-CoA synthetases (LACS 6 and 7) before entering the *β*-oxidation pathway (Theodoulou and Eastmond, 2012; Gram and Gram, 2005). These acyl-CoAs are used as starting materials of this pathway (Bewley and Black, 1994) and many enzymes and multifunctional proteins (*MFPs*) are required for its oxidation, hydration, dehydrogenation and cleavage (Figure 1) (Theodoulou and Eastmond, 2012; Graham, 2008). The initial oxidation of acyl-CoAs is catalyzed by acyl-CoA oxidase (*ACX*) and the next steps are catalyzed by MFP and the 3-ketoacyl- CoA thiolase (*KAT*) (Graham, 2008).

The produced Acetyl-CoA can undergo two different pathways. It can either enter the glyoxylate cycle for carbohydrate synthesis or it can be completely converted into citrate to be used for respiration via citric acid cycle (Penfield *et al*., 2005; Bewley and Black, 1994). The level to which Acetyl-CoA is converted to either carbohydrate or citrate depends on plant physiology. For instance in the case of castor bean, the oil molecules are mainly stored in the seed endosperm and the latter is completely degraded during germination. The demand of Acetyl-CoA for respiration in this tissue is negligible. Thus, the most part of products from oil breakdown are converted to carbohydrate required for seedling growth instead of the respiratory pathway. However, in other oilseed crops like Arabidopsis and sunflower, the oil reserves are mainly accumulated in the cotyledons. The latter persist after oil degradation and become photosynthetic active tissue. Thus, in these plants there is a high demand of organic compounds from FA degradation for respiration comparing to the molecules transported in cells for carbohydrate biosynthesis. In oilseed plants, FA β-oxidation continues until the complete degradation of fatty acids (Graham and Eastmond, 2002).

1.2.3. Glyoxylate cycle and gluconeogenesis

The glyoxylate cycle is a central and crucial pathway in TAG conversion to carbohydrate during seedling establishment in oilseed crops (Nakazawa e*t al.*, 2005; Runquist and Kruger, 1999). This occurs in both the glyxosomes and the cytosol (Graham, 2008) (Figure 1). The glyoxylate cycle converts the Acetyl-CoAs produced from β–oxidation into organic acids with four-carbon units such as malate (Graham, 2008; Nakazawa *et al.*, 2005). This pathway is performed by five key enzymes (Borek *et al.*, 2013; Graham, 2008). Two of them, isocitrate lyase (*ICL*) and malate synthase (*MLS*), are considered as unique enzymes of the glyoxylate cycle. *MLS* directly converts Acetyl-CoA into malate while *ICL* catalyzes the conversion of isocitrate into glyoxylate or succinate. Glyoxylate is subsequently also converted into malate by *MLS* (Graham, 2008; Nakazawa *et al*., 2005).

The other three enzymes, malate dehydrogenase (*MDH*), citrate synthase (*CSY*) and aconitase (*ACO*) function both in the glyoxyalate cycle and the tricarboxylic acid (TCA) cycle (Borek *et al.*, 2013; Graham, 2008). Malate is used as substrate for oxaloacetate (OAA) production by cytosolic *MDH* (Quettier and Eastmond, 2009; Graham, 2008; Penfield *et al*., 2005). OAA in turn can either enter gluconeogenesis or be converted into citrate by citrate synthase (*CSY*) and then transported to the mitochondria for respiration (Borek *et al.*, 2013). The glyoxylate cycle depends on substrates derived from FA oxidation and these are subsequently converted either into succinate or citrate by the TCA cycle or into phosphoenolpyruvate (PEP) by gluconeogenesis (Theodoulou and Eastmond, 2012). Usually, the TCA cycle for respiration is suppressed in favour of carbohydrate biosynthesis when the glyoxylate cycle is active in germinating seeds. During storage lipid breakdown, the seedling must adapt to the environmental conditions especially during the glyoxylate cycle to complete this mobilization efficiently and to reach the photoautrophic phase before the exhaustion of reserves (Eastmond and Graham, 2002).

The gluconeogenesis pathway occurs in the cytosol and its starting step is characterized by the conversion of oxaloacetate into phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (*PCK*), a key gluconeogenesis enzyme (Rylott *et al*., 2003). PEP is then converted into G3P which can be converted into soluble carbohydrates like hexose which can be used for cell wall synthesis or sucrose for the support of seedling development (Borek *et al.*, 2013; Graham, 2008; Kornberg and Beevers, 1957) (Figure 1).

1.3. Transition from germination to seedling establishment

As mentioned before, the transition from seed germination to seedling establishment is the crucial stage in the plant life cycle and its success in oilseed plants depends on the lipid mobilization which is performed by many enzymes and associated proteins (Graham, 2008). These enzymes and proteins require optimum environmental conditions for their structure and functional stability. Beside pH, the other most important factor affecting this stability is temperature. It is known that below the optimum temperature, the increase of temperature is associated with an increase of the functional rate of enzymes, while above this temperature there is enzyme denaturation accompanied with a decrease in reaction rate (Granjeiro *et al.*, 2004). Temperature also affects seed germination and plant physiology in a species specific manner (Bewley and Black, 1994). In this light it is clear why there is an optimum temperature for optimal seedling establishment in respect to enzymatic activity during seed germination and storage oil catabolism.

A previous study conducted at different temperatures (20, 25, 35°C) revealed that seed germination in castor bean was faster at higher temperature. However, seedling establishment was negatively affected by harsh temperature (personal communication Paulo, 2014) (Figure 2).

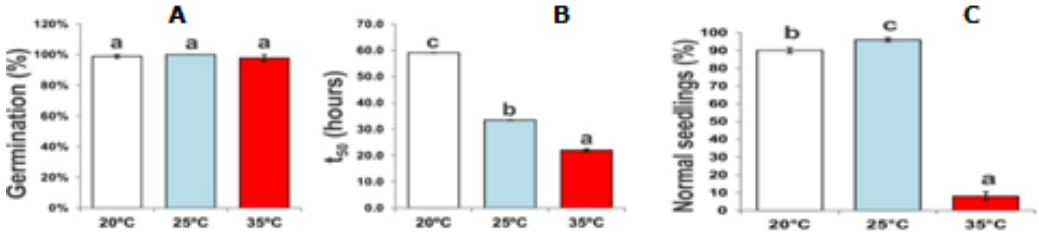


Figure 2. Effect of temperature on seed germination (percentage and speed) (A and B) and seedling health (C) in R. communis. Error bars were put though the averages of treatments and different letters indicate significant difference between means of treatments.

Beside seed germination and seedling health, different temperatures were also reported to affect the expression of genes which encode enzymes involved in storage oil mobilization to ensure the success of castor bean seedling development. Although the expression of genes encoding for *ICL*, *MFP*, *LACS6/7* and *KAT* enzymes were considerably similar at different temperatures during the post-germinative stage, other enzymes like *ACX*, *GLI1* or *GK1*, *MLS* and *PCK* were lower expressed at 35°C (Figure 3) (Personal communication Paulo, 2014).

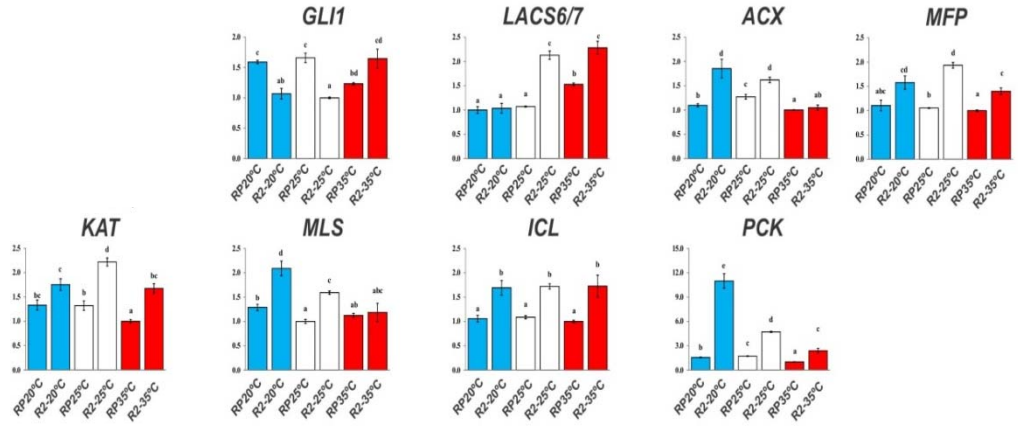


Figure 3. Effect of temperature on gene expression during the germintaion and postgerminative phase in R. Communis. Error bars were put through the averages of treatments and different letters indicate significant difference between means.

It is reported that all these enzymes are highly regulated at both transcriptional and posttranscriptional level (Graham, 2008). Some factors behind this regulation are known while others are not well understood. It is also reported that there is an interaction between specific transcription factors and the physiological signals (Baud and Lepiniec, 2010). Penfield and colleagues (2004) reported that a disruption of genes encoding *GK*, *MLS* and *PCK* resulted to low hypocotyl elongation. Although all these genes were shown to affect seedling performance, none of the studies describe the effect of temperature on expression level of these genes during germination and how they could affect seedling performance.

Therefore, the aim of this study is to clone and characterize the function of genes encoding *GK*, *MLS* and *PCK* enzymes. The study will focus on these three genes that are considered to be the main enzymes involved in the storage oil breakdown especially during the glyoxylate cycle and gluconeogenesis and that are also affected by different temperatures. Therefore, their expression and function are expected to have higher impact on seedling establishment and post-germinative stages of castor bean. The goals of this project will be achieved by assessing the germinative and post-germinative behaviour of castor bean, cloning of the genes coding for *GK*, *MLS* and *PCK* in *R. communis* and their functional characterization in transgenic *Nicotiana benthamiana* plants.

This experiment will contribute to an understanding of the mechanism behind the expression of these enzymes at different temperatures and their function. The final outcome will be useful for the improvement of castor bean cultivars that can survive the crucial growth stage of seedling establishment under harsh environmental conditions at different locations in the world. A high post-germinative growth leads to a better plant development in the later stages and ultimately to a higher seed yield. The adaptation potential to different environments will also enable extension of growing areas and increasing yields. All these aspects will contribute to a high seed production to meet the world demand.

2. Materials and methods

2.1. Germination and post-germinative behaviour analysis

This study was conducted in the Wageningen Seed Lab from the lab of Plant Physiology at Radix (Wageningen University) from February until August 2014. Seeds of *R. Communis* L. cv MPA11 were surface sterilized by dipping them in a solution of bleach and demi water (1:4 ratio). Subsequently, the seeds were germinated in trays containing white blotter filter-paper with addition of 30 ml of demi-water and covered with an extra filter-paper on which 20 ml of water was added. Seeds were incubated at three different temperatures (20, 25 and 35°C) in the dark. For every temperature, four replicates of fifteen seeds each were used. Watering and counting of germinated seeds was done twice a day for two weeks. The number of healthy seedlings was counted one and two weeks after the beginning of imbibition. After two weeks cotyledons and roots from the healthy seedlings were collected and kept at -80°C prior to further analysis. The analysis of data from seed germination and seedling health experiments was performed by excel and GenStat softwares for Analysis of Variance (ANOVA) and Student t-test to determine the difference in parameters due to treatments at 5% level of significance.

2.2. RNA extraction and DNAse treatment

Plant materials were freeze-dried for two days before RNA extraction. RNA extraction was performed with the hot-Borate protocol as described by Jawdat and Karajoli (2012). The hot-borate buffer was made with 50ml DEPC in which were added 3.81 g of 0.2M Na borate, 0.57 g of 30mM EGTA, 0.5 g of SDS and 0.5 g of Na-DOC. This buffer was used to make the extraction (XT) buffer by adding 264mg PVP and 8.8mg DTT to 4.4 ml of hot-borate buffer. The buffer was incubated at 80°C to enable complete dissolution. Ten milligrams of freeze-dried and ground roots or cotyledons were mixed with 800 µl XT-buffer. Then, after adding 4 µL proteinase K (ca. 0.3 mg/µL), samples were incubated at 42°C for 15 minutes. Subsequently, 64 µl of 2M KCl was added and samples were incubated on ice for 30 minutes followed by a centrifugation at 12000g at 4°C for 20 minutes. 700 µL of supernatant was transferred to a new tube and 259 µL of 8M LiCl was added and incubated overnight on ice.

Subsequently, samples were centrifuged at 12000g at 4°C for 20 minutes and the remaining pellet was washed with 750 µL of 2M LiCl. The pellet was resuspended in 100 µL DEPC-treated water. In order to remove genomic DNA, 10 µg of RNA was subjected to a DNAse treatment by adding 10 µL of DNAse buffer and 10 µL of DNAse enzyme (Table 1).

Table 1. Calculated volume (µL) of RNA based on concentration (ng/µL) and mass (10µg).

Treatment (°C)	Plant materials	RNA concentration (ng/ µL)	RNA mass (µg)	RNA volume (µL)
20	Roots	272.9	10	36.64
	Cotyledons	229.1	10	43.65
25	Roots	191.7	10	52.16
	Cotyledons	147.9	10	67.61

In the next step, samples were incubated at 37°C for 20 minutes. Then, we added 100 µL phenol chloroform and after a new centrifugation for 30 seconds. Approximately 90 µL of the upper phase was transferred to a new Eppendorf tube in which 9 µL of 3M NaAc and 225 µL of 100% ice-cold ethanol was added and RNA precipitated overnight at -20°C. The pellet was washed with 250 µL cold 70% ethanol and subsequently dissolved in 20 µL DEPC-treated MQ water. RNA concentration and quality control were assessed by using Nanodrop spectrophotometer and RNA integrity was checked by agarose gel electrophoresis.

2.3. cDNA synthesis

cDNA synthesis was performed by using a cDNA synthesis kit (Biorad) for first-strand cDNA synthesis. It contained the RNase H^+ iScript reverse transcriptase for sensitivity, a premixed RNase inhibitor to prevent indiscriminate degradation of RNA template. First, a reaction solution was prepared by mixing 4 µL 5x iScript reaction mix (buffer), 1 µL iScript Reverse Transcriptase enzyme, RNA template volume calculated from the RNA concentrations obtained on NanoDrop in order to use 1µg RNA and nuclease free water (DEPC) to get a total volume of 20 µL (Table 2).

Table 2. Composition of solution for cDNA synthesis

Treatment (°C)	Plant materials	RNA mass (µg)	RNA concentration (ng/ µL)	RNA template volume (µL)*	Nuclease free water (µL)
20	Roots	1	417.7	2.4	12.6
	Cotyledons	1	186.3	5.4	9.6
25	Roots	1	184.2	5.4	9.6
	Cotyledons	1	400.4	2.5	12.5

* Equation for calculating RNA template volume: **V=M /C** ; where V: Volume (µL), M: Mass (µg) and C: concentration (ng/µL)

Reverse transcription was performed at 37°C for 40min followed by 85°C for 5min. The cDNA was diluted 40 times and stored at -20°C prior to the analysis. *RcNIC1* primer was used as control to check the synthesis of cDNA (Table 3).

Table 3. Sequence of *R. Communis* nicotinamidase1 (*RcNIC1*), a primer used for cDNA amplification

Gene name	Primer number	Primer name	Annealing temperature (°C)	Primer sequence
GI:255586656||ref|AT2G22570.1| *Ricinus communis* nicotinamidase1	1529	RcNIC1_cloning F	59	ATG GTC TCC TCC ACG GTT GAA TTG
	1530	RcNIC1_cloning R	59	CTA TTG CAG TGC ACT AAC AGA CAC CTC
	Full length of coding region of *NIC1*=732 bp			

2.4. Primer design

The identification of candidate genes related to glycerol kinase (*GK*), malate synthase (*MLS*) and phosphoenolpyruvate carboxykinase (*PCK*) in *R. communis* and their DNA sequences was performed through NCBI software in which BLAST program was used to get candidate genes from those of *Arabidopsis thaliana* species. On the basis of the DNA sequences, forward and reverse primers without and with attB sites were designed (Table 4). Primer amplification was assessed at different temperatures by a Bio-Rad thermal cycler machine and agarose gel electrophoresis.

Table 4. Primer sequence without attB sites for normal PCR.

Gene name	Base pair (bp)		Primer number	Primer name	Annealing temperature (°C)	Primer sequence
	Full	Coding region				
>gi|255553276|ref|XM_002517635.1| *Ricinus communis* glycerol kinase, putative, mRNA	1566	1566	2169	RcGLI cloning_F	53.4-53.5	ATG GCA AAA CAG GAA CCA G
			2170	RcGLI cloning_R		TCA TAT TGA AAG ATC AGC CAA GC
>gi|255540320|ref|XM_002511179.1| *Ricinus communis* malate synthase, putative, mRNA	1812	1704	2171	RcMLS1 cloning_F	52.8-52.0	ATG ATG CGA TAT GAT ACT TAT GGT G
			2172	RcMLS1 cloning_R		ACT TAT TCA CAG CCT AGA TGA TC
>gi|21075|emb|X52806.1| *Ricinus communis* mRNA for malate synthase (EC 4.1.3.2)	1826	1704	2173	RcMLS2 cloning_F	53.7-53.4	TGA TGC GAT ATG ATA CTT ATG GTG A
			2174	RcMLS2 cloning_R		GAA ATC AAC ATT ATT CAC AGC CTA GA
>gi|255537863|ref|XM_002509951.1| *Ricinus communis* Phosphoenolpyruvate carboxykinase [ATP], putative, mRNA	2333	1986	2175	RcPCK1 cloning_F	54.2-52.2	ATG GCG GAG AAT GGA GAG
			2176	RcPCK1 cloning_R		TCA AAA ACC AAT CTG ATC AGA GAT A
>gi|255576016|ref|XM_002528858.1| *Ricinus communis* Phosphoenolpyruvate carboxykinase [ATP], putative, mRNA	2001	2001	2177	RcPCK2 cloning_F	58.2-57.5	ATG GCG ACC AAC GGC AAT
			2178	RcPCK2 cloning_R		TTA GAA ATT CGG ACC AGC TGC C

The sequences of primers attB sites which were used for DNA amplification were 5'-GGGG ACA AGT TTG TAC AAA AAA GCA GGC T-3' for forward direction and 3'-GGGG AC CAC TTT GTA CAA GAA AGC TGG GT-5' for reverse direction.

2.5. Gene amplification and purification

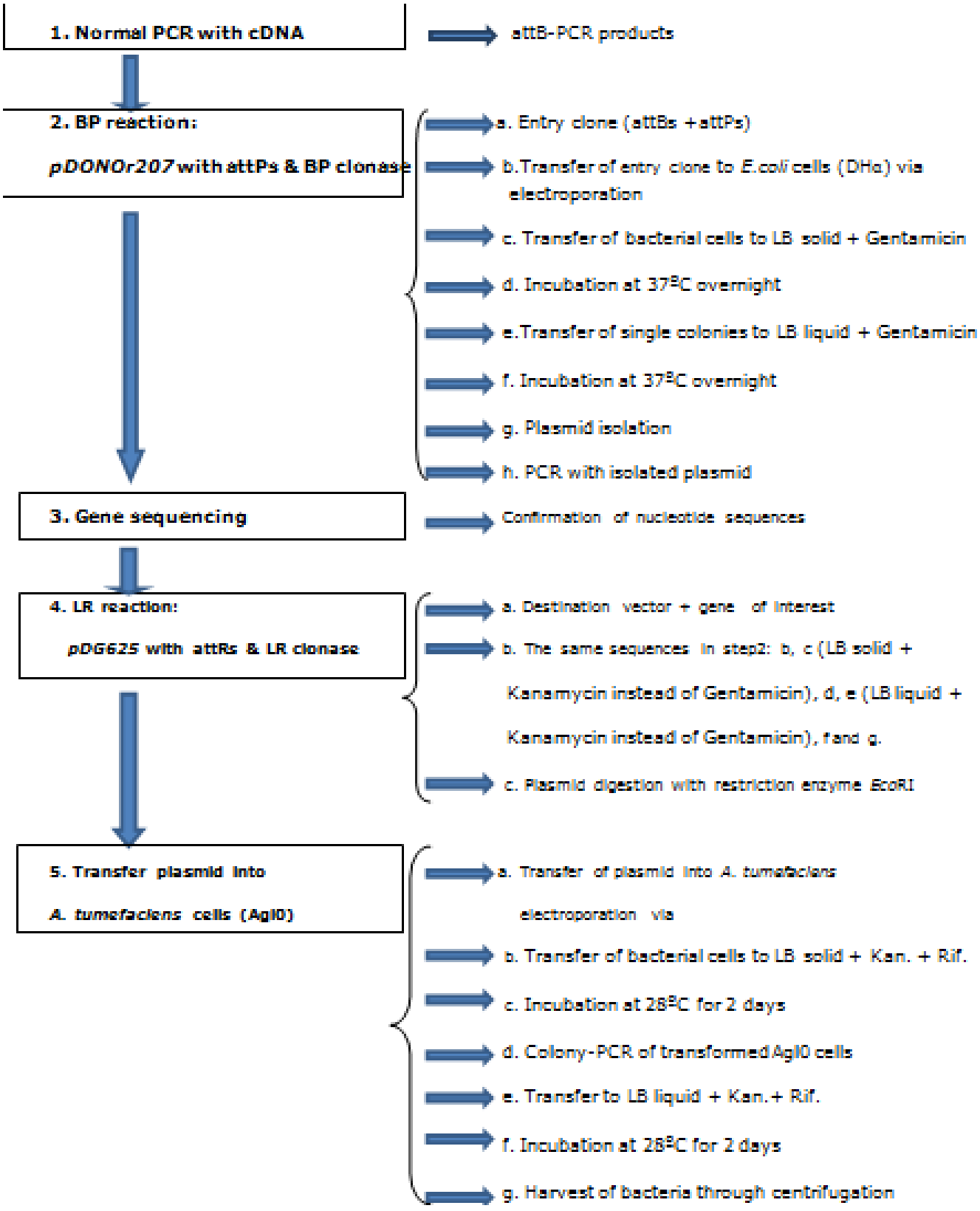
Amplification of the full length coding region of the candidate genes was done with a total volume of 15 µL solution for normal PCR reactions composed of 1.5 µL 10x Buffer B, 1.5 µL of 25mM MgCl2, 6.65 µL DEPC, 0.15 µL Tag polymerase enzyme (firepol) (5U/µL), 0.4 µL of 10mM dNTPs, 4.0 µL cDNA and 0.8 µL of primer (10µM). In addition, the PCR was also done by using phusion polymerase (2U/µL) enzyme and primers with attBs. PCR was first performed by firepol

polymerase, an enzyme with high thermo-stability and polymerization and then we used phusion because it is a High-Fidelity DNA Polymerases which is used to know if a correction is needed in DNA sequence. During PCR amplification, DNA denaturation, primer annealing and polymerase reaction were performed by different thermal conditions. The exposure of the solution to high temperature (95°C) allowed DNA denaturation into single-strands. When the temperature was decreased to an annealing temperature according to the gene, the primer annealed to specific nucleotides (their complementary sequences). Raising the temperature to 72°C enabled optimal functioning of the Tag polymerase or phusion polymerase by adding nucleotides for synthesis of the second DNA strand. After PCR amplification, samples were analysed by 1% agarose gel electrophoresis for cDNA fragment characterization and quality control.

Gene purification was performed by a QIAquick Gel Extraction Kit method following the manufacturer's instructions. First, amplified fragments with desired size were cut from the gel under UV light. Subsequently, an addition of 3 volumes of QIAquick Gel Extraction Kit buffer (QG) to 1 volume of gel slice was followed by incubation at 50°C and regular mixing for a complete dissolution. Subsequently, 500 µL of isopropanol was added and the solution was applied to the QIAquick column and centrifuged for 1 minute. The column was washed with 750 µL of buffer PE followed by centrifugation. Finally, the column was transferred to a new microcentrifuge tube and 20 µL of MQ water was added to the centre of membrane and centrifuged once to elute the plasmid. The concentration of purified fragment was measured with a NanoDrop spectrophotometer.

2.6. Gene cloning

Gene cloning was performed by Gateway technology. The production of clones was performed by enabling a rapid and efficient recombination of DNA fragments of target genes with the donor and destnation vectors (pDONr207 and pGD625 respectively) through a site-specific recombination property. Moreover, gene cloning ensured a transfer of recombinants DNAs into the host (*E.coli*) in which they replicated. With this technology four main steps were done: cloning of attB products via BP reaction, gene sequencing, LR reaction and transfer of targeted genes into *Agrobacterium tumefaciens* (Agl0 strain) (Figure 4).



Kan.= Kanamycin; Rif.= Rifampicin

Figure 4. Different steps in Gateway cloning technology

2.6.1. Cloning of attB products via BP reaction

During the BP reaction, we mixed 1 µL of donor vector (*pDONr 207*) (100ng/µL), 2 µL of TE buffer, 1 µL of attBs PCR product and 1 µL of BP clonase. The later catalyzed the recombination and insertion of attBs of the PCR product (expression clone) into attP recombination sites in the donor vector to make the entry clone with attL sites. The total volume of 5 µL was centrifuged for 20 seconds and incubated at 25°C overnight. This step was followed by an addition of 1 µL of Proteinase K (3-15U/mg) and incubation at 37°C for 10 minutes.

Once the entry clone was formed it was transferred to *E. coli* (DH5α) cells via electroporation. For this step, 1 µL of plasmid was mixed with electro competent *E.coli* cells followed by transformation by electroporation at 2.5 voltages (2 mm cuvette). The transformed cells were mixed with 400 µL of LB liquid medium and incubated at 37°C for 1 hour to allow bacteria from electroporation shock to recover. These cells were transferred to plates containing LB solid medium and gentamicin (25 µg/mL). This antibiotic ensures the growth of only transformed bacteria which contain plasmids able to resist to it. The next day, single colonies from each plasmid were transferred to both LB liquid and solid media also containing gentamicin. The mixtures were then incubated at 37°C overnight.

Before plasmid isolation, glycerol stock of each gene was made by adding 750 µL of transformed *E.coli* cells containing target gene to 250 µL of glycerol (60%) solution. Those glycerol stocks were immediately transferred to liquid nitrogen and stored at -80°C for further usages. Then, plasmid isolation was performed by QIAprep Spin Miniprep Kit High-Yield method. 3.25 mL medium from each plasmid was centrifuged at >8000rpm for 3 minutes followed by addition of 250 µL of both Buffer P1 and Buffer P2 to the pellet and resuspension of the pellet by mixing until no clumps of cells remained.

After incubation for 5 minutes at room temperature, 350 µL of Buffer N3 was added followed by vortexing, centrifugation for 10 minutes and transfer of the supernatant to a QIAprep spin column. After centrifugation the column was washed with 500 µL Buffer PB. Next, the column was transferred to a new microcentrifuge tube and 40 µL of MQ-water was added to its membrane. After incubation for 1 minute, the tubes were centrifuged for 1 minute to collect the plasmid and the concentration was measured by NanoDrop.

2.6.2. Gene sequencing and LR reaction

After a normal PCR with isolated plasmids, positive plasmids were sequenced by mixing 5 µL forward or reverse primer (10µM) for *pDONR207* with 5 µL of plasmid diluted with MQ water regarding the DNA concentrations to get 100ng of DNA. Sequencing was performed by Macrogen Europe and the results were compared with the sequences of the reference genes in the database with CLC bio. Positive plasmids were subjected to LR reaction. An LR reaction was used in a similar way as the BP reaction to generate a destination vector containing the gene of interest with the difference that we used LR clonase instead of BP clonase and the destination vector *pDG625* with attR sites instead of *pDONR207* with attP sites. Another difference was that after transferring plasmids from LR reaction into *E.coli* through electroporation, the bacterial cells were transferred to plate containing LB solid medium with kanamycin instead of gentamicin. This was also followed by transfer of transformed single colonies into LB liquid with kanamycin and plasmid isolation as described above.

2.6.3. Plasmid digestion with restriction enzyme

In the next step, different isolated plasmids containing target genes were digested with restriction enzyme (Table 5). Restriction enzyme used was EcoRI while the empty vector used as control was *pDG625*. In principle, this was performed by mixing XXXµL of MQ, 500ng of DNA, 1 µL of enzyme and 2 µL of Buffer CutSmart in order to get total volume of 20 µL. After mixing, the solution was kept at 37°C for 1 hour and heated at 65°C for 10 minutes. Plasmid fragments (number and size) of transformed cells and empty vector were confirmed through 1% agarose gel electrophoresis.

Table 5. Volume of different components used for gene digestion with restriction enzyme

Gene/vector	Clone	Concentration (ng/µL)	Volume of plasmid (µL)	MQ (µL)	Buffer (µL)	Enzyme (EcoRI) (µL)	Total volume (µL)
MLS1	*MLS1-3-1*	111.2	4.5	12.5	2	1	20
	MLS1-3-2	96.2	5.2	11.8	2	1	20
	MLS1-3-3	77.9	6.4	10.6	2	1	20
pDG625*	*pDG625-2*	83.9	5	12	2	1	20
GK	*GK-2-5*	31.4	10	-	1.2	1	12.2
	GK-3-2	21.0	10	-	1.2	1	12.2
PCK1	*PCK1-2-1*	27.6	10	-	1.2	1	12.2
	PCK1-2-2	27.5	10	-	1.2	1	12.2
pDG625*	*pDG625-2*	83.9	5	5	1.2	1	12.2

*: Plasmid which was used as control for digestion with restriction enzyme

2.6.4. Gene transfer into *Agrobacterium tumefaciens*

The last step of gene cloning concerned the transfer of plasmids from digestion into *Agrobacterium tumefaciens* (Agl0 strain) via electroporation 2.5V (2mm cuvette) after mixing 1 µL of plasmid with Agl0 competent cells. Then, the bacteria were kept at 28°C for 1 hour for recovery followed by transfer to plate with LB solid medium, kanamycin (100 µg/mL) and rifampicin (25 µg/mL) and incubation at the same temperature for 2 days. After colony-PCR which was done to confirm the presence of desired genes into *Agrobacterium* cells, positive bacterial colonies were transferred to LB liquid medium containing the same antibiotics and grown at 28°C for 2 days for further usages in plant transformation.

2.7. Transient expression

2.7.1. Bacteria harvesting

To characterize the function of the cloned genes, transient expression was performed in *N. benthamiana* leaves with the help of *Agrobacterium tumefaciens.* The bacteria containing the plasmids with targeted genes (Agl0-*GK,* Agl0-*MLS* and Agl0-*PCK*), Agl0-EV (empty vector *ImpactTim1.1)* and Agl0-*P19* were harvested from LB liquid medium by centrifugation at 5000g at 20°C for 15 minutes and Agro-infiltration buffer was added to each to obtain an OD_{600} of 0.5. This buffer was previously prepared by mixing 952.11mg $MgCl_2$ (500mM), 2132 mg of MES (500mM) and 1L of distilled water and adjusted to a pH of 5.7 with 1N KOH before adding 1 mL Acetosyringone (100mM).

After that, 4 mL of *P19* was mixed with 4 ml of *GK, MLS* and *PCK* plasmids while 8 mL was used for empty vector. *P19* is a plasmid of Cymbidium ringspot virus (CymRSV) which contains doubles-stranded siRNAs (small RNAs). The accumulation of doubles-stranded siRNAs in plant results to RNA silencing in infected plant and subsequently to repression of defence signalling. *P19* was reported to have the ability of suppressing the accumulation of siRNA produced in Agro-infiltration assays (Lakatos *et al*, 2004). Therefore, *p19* can prevent plant defence against transfer of genes into plant via *A. tumefaciens*.

2.7.2. Agro-infiltration in *Nicotiana benthamiana*

Agro-infiltration consists of injecting target genes in plants by using *Agrobacterium*. This was performed by infiltrating the bacteria into the 4-week-old *Nicotiana benthamiana* leaves. After that, plants were allowed to grow under greenhouse conditions for 1 week. Agro-infiltration was achieved by four treatments such as Agl0-EV (empty vector), Agl0-*PCK,* Agl0-*GK* and Agl0-*MLS* (leaves infiltrated with *Agrobacterium* Agl0 strain containing empty vector *ImpactTim1.1* used as control, *PCK*, *GK* and *MLS* cloned genes respectively). The empty vector used is *ImpactTim 1.1* which was previously modified to make it non-toxic to plant. Each treatment was replicated six times (six plants) and in each replication two leaves were infiltrated.

2.7.3. Metabolite extraction

Sampling was done one week after infiltration then leaf samples were put in freeze drier for two days, and after, they were ground. The metabolites were analysed in four samples such as Agl0-EV, Agl0-*PCK*, Agl0-*GK* and Agl0-*MLS.* The extracts from infiltrated leaves were used for analysis of metabolites such as glycerol, glycerol-3-phoshpate, glyoxylate, malate, pyruvate and carbohydrates which are the main metabolites directly correlate to the studied genes (*GK*, *MLS* and *PCK*) in lipid mobilization pathway. However, they are extra metabolites also analyzed such as putrescine, succinate, oxalate, myo inositol, ethanolamine, 4-trans-caffeolyquinate, 3-trans-caffeolyquinate, 3-cis-caffeolyquinate, GABA, glucuronate, threonate, ascorbate, citrate, glutamate, maleate, xylitol, proline and quinate. Glycerol, glycerol-3-phoshpate, glyoxylate, malate and pyruvate and most of carbohydrates were analyzed with GC-MS using the online derivatisation protocol while a Dionex HPLC system with an ED40-pulsed electrochemical detector was used for starch and some sugars.

GC-MS analysis

By GC-MS protocol, three different extraction mixes (A, B and C) were prepared. Mix A was composed by 400 µL of MeOH and 200 µL of $CHCL_3$. Mix B was composed by 130 µL MQ water and 20 µL of ribitol (1mg/ mL) while mix C was composed by (1:1) 400 µL MeOH and 400 µL of $CHCL_3$. Metabolite extraction was performed by mixing 5mg of ground materials with 175 µL of mix A followed by a brief vortex and addition of 37.5 µL of mixture B. After a new vortex, the solution was put in Ultrasonic bath for 10 minutes to get metabolites out of plant tissues. Then, an addition of 50 µL of MQ-water, vortex and spin for 5 minutes at 20°C at 15000rpm was achieved. This step was followed by the transfer of 150 µL of upper (polar) phase to a 2 mL

Eppendorf tube followed by an addition of 125 µL of mix C. After vortex, the samples were kept on ice for 10 minutes and 50 µL of MQ-water was added. Centrifugation for 5 minutes at 15000rpm at 20°C was achieved followed by a new collection of 90% upper phase and mixing it with the previously collected one. Then, 20 µL of polar phase was transferred to 1.5 mL glass GC-vials and dry overnight.

Dionex HPLC analysis

Dionex HPLC system was mainly used for starch quantification and this was achieved from the remaining pellets after extracting some sugars like sucrose, fructose and glucose. The remaining pellets were washed 4 times with 1 mL of MQ water (addition of 1 mL MQ water followed by a centrifugation at 14000rpm for 5 minutes and removal of supernatant). The residual water was removed from the pellets prior to further analyses. In the next step, in dry pellets we added 50 µL of 8N HCl and 200 µL of DMSO and we put the solution in the shaker at 60°C for 1 hour for homogenisation of the mixture. After that, we added 150 µL of MQ water, 40 µL of 5N NaOH and 185 µL of citric acid buffer and we vortexed.

Then, this mixture was centrifuged at 14000rpm for 5 minutes and 100 µL of supernatant was transferred to new tubes. This was followed by an addition of 20 µL of AGS enzyme. Next, the samples were put in water bath at 40°C for 1 hour. After that, 95 µL of supernatant was transferred to Eppendorf vials in which we added 5 µL of lactose which served as internal standard to make a correction of any problem that could happen during the procedure and after a vortex, the samples were put in Autosampler which is connected to Dionex HPLC system for starch abundance analysis. The total number of samples was 12 (4 samples replicated three times: empty vector, *PCK*, *GK*, *MLS*). In this extraction we used 10 different concentration of starch (2, 4, 6, 8, 10, 20, 40, 60, 90, 100 µg/mL) as calibration curves for the calculation of absolute starch concentration in our samples and we also used 12 blanks (used as reference to set the measurement to zero in order to prevent any errors that could happen during measurements. The analysis of data from metabolite extraction was performed by excel and Student t-test to determine the difference in parameters due to treatments at 5% level of significance.

3. Results

3.1. Seed germination and seedling performance

Castor seed germination percentage was significantly affected by the temperature (P = 0.022) (Appendices). It increased with increasing temperature. Thus, seed incubation at 20°C led to the lowest germination percentage (73.3%), while incubation at 25 and 35°C resulted to the highest germination percentage (91.67 and 90.00%, respectively). Therefore, there was a significant difference between seed germination percentage at 20 and 25°C and between 20°C and 35°C. However, no significant difference of germination percentage between 25 and 35°C was observed (Figure 5). From this result, both 25 and 35°C are reported to be the optimum temperature in respect to germination percentage in castor bean.

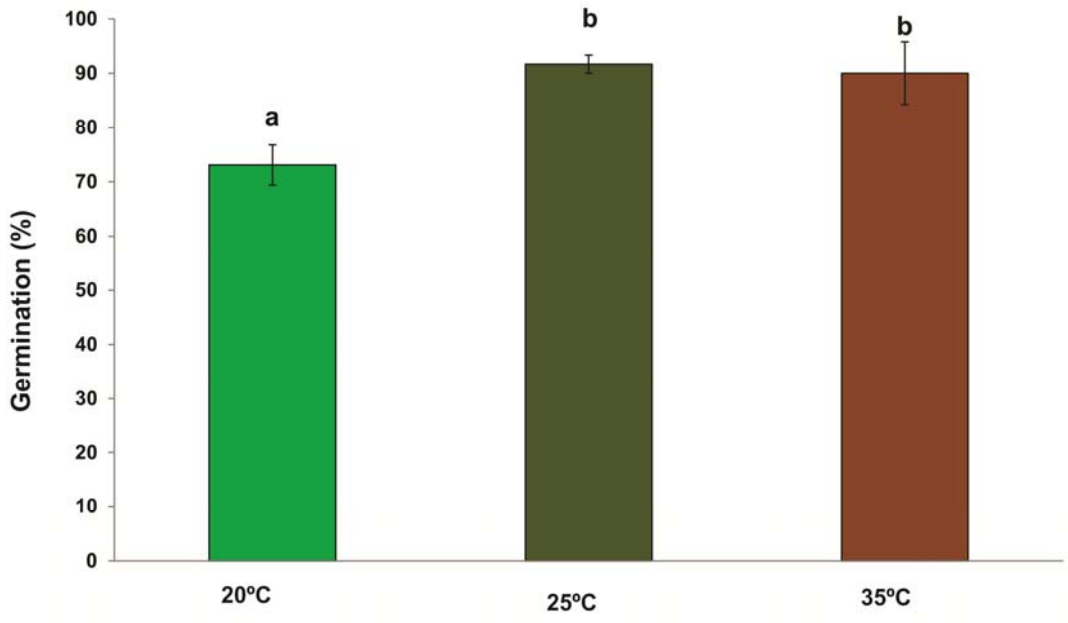


Figure 5. Temperature effect on castor seed germination percentage. Seeds germinated at 20, 25 and 35°C. Average and standard deviation are presented. Different letters represent significant difference between treatments.

Temperature also affected *R. communis* seedling estblishment for both one week after incubation (1WAI) and two weeks after incubation (2WAI) (*P<.001*) (Appendices). Percentage of normal seedlings decreased with increasing temperature. Therefore, the lowest normal seedling percentage was found at 35°C (66.7 and 0% for 1WAI and 2WAI respectively) followed by 25°C (76.4 and 67.3% for 1WAI and 2WAI respectively) while seedling growth at 20°C resulted in the

highest percentage of normal seedlings (97.7 and 84.1% for 1WAI and 2WAI respectively) (Figure 6).

Percentage of normal seedlings decreased between 1WAI and 2WAI samples (P*<.001*) (Appendices). Especially, all seedlings growth at 35°C died two weeks after incubation. Temperature of 20°C results in healthier seedlings during early stages following the seed germination in *R. communis* followed by 25°C while 35°C is the harshest temperature for seedling establishment. As discussed previously, key enzymes involved in lipid breakdown (*GK, MLS* and *PCK*) are necessary to provide energy required in early seedling growth and the expression of these genes are affected by temperature. Thereby, they require optimum temperature for maximum activity and this could explain why high temperature resulted in low seedling performance.

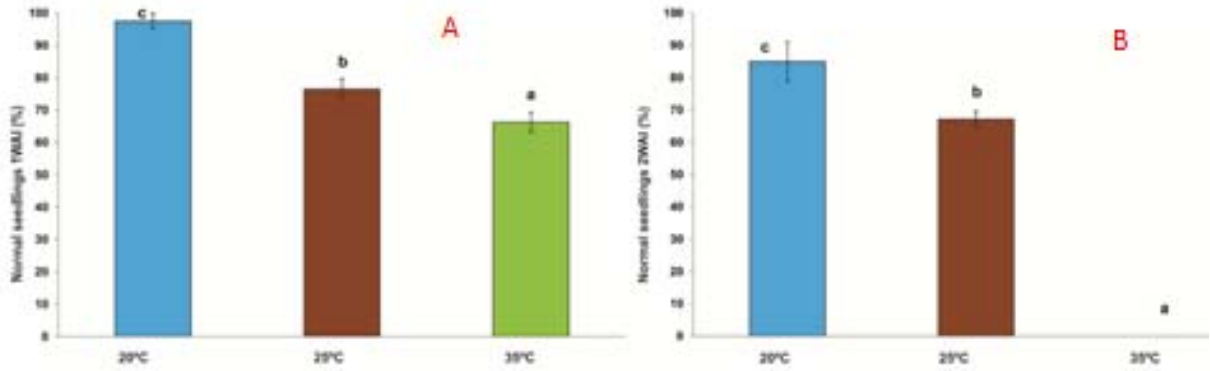


Figure 6. Temperature effect on seedling performance in R. communis. The percentage of normal seedlings was calculated in three different treatments (20, 25 and 35°C) one week after incubation (1WAI) (A) and two weeks after incubation (B). Average and standard deviation are presented. Different letters represent significant difference between treatments.

Although we did not measure root weight, it seems that 1WAI seedlings grown at 25 and 35°C produced higher root mass compared to the ones grown at 20°C. 2WAI seedling grown at 25°C produced higher root biomass compared to 20 and 35°C (Figure 7), suggesting that 25°C is the best temperature for seed germination and seedling growth in terms of biomass production.

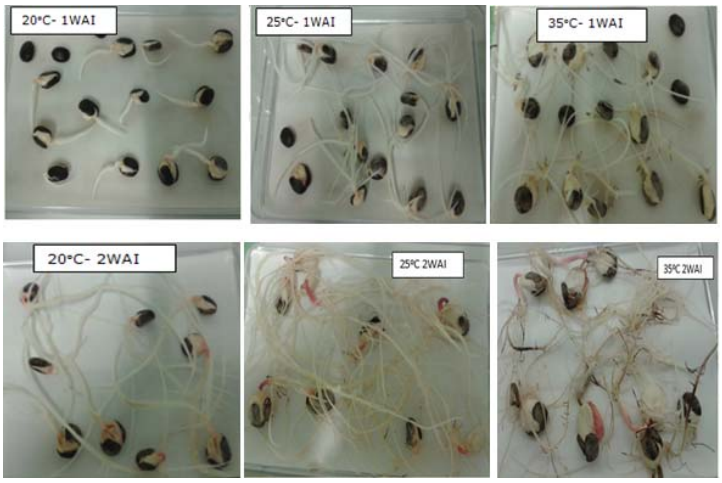


Figure 7 . Temperature effect on post-germination behavior of castor seedlings. Seedlings physiology was checked in three different treatments (20, 25 and 35°C) and also the difference between treatments over time (1WAI and 2WAI: One and two weeks after incubation).

3.2. RNA extraction

Agarose gel electrophoresis with extracted RNA showed visible RNA bands in plant materials (roots and cotyledons) in all treatments (20, 25 and 35°C). However, higher intensity of RNA bands were produced from roots which were grown at 20°C compared to that of plant materials which were collected from 25°C (Figure 8).

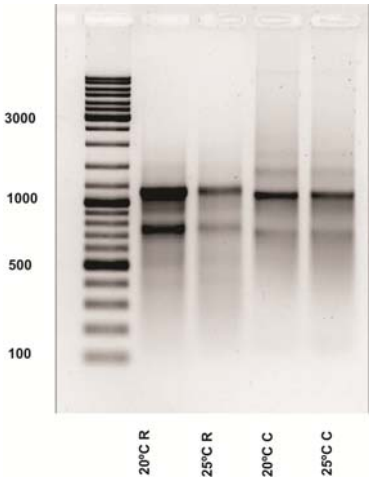


Figure 8. RNA bands in roots (R) and cotyledons (C) of R. communis by agarose (1%) gel electrophoresis. Plant materials were collected from seedlings of two weeks old which were grown at 20 and 25°C.

Further RNA quality control was verified by NanoDrop spectrophotometer (Table 6). A260/A280 and A260/A230 ratios are considered as the most important parameters to analyze RNA purity (Carvalhais *et al.,* 2013; Chedea *et al.*, 2010). These ratios varied between 1.96 and 2.23 for root samples collected at 20 and 25°C respectively. Another parameter for RNA quality evaluation was RNA concentration. This varied between 184.2 and 417.7ng/µL for root samples collected from seedlings grown at 25 and 20°C respectively (Table 6). From absorbance ratios and RNA concentration, all plant materials contained pure RNAs and were used in further gene cloning.

Table 6. RNA concentration (ng/µL) and purity (Absorbance ratios) of roots and cotyledons samples

Treatment (°C)	Plant materials	RNA concentration (ng/µL)	RNA purity (Absorbance ratio)	
			260/280	260/230
20	Roots	417.7	1.96	2.01
	Cotyledons	186.3	2.08	2.16
25	Roots	184.2	2.17	2.13
	Cotyledons	400.4	2.09	2.23

3.3. cDNA synthesis, gene amplification and purification

3.3.1. Gene amplification and test of primer performance

Initial, gene amplification was performed by using a gradient temperature and with primers which were specific to the target genes. In this study, we used primers for genes encoding glycerol kinase (*GK*), malate synthase (*MLS1* and *MLS2*) and phosphoenolpyruvate carboxykinase (*PCK1, PCK2* and *PCK3*) (Figure 9A and B). *GK* and *MLS1* were amplified at all temperatures except 60°C for *GK* and 52°C for *MLS1*. Moreover, *MLS2* was amplified at all temperatures but with intense DNA bands at 58.5, 53.9 and 52°C compared to 60°C. *PCK1* and *PCK2* were amplified at all temperatures, while no amplification of *PCK3* was observed. This indicates that both 58.5 and at 53.9°C temperatures enable amplification of all genes except *PCK 3*. We decided to skip *PCK3* candidate which did not show any amplification in all tested temperatures.

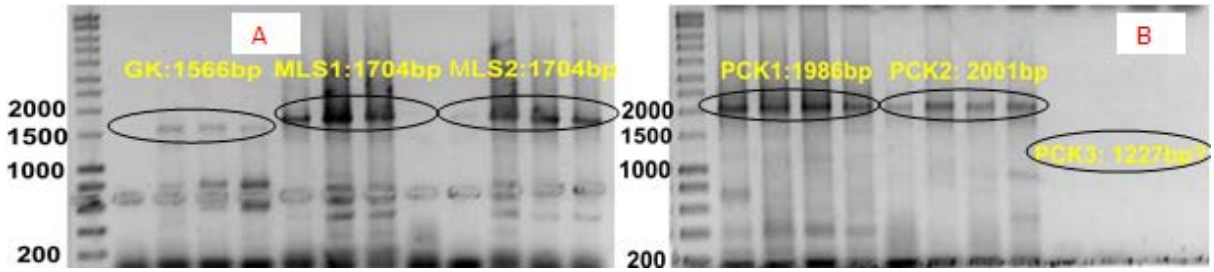


Figure 9. DNA amplification of GK, MLS1, MLS2 (1566, 1704, 1704bp respectively) (A) and PCK1, PCK2 and PCK3 (1986, 2001 and 1227bp respectively) (B) in R. communis. Normal PCR at gradient temperatures (60, 58.5, 53.9 and 52°C) was performed with specific primers. DNA used as template for amplification was the one from cotyledons at 20°C. Results from agarose (1%) gel electrophoresis.

PCR was also performed with phusion polymerase and primers which contained attB sites (Figure 10A and B). The primers with attB sites enable first the amplification of target genes and later these attBs serve as binding sites which help in the combination of PCR product with attP sites contained by donor vector during BP reaction in gene cloning to make an entry clone. Amplification of all target genes was observed.

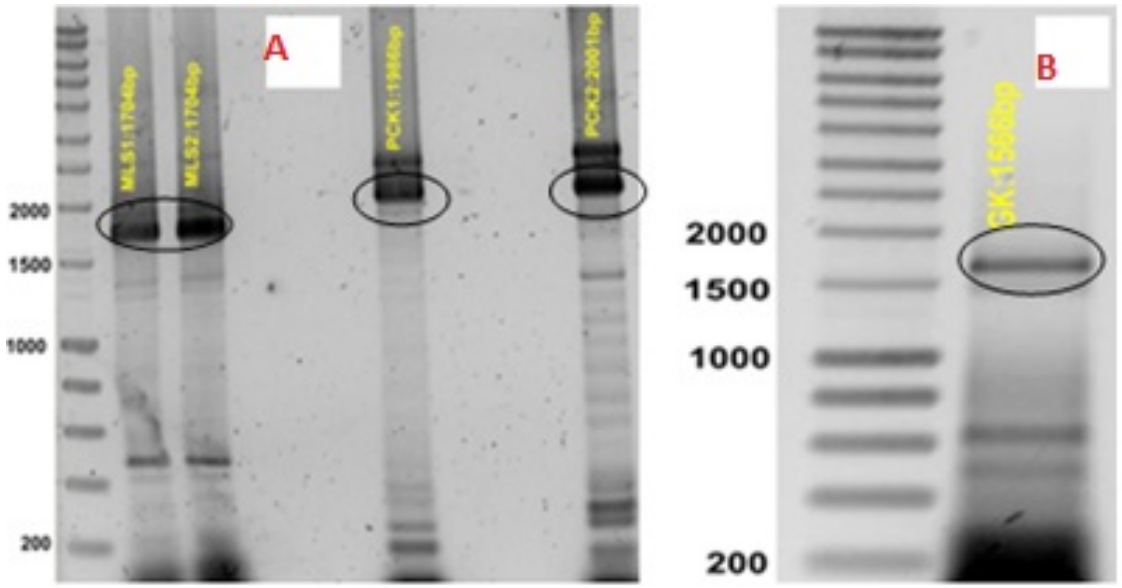


Figure 10. DNA amplification with phusion polymerase and primers with attB sites for MLS1, MLS2 (1704bp), PCK1 (1986bp) and PCK2 (2001bp) (A) and GK (1566,bp) (D). Annealing temperature was 58.5°C for both GK and PCK2, 52°c for both MLS2 and PCK1 and 60°C for MLS1. DNA used as template for amplification was the one in cotyledons from 20°C.

3.3.2. DNA fragments isolation and gene purification

Agarose gel fragments containing the targeted genes were used for purification of the amplified full-length sequence by using QIAquick kit according to the manufacturer's instructions. Concentration of isolated fragments ranged from 7.3 to 21.5 ng/µL (Table 7).

Table 7. Plasmid concentration (ng/µL) of purified gene measured in NanoDrop spectrophotometer.

Gene	Concentration of isolated plasmid (ng/µL)
GK	7.3
PCK1	9.0
PCK2	13.0
MLS1	16.1
MLS2	21.5

3.4. Gene cloning

3.4.1. BP reaction and gene transfer to *E. coli* competent cells

In gene cloning, plasmids are also called vectors (*pDONr207* in our case) and they contains a special region called multiple cloning site (attP sites) which ensures its recombination with DNA fragment and facilitates the transfer of plasmid into host cells (*E.coli*) (Hartley *et al*, 2000). Moreover, it contains a gene for resistance to specific antibiotic (gentamicin in our case) as one selectable marker and also the *CcdB* gene which is lethal to *E.coli* host cells as a second selectable marker (Walhout *et al*, 2000; Phillipe, 1996), since recombination of this vector with DNA fragment via BP reaction inactivates this toxic gene (Phillipe, 1996). Therefore, only bacterial cells which contained successful recombinant DNAs grew in different single colonies on the plate with gentamicin (Figure 11).

The isolated coding region sequences containing attB sites were subjected to a BP reaction (recombination of attB sites with attP sites). This reaction is responsible to transfer these fragments with attB sites into the donor vector *pDONr207* which contained attP sites. The recombinants were subsequently transferred into *E.coli* competent cells (DH5α strain) via electroporation and grown on plate with gentamicin for selection of the transformants. The highest number of transformed colonies was found in A (*GK*), B (*MLS1*), D (*PCK1*) while few transformed colonies were found in C (*MLS2*) and E (*PCK2*).

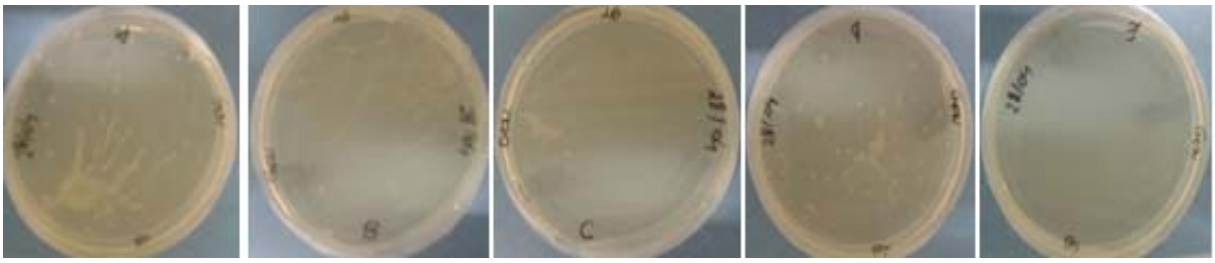


Figure 11. Single colonies of transformed genes with E. coli (DH5α strain) competent cells after BP reaction. Plates A, B, C, D and E contained transformed GK, MLS1, MLS2, PCK1 and PCK2 respectively.

Transformants were grown in LB liquid media with gentamicin. Then, after harvesting the cells by centrifugation, plasmids were extracted by using the QIAprep Spin Miniprep Kit High-Yield kit (2.6 section). Plasmid concentration was measured by NanoDrop and varied from 15.1 and 299.7 ng/μL (Table 8).

Table 8. Plasmid concentration (ng/μL) after BP reaction.

Gene	Clone	Plasmid concentration (ng/μL)
	GK_1	192.0
GK	*GK_2*	257.1
	GK_3	320.9
	MLS1_1	227.0
MLS1	*MLS1_2*	274.4
	MLS1_3	216.3
	MLS2_1	299.7
MLS2	*MLS2_2*	268.2
	MLS2_3	242.3
	PCK1_1	15.1
PCK1	*PCK1_2*	233.8
	PCK1_3	234.2
	PCK2_1	38.3
PCK2	*PCK2_2*	113.4
	PCK2_3	34.8

Moreover, a normal PCR with pDONR207 primer to amplify the coding region in isolated plasmids and agarose gel electrophoresis were performed to analyze the size of isolated plasmids. This was done to verify if the plasmids contained the genes of interest. All isolated plasmids except *PCK2*, one clone in *MLS1* and two clones in *PCK1* contained the target genes which were ready to be used for sequencing (Figure 12), although plasmid concentration varied

between samples. Plasmid concentration varied between 192.0 to 320.9 ng/µL in *GK*, 216.3 to 274.4 ng/µL in *MLS1*, 242.3 to 299.7 ng/µL in *MLS2*, 15.1 to 234.2 ng/µL in *PCK1* and 34.8 to 113.4 ng/µL in *PCK2* (Table 8).

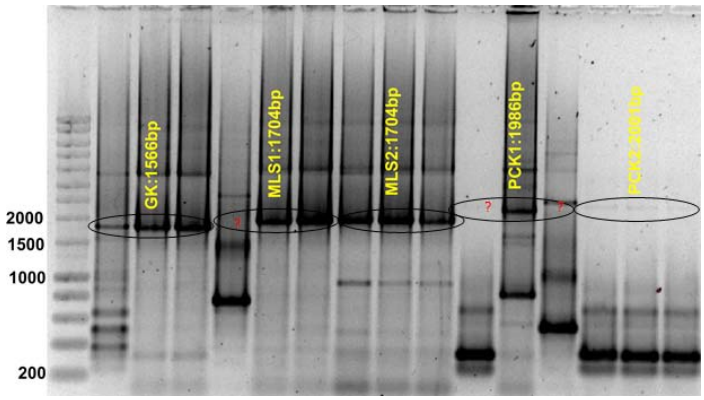


Figure 12. DNA bands of Isolated Plasmids of five genes (GK, MLS1, MLS2, PCK1 and PCK2) in R. communis. Three clones for each gene were used. DNA amplification with pDONr207 primer was performed through normal PCR and the results were shown via agarose gel electrophoresis.

3.4.2. Gene sequencing and LR reaction

Sequencing of the isolated plasmids was performed by Macrogen Europe Company. Positive results were found for pDONr207-*MLS1* and pDONr207-*PCK1*. It was observed that pDONr207-*MLS1* and pDONr207-*PCK1* and reference genes contained the same nucleotide sequences (1704bp and 1986bp respectively). Furthermore, translation to protein was also performed and amino acid sequence of cloned and reference genes was identical (Appendices).

Moreover, the nucleotide sequence of cloned pDONr207-*GK* gene was also identical to the reference gene except for a mutation in the nucleotide at 1354th position. The plasmid isolated from the transformants has a G (Guanine), while the reference sequence has a T (Thymine). In order to check whether this mutation would affect the amino acid sequence of the translated protein, we checked the translated amino acid sequences. No mutation in the amino acid sequence was found after translation of the nucleotide sequences.

The isolated plasmids (pDONr207-*MLS*, pDONr207-*GK* and pDONr207-*PCK*) were recombined with expression vector (pDONr207-*pDG625*) by LR clonase. Subsequently, these genes were transferred to *E.coli* competent cells and grown on plate with kanamycin. The expression vector contained a gene that make *E.coli* cells resistant to kanamycin and also the *CcdB* gene lethal to

these cells as selectable marker. It is also known that a successful recombination between two plasmids (entry clone and destination vector) results in inactivation of this *CcdB* harmful gene. Therefore, only recombined plasmids grew on plates with kanamycin. Two clones of *GK* (*GK_2* and *GK_3*), one of *MLS1* (*MLS1_3*) and one of *PCK1* (*PCK1_2*) were produced (Appendices).

Single colonies were transferred to LB liquid medium containing kanamycin and incubated at 37°C overnight. The concentration of the isolated plasmids varied from 18.9 to 111.2 ng/µL (Table 9).

Table 9. Plasmid concentration (ng/µL) of different genes from LR reaction.

Gene	Clone	Sub-clone	Plasmid concentration (ng/ µL)
GK	*GK_2*	*GK_2_1*	26.5
		GK_2_2	23
		GK_2_3	27.1
		GK_2_4	26.7
		GK_2_5	31.4
	GK_3	*GK_3_1*	44.8
		GK_3_2	21
		GK_3_3	20.3
		GK_3_4	28.2
		GK_3_5	28.3
MLS1	*MLS1_3*	*MLS1_3_1*	111.2
		MLS1_3_2	96.2
		MLS1_3_3	77.9
PCK1	*PCK1_2*	*PCK1_2_1*	27.6
		PCK1_2_2	27.5
		PCK1_2_3	20.6
		PCK1_2_4	18.9
		PCK1_2_5	26.6

3.4.3. Plasmid digestion with restriction enzyme and *A. tumefaciens* colony-PCR.

Plasmid digestion with restriction enzyme was used to confirm the presence of target genes in isolated plasmids after LR reaction. The digestion of cloned genes *GK* (*GK_2* and *GK_3*), *MLS1_3* and *PCK1_2* and empty vector (*pDG625*) with *EcoRI* restriction enzyme gives the following restriction fragments (Table 10 and Appendices).

Table 10. Number and size of plasmid fragments of transformed genes and empty vector with restriction enzyme (EcoRI).

Gene or vector	Restriction sites	Size of fragments (bp)
MLS1_3	2	12371 2952
GK_2	4	12371 1225
GK_3		1043 554
PCK1_2	3	12371 2155 1083
pDG625*	3	12371 1551 1524

* Used as empty vector for control

Plasmid digestion and fragment production were analysed by electrophoresis in agarose gel (1%) (Figure 13A and B). Therefore, all plasmids contained *MLS1* (Figure 13A) produced two fragments with 2952bp and 12371bp, nine out of ten plasmids of *GK* also produced four desired fragments with 554, 1043, 1225, and 12371bp while four out of five plasmids of *PCK1* also were positive with three desired fragments of 1083, 2155 and 12371bp. Hence, only one plasmid of *GK* and *PCK1* did not produce the desired fragments.

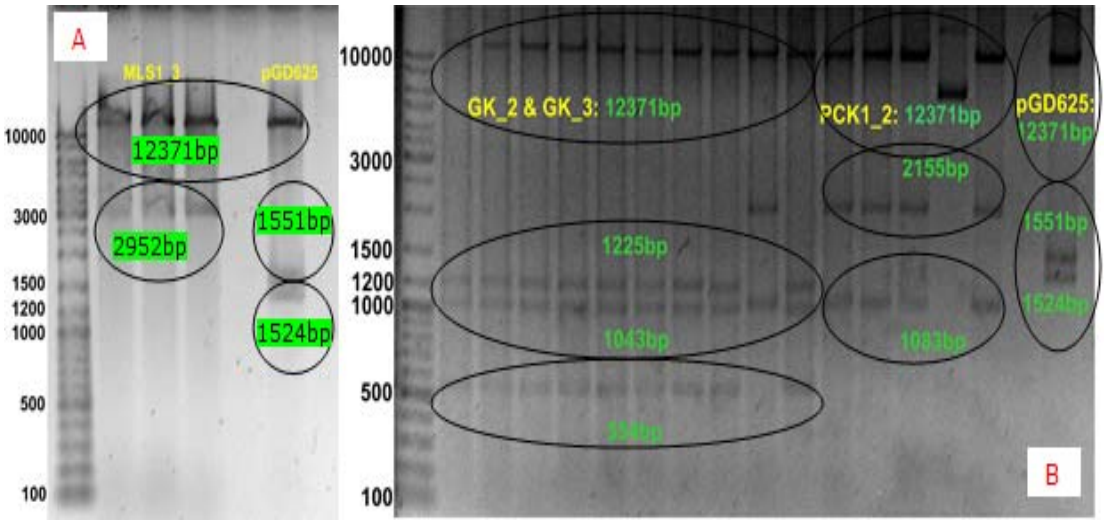


Figure 13. Restriction sites of MLS1_3 and pDG625 an empty vector used as positive control (A). DNA bands of GK_2, GK_3, PCK1_2 and pDG625 (B) after the digestion with EcoRI restriction enzyme. The results were shown via agarose gel electrophoresis after a normal PCR.

With a colony-PCR, all colonies presented the target genes (Figure 14).

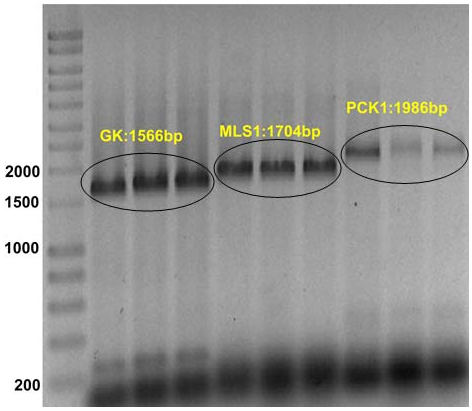


Figure 14. DNA bands of cloned GK_3_2, MLS1_3_2 and PCK1_2_1 after transformation with Agrobacterium tumefaciens (Agl0 strain). Three clones from each gene were used. The results were shown via agarose gel electrophoresis after a normal PCR. The specific primers without attB sites were used.

3.5. Metabolite profiling in transgenic *Nicotiana benthamiana*

Metabolite profiling in transgenic *Nicotiana benthamiana* showed that significant differences were only observed for a few metabolites: glucose, ethanolamine, myo inositol and starch (Figure 15). Glucose was accumulated in leaves infiltrated with Agl0-EV compared to leaves infiltrated with Agl0-*PCK*, Agl0-*GK* and Agl0-*MLS* cells. However, no difference in glucose was observed between leaves infiltrated with Agl0-*PCK*, Agl0-*GK* and Agl0-*MLS* cells. Higher content of starch was found in leaves infiltrated with Agl0-*GK* and Agl0-*MLS* while the lower level was observed in leaves infiltrated with Agl0-EV and Agl0-*PCK*. Ethanolamine was accumulated in Agl0-EV compared to the extracts from leaves injected with Agl0-*MLS*. However, no significant differences were observed between Agl0-*MLS* and the leaves infiltrated neither with both Agl0-*PCK* and Agl0-*GK* nor between Agl0-*PCK* and Agl0-*GK* and Agl0-EV. Myo inositol was accumulated in leaves infiltrated with Agl0-EV whereas the lowest level of myo inositol was found in leaves infiltrated with Agl0-*PCK* cells. However, no difference was observed between Agl0-*PCK* and the extracts from the leaves infiltrated with Agl0-*GK* and Agl0-*MLS* cells. Moreover, no difference in myo inositol was found between leaves infiltrated with Agl0-EV, Agl0-*GK* and Agl0-*MLS* cells.

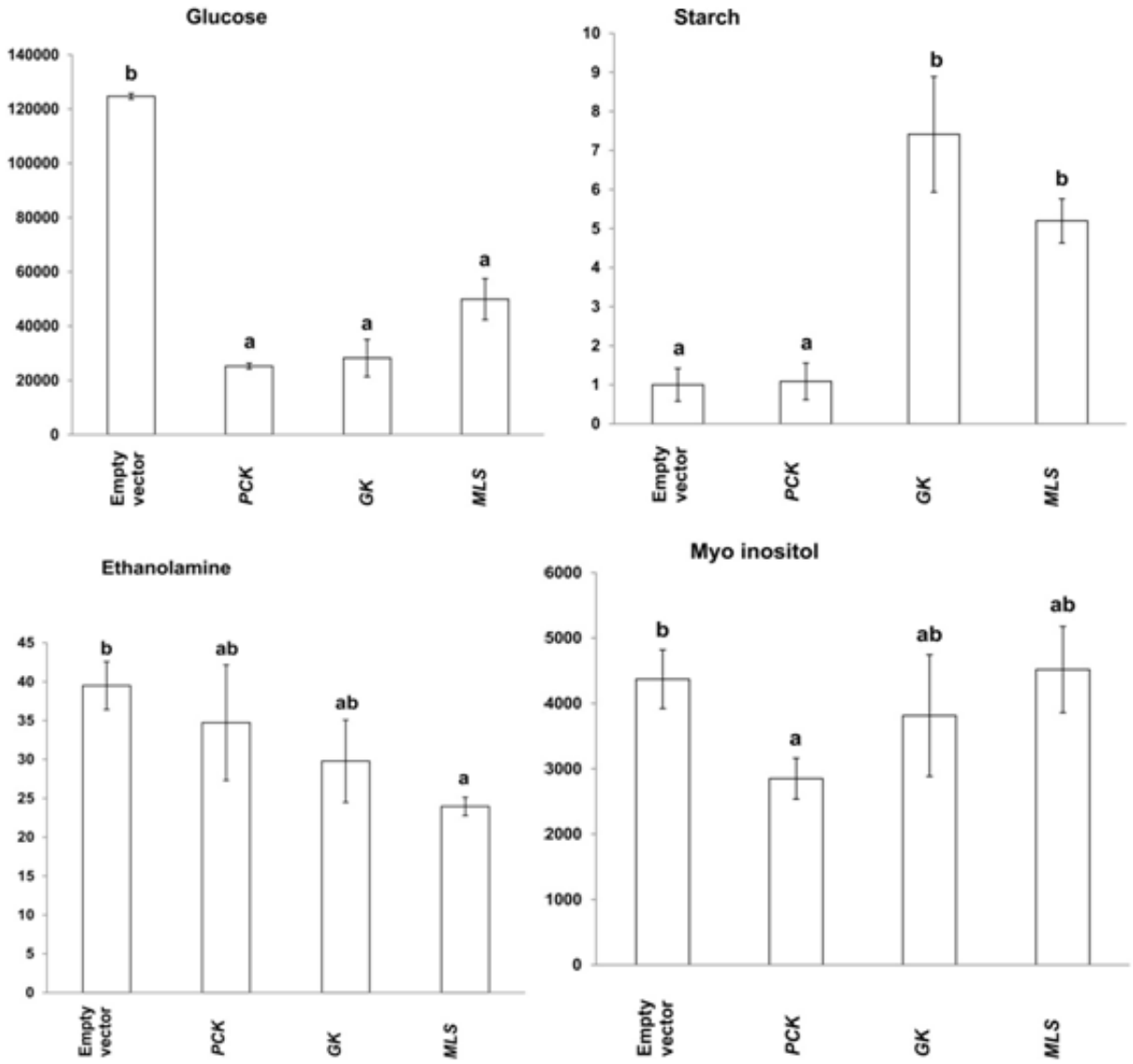


Figure 15. Metabolite profile in transgenic leaves of Nicotiana benthamiana. Agro-infiltration of Nicotiana seedlings of 1 month old was performed with Agrobacterium tumefaciens. Metabolite extraction was achieved 1 week after leaf infiltration through GC-MS and Dionex HPLC protocols. Empty vector was used as control and compared with extracts from infiltrated leaves with PCK, GK and MLS genes. Error bars were added via standard deviation between treatments. The different letters showed a significant difference between treatments at 5% level.

4. Discussion

4.1. Seed germination

Seed germination begins with water uptake (seed imbibition) followed by enzyme activation, initiation of embryo growth, rupture of the seed coat and radicle protrusion (Miller, 2008). Seed germination is also associated with other important cellular events such as cell division and elongation, respiration, RNA and protein biosynthesis (Miller, 2008; Bewley and Black, 1994). After seed imbibition, the mRNAs which were synthesized during seed development are translated into proteins/enzymes required for germination, storage lipid breakdown and seedling growth (Miller, 2008; Eastmond and Graham, 2001). Proper seed germination provides the necessary elements to early seedling growth and development before they become autotrophic. At this stage, seedlings are able to synthesize their own carbohydrates through photosynthesis to provide energy required for growth (Theodoulou and Eastmond, 2012).

Seed germination is governed by environmental factors among which temperature and water availability are the most important factors (Cheema *et al*., 2010; Mahan, 2005). Temperature affects metabolic processes during seed germination and seedling establishment, especially enzyme activation (Miller, 2008; Mathewson, 1998; Bewley and Black, 1994). Cardinal temperature is defined as the minimum, maximum and optimum temperature in which plant is able to grow and develop (Shaban, 2013). At minimum temperature, seed germination can occur but at low percentage and slow rate, while at the optimum temperature the germination percentage is at the highest level in the shortest time. Seedling vigour and health is related to the time that it takes between planting and seed germination or seedling emergence (Mahan and Gitz, 2007). However, maximum or high temperature results to an inhibitory effect on essential proteins required for germination and the subsequent physiological processes (Cheema, 2011; Shyam et al, 2011; Cheema et al, 2010).

Germination of castor bean seeds at different temperature (20, 25 and 35°C) was strongly affected in terms of germination percentage. Percentage of germination increased significantly with increasing temperature. This study reported 20°C as temperature which led to lower castor seed germination (variety MPA11), while both 25 and 35°C gave higher seed germination. Cheema and colleagues (2010) reported that although seeds can germinate at 10°C, the faster germination occurs at 25°C temperature. In another study conducted by Cheema (2011), temperature response in castor bean varied depending on cultivar. Thus, the lowest and highest seed germination index (GRI) was found at 10 and 25°C respectively in Bahawalpur seeds, at 10 and 30°C respectively for Tandojam seeds and at 10 and 35°C respectively for Faisalabad seeds.

From the experiment, the temperature increase or decrease from the optimum level resulted in slow germination. Severino and colleagues (2012) also reported that low temperatures extend the time for germination and results in irregular seed germination. Temperature of 10°C is considered as cold stress and in our case seeds were not submitted to cold stress but were at least at 20°C.

4.2. Role of studied genes in seedling performance

Castor bean originates in Ethiopia and prefers semi-tropical and tropical conditions for growth (Miller, 2008). Generally, these regions have high temperatures and dry climates. Therefore, Castor bean can survive an environment with high temperature. However, in our case, seedling performance decreased significanlty with the increasing temperature until it led to the death of all seedlings grown at 35°C two weeks after incubation. This could be due to the fact that high temperature during germination led to down regulation of important metabolic processes for seedling establishment.

It is already known that for seedling establishment, lipid breakdown is the crucial pathway to provide energy for seedling growth in Castor bean. It is also known that GK, MLS and PCK are the key enzymes involved in this pathway (Mahan, 2000) (Figure 16).

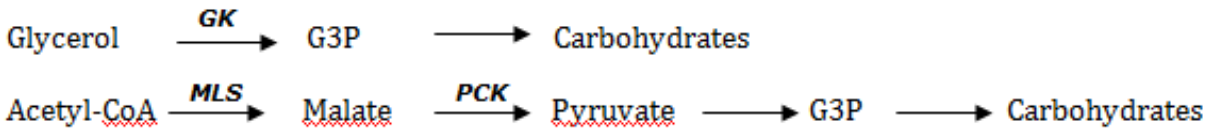


Figure 16. Correlation between genes encoding glycerol kinase (GK), malate synthase (MLS) and phosphoenolpyruvate carboxykinase (PCK) and carbohydrates biosynthesis. G3P means glycerol-3-phosphate.

Many studies reported the role of these enzymes in carbohydrate production and seedling establishment. For instance, Mahan and Gitz (2007) conducted a study in which they used soil temperature in field to predict the emergence of cotton and thermal dependence on velocity of MLS by using a enzymatically-based mechanistic model. With this model, they found that the rapid reaction of malate synthase over time correlates with the synthesis of carbohydrates from lipid reserves and the rate of seedling emergence in sunflower and cotton. In addition, Mahan (2000) reported that MLS velocity is correlated to glyoxyalate cycle and this enzyme responds differently on temperatures according to plant species. For instance, he revealed that the maximum velocity in cotton is reached at 35°C while in sunflower it requires 28°C.

In addition, Eastmond (2004) conducted a study on glycerol insensitive phenotypes in Arabidopsis and they observed that a *gli1* mutant (lacking *GLI1*, a gene which encodes glycerol kinase) was not able to breakdown glycerol and this led to the accumulation of glycerol and the root growth inhibition. This inhibitory effect was suppressed by applying exogenous sucrose in the medium. This indicates that a disruption of *GLI* activity required for glycerol catabolism results in glycerol insensitivity and prevention of sucrose production for root growth.

Cornah and colleagues (2004) observed that the expression of *MLS* is highly correlated with lipid catabolism during post-germination growth in Arabidopsis. This was shown by a blockage of carbohydrate biosynthesis in an Arabidopsis *mls* mutant which resulted in the inhibition of hypocotyl elongation. This indicates that the glyoxylate cycle is a vital pathway and required for lipid breakdown in oilseed plants. Graham (2008) considered glyoxylate cycle as a central pathway in carbohydrates synthesis as it links β-oxidation and gluconeogenesis. Nakazawa and colleagues (2005) ensured that glyoxylate cycle is the absolute pathway in oil catabolism especially under carbon deficiency conditions.

Runquist and Kruger (1999) observed a strong correlation between the lipid breakdown and expression of *MLS* as its expression peaked at the stage of higher rate of lipid catabolism. They concluded that effective lipid catabolism depends on *MLS* expression. It is known that the expression of the gene encoding *MLS* is governed by transcription during seed germination and post-germination growth and that their induction is a metabolic process that ensures the rapid lipid mobilization (Ettinger and Harada, 1990). In addition, this induction is associated with the one of genes encoding β-oxidation and gluconeogenesis enzymes (Eastmond and Graham, 2002).

Moreover, a study conducted by Penfield and colleagues (2004) in Arabidopsis showed that not only a disruption of genes encoding glyoxylate cycle enzymes affect carbohydrates production and reduction of hypocotyl elongation, but also a down-expression of *PCK1,* a gene which encodes phosphoenolpyruvate carboxykinase (*PCK*), prevents the gluconeogenesis for carbohydrate synthesis in dark conditions. PCK enzyme catalyses the conversion of oxaloacetate into phosphoenolpyruvate (Leegood and Walker, 2003; Matsuba *et al*., 1997). In a study they conducted on *Cucurbita pepo* seedlings, they revealed that a disruption of gluconeogenesis results to the conversion of oxaloacetate into malate, aspartate and TAC (tricarboxylic acid) cycle (required in biosynthesis of photosynthetic tissues) instead of phosphoenolpyruvate. Thereby, they concluded that production of the later metabolite from oxaloacetate with *PCK* is a crucial step in gluconeogenesis.

Briefly, GK, MLS and PCK are the key enzymes required in glycerol catabolism, glyoxylate cycle and gluconeogenesis respectively for carbohydrate production required during early seedling growth. In addition to the role of these enzymes, it was also observed that the expression of genes that encode these enzymes is strongly affected by temperature. For instance, in an experiment conducted in our lab on castor been seedlings, we showed that some important metabolic enzymes (GK, MLS and PCK) were down-regulated at 35°C when compared to 20 and 25°C. In fact, expression levels of genes encoding GK, MLS and PCK enzymes decreased with the increasing temperature. However, it was reported that a disruption of expression of these enzymes results to low seedling establishment. Thereby, downregulation of these genes might be the cause of low seedling performance observed at high temperature in our case (Personal communication Paulo, 2014).

In the present study, the deleterious thermal response increased over time. Therefore, percentage of normal seedlings decreased from 1WAI to 2WAI. It is known that castor bean uses lipid reserve for germination and that the mobilization of this reserve is necessary for seedling establishment before the biosynthesis of photosynthetic metabolites (Theodoulou and Eastmond, 2012). During the first days after incubation, germination processes (water uptake, rupture of the seed coat and radicle protrusion) and little enzyme activation take place and the lipid mobilization which is a post-germination process occurs after. Therefore, even lipid mobilization can start before complete seed germination, the function of enzymes involved in this pathway is mainly required in post-germination events (Graham, 2008; Bewley and Black, 1994).

It was also reported that many genes encoding enzymes involved in lipid mobilization are highly expressed 2 to 5 days after imbibition and that complete lipid mobilization occurs after day 5 to 10 after imbibition (Eastmond, 2004; Rylott *et al*., 2003; Bewley and Black, 1994; Huang, 1992). Leegood and Walker (2003) conducted a study on *Cucurita pepo* seedlings and they observed that gluconeogenesis reaches a peak 5 to 8 days after planting. Moreover, it is mentioned above that the expression of genes encoding GK, MLS and PCK enzymes decreased with temperature increase. Thereby, gene expression in some days later after incubation and negative effect of temperature on gene expression might be the causes of the increasing of deleterious thermal response over time.

4.3. Gene cloning and sequencing

We showed how genes encoding GK, MLS1 and PCK1 enzymes correlate with carbohydrate production and how a down regulation of these genes at high temperature correlates with low seedling performance in castor bean. Although these genes were revealed to be important in lipid breakdown, there was an misunderstanding on how their expression responds to temperature conditions and how this affects physiological, biochemical and molecular aspects in castor bean. Thereby, with this study we isolated and cloned coding regions of these three candidate genes for further analyses.

In this experiment, gene sequencing (nucleotide sequence alignment) revealed that plasmids contained *GK*, *MLS1* and *PCK1* genes were positive because their nucleotide and amino acids sequences matched the sequence of desired genes. Although cloned *GK* contained one different nucleotide (G) compared to the reference gene (T), this mutation occurring during cloning processes did not affect its amino acid sequence. That is what so called' silent single nucleotide polymorphism' (silent SNP). This occurs when a change in single nucleotide does not affect the amino acid composition (Komar, 2007). This might be explained by the fact that the substitution of one base in DNA sequence usually is considered as small genetic change which does not have a discriminate effect on gene stability and function (Komar, 2007; Wang *et al.*, 2010; Clancy, 2008).

4.4. Metabolite profile in transgenic *Nicothiana benthamiana*

Briefly, many metabolites were identified either by GC-MS or Dionex HPLC techniques. However, only glucose, starch, ethanolamine and myo inositol levels varied between different samples. Glucose content was lower in leaves infiltrated with target genes (*PCK*, *GK* and *MLS*). However, starch was highly accumulated in both *GK* and *MLS* samples. This explains that glucose was converted into starch in leaves infiltrated by these genes. The surprising result is that both glucose and starch contents were low in sample from *PCK.* In this study, we used *AtPCK* (*A. thaliana PCK*) gene as reference to have *RcPCK* (*R. communus PCK*). We have got three putative *RcPCKs (PCK1, PCK2* and *PCK3).* However, during cloning, only cloned *PCK1* expressed identical nucleotide sequence compared to reference *PCK1* gene. Therefore, the mechanism behind the low level of both glucose and starch in leaves infiltrated with *PCK1* could be due to the fact that among three *PCKs*, this was not the exact gene that should result to proper gluconeogenesis.

Ethanolamine and myo inositol content was low in leaves infiltrated with target genes. Ethanolamine is organic compound which contains both amine and alcohol chemical. In plant, it is synthetized from serine or phosphatidylserine and it is important for synthesis of choline and membrane lipid likephosphatidylethanolamine and phosphatidylethanolamine (Kwon *et al.*, 2012). Unfortunately, there is no clear relationship between this metabolite and the function of studied genes. Myo inositol is a carbohydrate classified in sugar alcohols (Gupta *et al*., 2010) which can be synthesized from glucose (Sasaki and Taylor, 1986). As a carbohydrate, it must be highly accumulated in leaves infiltrated by studied genes especially bacterial cells contained *PCK* genes.

Conclusion

The aim of the present study was to clone and functional characterize temperature responsive genes encoding glycerol kinase (GK), malate synthase (MLS) and phosphoeonylpyruvate corboxykinase (PCK) enzymes which are involved in lipid mobilization in Castor bean (*Ricinus communis*).

From this experiment the following conclusions are made:

1. Castor bean germination and seedling performance are strongly affected by the temperature. Seed germination percentage increased with the increasing temperature. However, a decrease in number of normal seedlings was associated with increasing temperature. Therefore, 25 and 35°C are the best temperatures for seed germination followed by 20°C. However, 20°C led to higher seedling performance followed by 25°C, while 35°C is a harsh temperature which resulted in low percentage of normal seedlings. In conclusion, 25°C is the best temperature for both germination and seedling establishment in castor bean.

2. Seedling performance correlates with the expression and function of genes that encode GK, MLS and PCK enzymes. The expression of these enzymes is negatively affected by the temperature, which might explain the low seedling survival of 2WAI samples at 35°C.

3. Transient expression in *N. Benthamiana* leaves showed that an initial reduction in glucose levels was followed by an increase in the content of starch in leaves that were infiltrated with Agl0-*MLS* and Agl0-*GK* as compared to infiltration with the empty vector (Agl0-EV). This supports our hypothesis that these genes are required for *R. communis* growth at the early of seedling establishment.

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Appendices

Daily seed germination (20 and 25°C). Seed incubation was done on 11th-February-2014.

Date	Treatment (°C)	Replication				Total	Germination rate (%)
		1	2	3	4		
12-Feb	20	0	0	0	0	0	0
	25	0	0	0	0	0	0
13-Feb	20	3	4	1	0	8	13.3
	25	10	10	13	13	46	76.7
14-Feb	20	9	10	12	12	43	71.7
	25	14	13	13	14	54	90.0
17-Feb	20	10	12	12	10	44	73.3
	25	14	14	14	13	55	91.7

Daily seed germination (35°C). Seed incubation was done on 17th February-2014.

Date	Replication				Total	Germination rate (%)
	1	2	3	4		
17-Feb	0	0	0	0	0	0
18-Feb	6	3	4	2	15	25
19-Feb	12	11	10	13	46	76.7
20-Feb	15	15	11	11	52	86.7
21-Feb	15	15	11	11	52	86.7
24-Feb	15	15	12	12	54	90.0

Normal seedlings one week after seed incubation

Treatment (°C)	Replication				Total	Normal seedling (%)
	1	2	3	4		
20	9	12	12	10	43	97.7
25	10	10	11	11	42	76.4
35	10	11	7	8	36	66.7

Normal seedlings two weeks after seed incubation

Treatment (°C)	Replication				Total	Normal seedling (%)
	1	2	3	4		
20	9	9	9	10	37	84.1
25	10	9	10	8	37	67.3
35	0	0	0	0	0	0

ANOVA table for seed germination (%)

Source of variation	df	s.s	m.s	v.r	F pr.
Tr	2	822.22	411.11	6.05	0.022*
Residual	9	611.11	67.90		
Total	11	1433.33			
		* Significant difference (*P <0.05*)			

Mean separation of germination rate (%)

Treatment (°C)	Mean
20	73.3 a
25	91.7 b
35	90.0 b
D (Least Significant Difference of means (5% level)=13.18	

ANOVA table of Normal seedling (%) one week after seed incubation

Source of variation	df	s.s	m.s	v.r	F pr.
Tr	2	2029.85	1014.93	29.49	<.001***
Residual	9	309.78	34.42		
Total	11	2339.63			
		*** Very High significant difference (*P*<.001)			

Mean separation (%) of Normal seedling (%) one week after seed incubation

Treatment (°C)	Mean
20	97.7c
25	76.4 b
35	66.7 a
LSD=9.385	

ANOVA table of Normal seedling (%) two weeks after seed incubation

Source of variation	df	s.s	m.s	v.r	F pr.
Tr	2	16073.0	8036.50	137.43	<.001***
Residual	9	526.30	58.48		
Total	11	16599.30			
		*** Very High significant difference (*P*<.001)			

Mean separation (%) of Normal seedling (%) two weeks after seed incubation

Treatment (°C)	Mean
20	84.1c
25	67.3 b
35	0 a
LSD=12.232	

ANOVA table of Normal seedling (%) over time

Source of variation	df	s.s	m.s	v.r	F pr.
Tr	2	14011.04	7005.52	150.82	<.001***
Tme	1	5173.31	5173.31	111.38	<.001***
Trt. Tme	2	4091.81	2045.91	44.05	<.001***
Residual	18	836.09	46.45		
Total	23	24112.25			

*** Very High significant difference (*P*<.001)

DNA sequence alignment of the coding region of cloned *GK*. Highlighted nucleotide in yellow shows the difference in position between cloned and reference genes.

ATGGCAAAACAGGAACCAGCTTTCATTGGAGCCATTGATCAAGGCACAACTAGCACCAGATTCATAATCTACAATCGCCATGCCAACTCTATTGGAT
CTCACCAGGTTGAATTCACCCAGTTCTACCCTCAAGCCGGATGGGTGGAGCATGATGCTATGGAGATACTTGAGAGCGTCAAGGTGTGTATGGCCAA
GGCAGTGGATAAAGCTACTGCTGATGGACATAATGTTGATGGTTTATTGAAGGCTATTGGGCTAACTAATCAGAGAGAGACCACTGTTATTTGGAGC
AAATCAACTGGTGTTCCTCTTTATAATGCCATTGTTTGGATGGATGTTCGTACCAGTTCTATTTGCAGGAAATTGGAGAAAGAATTACCAGGTGGAA
GGACTCATTTCATTGAGACTTGTGGTCTGCCATTAAGTACTTATTTCAGTGCAGTGAAGATACTGTGGTTGATGGAAAATGTTGATGCTGTTAAAGA
GGCTATCAAGAAAGGGGATGCTCTGTTTGGAACTATAGACTCTTGGTTAATATGGAATTTAACTGGTGGAGTGAAGGGTGGCTTGCATGTCACTGAT
GTTTCTAATGCATCTCGAACCATGCTCATGAATATCAAAACCCTTGAATGGGATAAACCTACATTGAATACTTTGGGAATTCCTGCTGAAATTCTGC
CCAAAATTATAAGCAACTCTGAGGTTATTGGAAAAATTGCAAAGGGGTGGCCAGTTACTGGCATTCCGATTGCAGGGTGTCTTGGAGACCAACATGC
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CAGTTCAATGGCTTAGAGATGGCCTTGAGCTAATTAGTAGTGCAAGTGAAATCGAGGAACTTGCCAAGCAGGTTGACTCGACTGGTGGTGTTTATTT
TGTACCTGCTTTTAATGGACTGTTTGCCCCATGGTGGCGTGATGATGCCCGTGGGGTCTGCATTGGGATCACAAGGTATACTAATAAGTCCCACATTG
CTCGAGCTGTGCTTGAGAGCATGTGTTTCCAAGTTAAAGATGTATTGGATTCAATGCACAAAGATAGAGAGGAAAAGCATAAGGACACTAAGAGGG
AGTTCCTGCTAAGAGTAGATGGTGGTGCAACTATTAACAACCTCTTGATGCAGATTCAGGCAGACTTGGTGGGACACCCAGTTGTGAGGCCAGCTGA
TATAGAAACAACAGCTCTTGGAGCAGCCTATGCTGCTGGTTTGGCTGTTGGGATTTGGACAGAGAAGGAGATTTTTGCTTCAGGAGAAAAGGCTAAG
ACAGATACCATCTTCTGTCCGAAATTAGATGAAGAACTGAGAAAGAAAAAGGTGGAATCTTGGTGCAAAGCTGTTGAAAGAACTTTTGGCTTGGCTG
ATCTTTCAATATGA

DNA sequence alignment of the coding region of *GK* reference. Highlighted nucleotide in yellow shows the difference in position between cloned and reference genes.

ATGGCAAAACAGGAACCAGCTTTCATTGGAGCCATTGATCAAGGCACAACTAGCACCAGATTCATAATCTACAATCGCCATGCCAACTCTATTGGAT
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AAATCAACTGGTGTTCCTCTTTATAATGCCATTGTTTGGATGGATGTTCGTACCAGTTCTATTTGCAGGAAATTGGAGAAAGAATTACCAGGTGGAA
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TATAGAAACAACAGCTCTTGGAGCAGCCTATGCTGCTGGTTTGGCTGTTGGGATTTGGACAGAGAAGGAGATTTTTGCTTCAGGAGAAAAGGCTAAG
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ATCTTTCAATATGA

Alignment of amino acids sequences of cloned *GK*

MAKQEPAFIGAIDQGTTSTRFIIYNRHANSIGSHQVEFTQFYPQAGWVEHDAMEILESVKVCMAKAVDKATADGHNVDGLLKAIGLTNQRETTVIWSK
STGVPLYNAIVWMDVRTSSICRKLEKELPGGRTHFIETCGLPLSTYFSAVKILWLMENVDAVKEAIKKGDALFGTIDSWLIWNLTGGVKGGLHVTDVSN
ASRTMLMNIKTLEWDKPTLNTLGIPAEILPKIISNSEVIGKIAKGWPVTGIPIAGCLGDQHAAMVGQGCKRDEAKSTYGTGAFILLNTGDHIVPSKHGLL
TTLAYKLGPKAPTNYALEGSIAIAGAAVQWLRDGLELISSASEIEELAKQVDSTGGVYFVPAFNGLFAPWWRDDARGVCIGITRYTNKSHIARAVLESM
CFQVKDVLDSMHKDREEKHKDTKREFLLRVDGGATINNLLMQIQADLVGHPVVRPADIETTALGAAYAAGLAVGIWTEKEIFASGEKAKTDTIFCPKL
DEELRKKKVESWCKAVERTFGLADLSI*

Alignment of amino acids sequences of *GK* reference

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STGVPLYNAIVWMDVRTSSICRKLEKELPGGRTHFIETCGLPLSTYFSAVKILWLMENVDAVKEAIKKGDALFGTIDSWLIWNLTGGVKGGLHVTDVSN
ASRTMLMNIKTLEWDKPTLNTLGIPAEILPKIISNSEVIGKIAKGWPVTGIPIAGCLGDQHAAMVGQGCKRDEAKSTYGTGAFILLNTGDHIVPSKHGLL
TTLAYKLGPKAPTNYALEGSIAIAGAAVQWLRDGLELISSASEIEELAKQVDSTGGVYFVPAFNGLFAPWWRDDARGVCIGITRYTNKSHIARAVLESM
CFQVKDVLDSMHKDREEKHKDTKREFLLRVDGGATINNLLMQIQADLVGHPVVRPSDIETTALGAAYAAGLAVGIWTEKEIFASGEKAKTDTIFCPKL
DEELRKKKVESWCKAVERTFGLADLSI*

DNA sequence alignment of the coding region of cloned *MLS1*

ATGATGCGATATGATACTTATGGTGACTCTGCCCCGATCAAGAAGACGGGTGCAGGCTATGATGTTCCTGAAGGAGTGGACATTCGAGGTCGGTACG
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DNA sequence alignment of the coding region of *MLS1* reference

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TAGATGCCTATAATAACATTGTGATTCATTATCCAAAGGGATCATCTAGGCTGTGA

Alignment of amino acids sequences of cloned *MLS1*

MMRYDTYGDSAPIKKTGAGYDVPEGVDIRGRYDGEFAKILTRDALQFVADLQREFRNRIRYAIECRKEAKSRYNAGALPGFDPATKYIREGEWTCAPV
PPAVADRKVEITGPVERKMIINALNSGAKVFMADFEDALSPSWENLMRGQVNLRDAVNGTISFHDKARNRVYKLNDQIAKLFVRPRGWHLPEAHILI
DGEPATGCLVDFGLYFYHNYAAFRRNQGAGYGPFFYLPKMEHSREAKIWNCVFEKAEKMAGIERGSIRATVLIETLPAVFQMNEILYELRDHSVGLNC
GRWDYIFSYVKTFQAHPDRPLPDRVQVGMTQHFMKSYSDLLVWTCHRRGVHAMGGMAAQIPIRDDPAANKAALELVRKDKLREVKAGHDGTWAA
HPGLIPACMEVFANNMGNTPHQIQAMKREDAANITEEDLIQRPRGVRTLEGLRLNTRVGIQYLAAWLTGTGSVPLYNLMEDAATAEISRVQNWQWL
KYGVELDGDGLGVKVTFDLLGRVVEDEMARIEREVGKEKFKKGMYKEACKMFVRQCAAPTLDDFLTLDAYNNIVIHYPKGSSRL*

Alignment of amino acids sequences of *MLS1* reference

MMRYDTYGDSAPIKKTGAGYDVPEGVDIRGRYDGEFAKILTRDALQFVADLQREFRNRIRYAIECRKEAKSRYNAGALPGFDPATKYIREGEWTCAPV
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GRWDYIFSYVKTFQAHPDRPLPDRVQVGMTQHFMKSYSDLLVWTCHRRGVHAMGGMAAQIPIRDDPAANKAALELVRKDKLREVKAGHDGTWAA
HPGLIPACMEVFANNMGNTPHQIQAMKREDAANITEEDLIQRPRGVRTLEGLRLNTRVGIQYLAAWLTGTGSVPLYNLMEDAATAEISRVQNWQWL
KYGVELDGDGLGVKVTFDLLGRVVEDEMARIEREVGKEKFKKGMYKEACKMFVRQCAAPTLDDFLTLDAYNNIVIHYPKGSSRL*

DNA sequence alignment of the coding region of cloned *PCK1*

ATGGCGGAGAATGGAGAGTTCAGCTTTAAGAGTAACAGCAGCGGACGCAACGGCCTGCCCAAGATTGTTACTCAGAAGAATGATGTTTGTCAGGATG
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DNA sequence alignment of the coding region of *PCK1* reference

ATGGCGGAGAATGGAGAGTTCAGCTTTAAGAGTAACAGCAGCGGACGCAACGGCCTGCCCAAGATTGTTACTCAGAAGAATGATGTTTGTCAGGATG
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Alignment of amino acids sequences of cloned PCK

MAENGEFSFKSNSSGRNGLPKIVTQKNDVCQDDSGPTVKAKTIDELHSLQRKKSAPTTPIKGGAPPSPLSEEQRQKQQLQSISASLASLTRETGPKVVKG
DPARKAEGQTVTHVEHPPIFAPAISVSDSSLKFTHVLYNLSPAELYEQAIKYEKGSFITSNGALATLSGAKTGRSPRDKRVVRDSTTEGELWWGKGSPNI
EMDEHTFLVNRERAVDYLNSLDKVFVNDQFLNWDPEHRIKVRIVSARAYHSLFMHNMCIRPTPEELEDFGTPDFTIYNAGQFPCNRYTHYMTSSTSID
INLARREMVILGTQYAGEMKKGLFGVMHYLMPMRQILSLHSGCNMGKDGDVALFFGLSGTGKTTLSTDHNRYLIGDDEHCWSENGVSNIEGGCYAKCI
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VAGTEDGIKEPQATFSACFGAAFIMLHPTKYAAMLAEKMQKHGATGWLVNTGWSGGSYGSGKRMKLAYTRRIIDAIHSGDLLRANYRKTEVFGLEIPI
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Alignment of amino acids sequences of *PCK1* reference

MAENGEFSFKSNSSGRNGLPKIVTQKNDVCQDDSGPTVKAKTIDELHSLQRKKSAPTTPIKGGAPPSPLSEEQRQKQQLQSISASLASLTRETGPKVVKG
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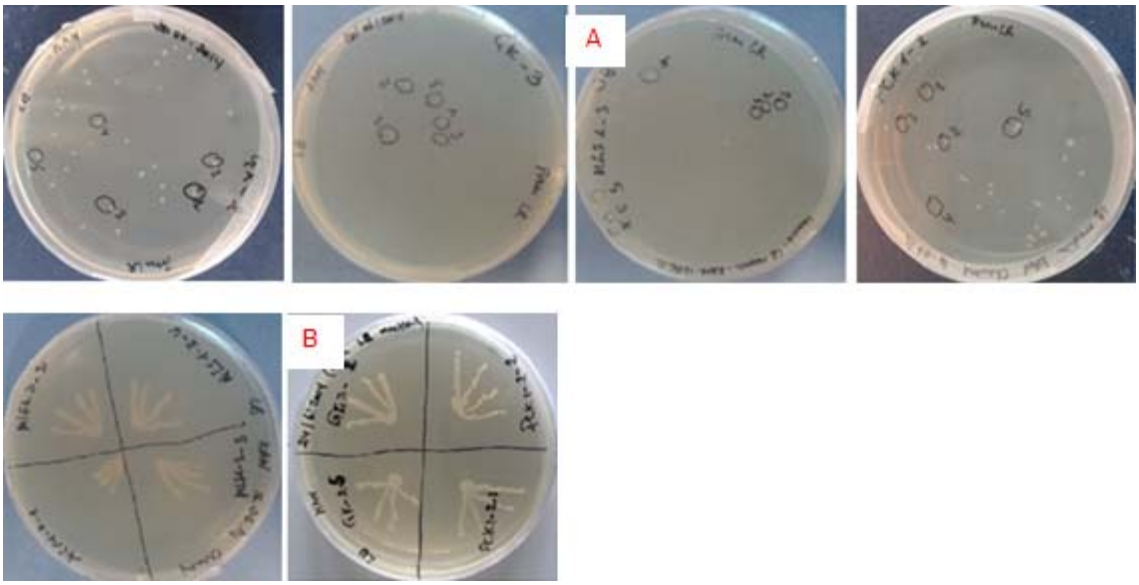
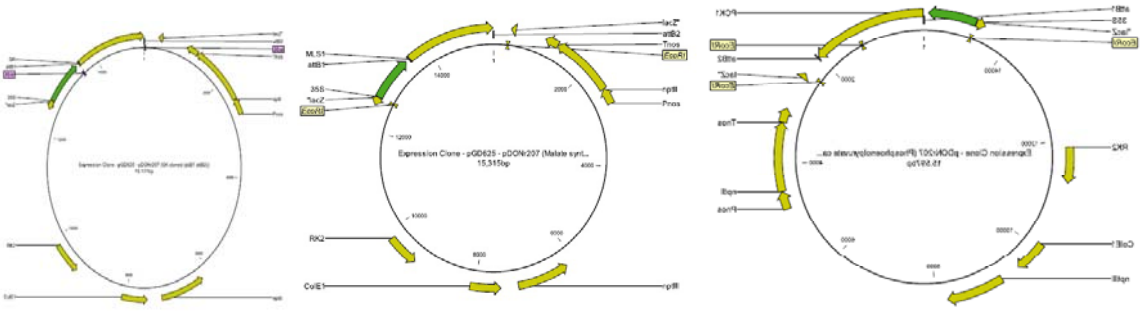


Single colonies of transformed genes with E. coli (DH5α strain) competent cells after LR reaction. Plates contained GK_2, GK_3, MLS1_3 and PCK1_2 respectively (A). Colonies transferred and grew into LB solid medium with kanamycin for further experiments (B).



Number and size of restriction fragments of cloned genes from the digestion with EcoRI.

DNA sequence alignment *ImpactTim1.1* (empty vector)

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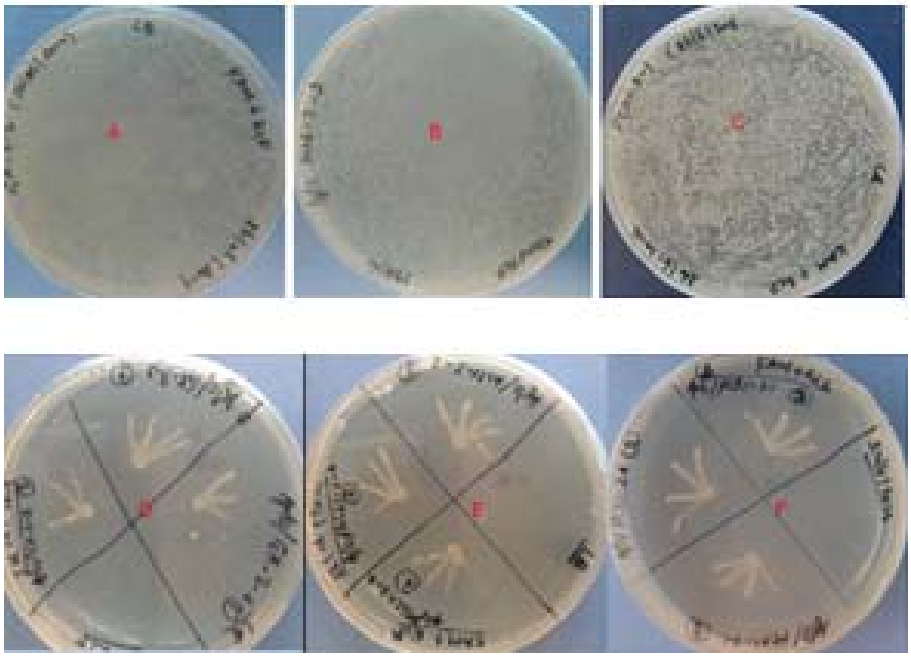

Transformed A. tumefaciens colonies (Agl0 strain) with cloned genes. Plates with kanamycin and rifampicin antibiotics contained cloned genes such as GK, MLS1 and PCK1 after transformation via electroporation (A, B and C respectively) and after transfer to LB solid medium with the same antibiotics (D, E and F respectively).