Biochemical, physiological and molecular responses of *Ricinus communis* seeds and seedlings to different temperatures: a multi-omics approach

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Contents

Chapter 1

General Introduction

Abiotic stresses in plants

The development of a seed into a mature plant is a remarkable process, involving growth by cell division, growth by expansion, and differentiation of organs such as roots, stems, leaves, and flowers. These processes are ultimately coordinated with the inherent genetic information present in each cell and the modifying effects imposed by the environment in which plants grow (Bewley et al., 2013; Han and Yang, 2015; Nonogaki, 2006; Rajjou et al., 2012; Vázquez-Ramos and Sánchez, 2003; Weitbrecht et al., 2011). In this context, stress is defined as an adverse condition which negatively affects seed germination, plant growth, plant development, and/or productivity (Bray et al., 2000).

Stresses can be caused by either abiotic or biotic factors. Biotic stresses are imposed by other living organisms, such as bacteria, viruses, fungi, parasites, insects, and other plants (Sergeant and Renaut, 2010). Abiotic stresses are caused by adverse environmental factors and it is usually associated with excess or deficit of water (Farooq et al., 2009), light (Niinemets and Valladares, 2004; Yamamoto et al., 2014), nutrients (Aleksandrov et al., 2014; Nenova and Stoyanov, 1999; Trubat et al., 2006), or by low or high temperatures (Hasanuzzaman et al., 2013; Zhou et al., 2011).

Abiotic stress is considered the main factor compromising crop growth and productivity worldwide (Boyer, 1982; Gao et al., 2007). For example, drought stress may impair seed germination leading to poor stand establishment, as well as reducing dry weight of shoot and roots (Okçu et al., 2005; Zeid and Shedeed, 2006). Drought stress has significant economic, environmental, and social impacts, both direct and indirect, and it is increasing in frequency and severity given the changing climatic conditions worldwide. In *Ricinus communis* (Castor bean), for example, withholding irrigation from 45-120 days after sowing or 65-120 days after sowing leads to a significant reduction of total dry matter and seed yield. Control plants produced 62.2 g of seeds per plant, whereas stressed plants produced around 42 g of seeds per plant (Lakshmamma et al., 2009). Heat stress can also reduce crop yield considerably, thereby causing low resource use efficiency. In soybean, for example, aboveground dry matter was significantly reduced by increasing temperatures. Reduced pod and seed number, and smaller seeds were also observed with increasing temperatures. These phenomena might be associated with the delayed pod set and lower seed growth rate under the warmer conditions (Tacarindua et al., 2013).

In order to cope with abiotic stresses, plants activate specific responses tailored to each environmental condition. Recent studies have shown that molecular and biochemical responses of plants to a combination of abiotic stresses might require a unique response (Prasch and Sonnewald, 2014;

Rasmussen et al., 2013; Rivero et al., 2014; Zhao et al., 2009). However, understanding the mechanisms for acclimation or acquisition of tolerance by plants to a combination of various abiotic stresses would first require a proper understanding of the responses to each individual environmental condition (Mittler, 2006). Drought and heat stress represent an excellent example of two different abiotic stress conditions that often occur simultaneously in the field (Ananda et al., 2011; Craufurd and Peacock, 1993). Physiological characterization of plants subjected to drought, heat stress or their combination has revealed that the stress combination has several unique aspects, combining highly active respiration with low photosynthesis, closed stomata and high leaf temperature (Rizhsky et al., 2002; Rizhsky et al., 2004a).

Heat stress can be defined as a rise in temperature beyond a threshold level for a period of time long enough to cause irreversible damages to plant growth and development (Wahid et al., 2007). In this context, heat stress can be seen as a relative concept, which depends on the ability of each individual plant to perceive and cope with a rise in temperature and therefore, handling complex perturbations on its homeostasis (Kotak et al., 2007; Yamori et al., 2014). In general, plants display a considerable plasticity to respond to short-term fluctuations of environmental factors (Callaway et al., 2003; Nicotra and Davidson, 2010; Valladares et al., 2007). Heat stress alters membrane fluidity (Kotak et al., 2007; Wahid et al., 2007), which may lead to disruption of cellular homeostasis and membrane related processes, such as trans-membrane transport (Dhanda and Munjal, 2009). Furthermore, heat stress can cause disturbance of enzyme activity leading to disruption of metabolic pathways (Kotak et al., 2007; Wahid et al., 2007).

Thermotolerance is the ability of plants to prevent or repair damages imposed to heat-sensitive structures and macromolecules within the cell, organ or entire plant. Basal thermotolerance is defined as the inherent ability of the plant to survive in temperatures above the optimal temperature for growth, and still be able to complete their lifecycle, whereas acquired thermotolerance is induced upon a short period of acclimation under elevated temperatures, after which plants can sustain otherwise lethal temperatures (Larkindale et al., 2005). Acquired thermotolerance functions through the production of heat shock factors that induces production of heat stress proteins. The most predominant function of the acquired thermotolerance is to stabilize proteins due to chaperone functions of the heat shock proteins (Kregel, 2002; Wahid et al., 2007), whereas basal thermotolerance triggers heat-stress signal transduction, phytohormone regulation and production of ROS-scavenging enzymes (Larkindale et al., 2005; Miller et al., 2008; Suzuki and Mittler, 2006).

Effect of temperature on seed germination and seedling establishment

Seed germination is a crucial process in the life cycle of higher plants and determines whether seedling establishment will be successful or not. Germination begins with the uptake of water by the seed (imbibition), is followed by reactivation of metabolism, and ends with visible protrusion of the radicle

through the surrounding layers (Bewley et al., 2013). Seed reserves are mobilized upon germination and the resultant carbohydrates, lipids, and proteins are utilized as energy resources and new building blocks to support the development of the seedling until it becomes photo-autotrophic (Bewley et al., 2013; Rosental et al., 2014). Mobilization of seed storage compounds upon imbibition is a crucial process to set proper conditions for seed germination and seedling establishment (Weitbrecht et al., 2011). Mature drying seeds accumulate mRNAs and functional proteins, which are involved in early physiological responses upon imbibition. Triacylglycerol (TAG) is a major seed storage reserve in oilseed plant species which accumulates during seed development. TAG is stored in the seed until germination, after which it is used to fuel initial seedling growth (Graham, 2008).

Temperature is one of the most important environmental factors that affect seed imbibition and germination. It plays an important role in water uptake and reactivation of metabolism (Bewley et al., 2013; Weitbrecht et al., 2011). In general, a progressive increase in temperature shortens the time required for germination to occur until it reaches an optimum temperature and the maximum final germination percentage, which varies between species (Cochrane et al., 2014; Da Mota and Garcia, 2013). Higher plants exhibit a maximum rate of growth and development at an optimum temperature for which they have become adapted (Fitter and Hay, 1981). For example, speed of germination and final germination percentage of *Calendula officinalis* seeds are optimized at temperatures around 16ºC. As temperature increased to above 17ºC, germination was observed to decrease (Eberle et al., 2014). Additionally, field studies indicated that *C. officinalis* stand establishment is susceptible to high soil temperatures immediately after planting (Gesch, 2013). *Borago officinalis* seeds are able to germinate in temperatures ranging from 9 to 37ºC, with an optimum of 23ºC (Gilbertson et al., 2014). As temperature deviates from optimal, several physiological, biochemical, and molecular changes occur within plants in order to maintain growth and developmental processes and, most likely, to maintain cellular homeostasis (Fitter and Hay, 1981). Most plants show considerable capacity to adjust their characteristics to their growth temperatures, allowing plants to more efficiently develop at new growth temperatures.

Metabolomics is an important tool to investigate abiotic stress tolerance in plants

Metabolite profiling has been used to dissect seed germination and seedling establishment under normal and stressed conditions (Fait et al., 2006; Howell et al., 2009; Na Jom et al., 2011; Rosental et al., 2014; Seo et al., 2011; Shu et al., 2008; Weitbrecht et al., 2011; Zhao et al., 2014; Zhao et al., 2013). For example, metabolite profiling of rice embryos during germination showed that one hour after imbibition changes in metabolism occurred, including increases in hexose phosphates, tricarboxylic acid cycle intermediates, and γ-aminobutyric acid (GABA). Later changes in carbohydrate-, amino acid- and cell wall metabolism appeared to be driven by increases in transcript levels observed to increase from 12 hours

after imbibition onwards (Howell et al., 2009). Fait et al. (2006) studied the metabolite profile of *Arabidopsis thaliana* seeds throughout development and germination under different temperatures. During seed maturation a significant reduction in the contents of sugars, organic acids and amino acids was observed, which suggested their efficient incorporation into storage reserves. During the transition from reserve accumulation to seed desiccation a major metabolic switch was observed, resulting in the accumulation of sugars, organic acids, nitrogen-rich amino acids, and shikimate-derived metabolites. In contrast, seed stratification to break dormancy was associated with a decrease in the contents of several of the metabolic intermediates that had accumulated during seed desiccation, implying that these intermediates might support the metabolic reorganization needed for seed germination. Concomitantly, the levels of other metabolites significantly increased during stratification and were boosted further during germination (Fait et al., 2006).

Metabolite profiling of plants growing under abiotic stress conditions has provided important information regarding biochemical and molecular changes related to adaptation to different environmental conditions such as drought (Figueroa-Pérez et al., 2014; Wenzel et al., 2014), salt (Canam et al., 2013; Wu et al., 2013; Zhao et al., 2014) and temperature (Guy et al., 2008; Krasensky and Jonak, 2012; Zhou et al., 2011). Carbohydrate and amino acid metabolism appear to be part of the mechanisms by which plants adapt to changes in temperature (Diamant et al., 2001; Gray and Heath, 2005; Kaplan et al., 2004; Panikulangara et al., 2004; Rizhsky et al., 2004). Kaplan et al. (2004) performed a metabolite profiling study to determine temporal metabolite dynamics associated with the induction of acquired thermotolerance in response to heat shock and acquired freezing tolerance in response to cold shock. Curiously, cold shock had a greater influence on metabolism than heat shock: the steady-state pool sizes of 311 and 143 metabolites (or mass spectral tags) varied in response to cold and heat shock, respectively (Kaplan et al., 2004). A coordinated increase was observed in the pool sizes of amino acids derived from pyruvate and oxaloacetate, polyamine precursors, and compatible solutes during both heat and cold shock. Gray and Heath (2005) examined the effects of cold acclimation on the Arabidopsis metabolome using a non-targeted metabolic fingerprinting approach. Cold acclimation turned out to involve many complex biochemical changes at the level of the metabolome (Gray and Heath, 2005).

Although metabolomics has been used to dissect plant responses to abiotic stresses, most of the studies regarding the temperature effect on seedling performance have focused on the ability of plants to maintain homeostasis at chilling temperatures (0 to 15ºC) or have investigated plant responses to high-temperature stress, mostly using Arabidopsis as model species (Arbona et al., 2013; Guy et al., 2008; Obata and Fernie, 2012). Plant metabolic plasticity in response to mildly elevated temperatures (20 to 35ºC) has received much less attention although it is an important trait for crop species (Lewicka and Pietruszka, 2006).

***Ricinus communis*: an oilseed crop with great agricultural and biotechnological potential**

Ricinus communis L. is a member of the Euphorbiaceae and an important oilseed crop. This species is predominantly cultivated throughout the tropical and sub-tropical regions of the world. Despites what the common name suggests, its seeds are not real beans and they are not edible. In fact, *R. communis* seeds contain high levels of ricin, ricinine and certain allergens that are highly toxic to humans and animals (Ogunniyi, 2006, Severino et al., 2012). The exact origin of the species is uncertain, but it is generally assumed that *R. communis* originates from eastern Africa where the plants still occur in the wild with high genetic diversity (Anjani, 2012; Foster et al., 2010). This region is also considered to be the origin of domestication, because *R. communis* seeds dating from 4000 BP have been found in Egyptian tombs (Purseglove, 1976). Ethno-botanical information dating back to times as early as 500 BP shows that *R. communis* was used in vast areas of the world (including India, China, tropical Africa and later, around 1500 AD, in Europe) where it had a wide array of uses in traditional medicine ranging from healing inflammatory to cardiovascular diseases (Scarpa and Guerci, 1982). The oil extracted from its seeds is mainly used for pharmaceutical and industrial applications due to its unique chemical composition (Ogunniyi, 2006; Severino et al., 2012).

The Brazilian National Program for Production and Use of Biodiesel has identified *R. communis* as the ideal oil crop to promote social development in the semi-arid region of Brazil because of its versatility as a productive (oil) crop in various environments (Cesar and Batalha, 2010). For this reason *R. communis* is currently grown in the arid zones of Northeastern Brazil (Sausen and Goncalves Rosa, 2010). Genotypes MPA11, MPB01 and IAC80 were developed by the breeding program of the Campinas Agronomic Institute and by the *Empresa Baiana de Desenvolvimento Agrícola S.A* (EBDA), aiming at finding alternative high yielding crops for family farmers in the semi-arid region of Brazil.

R. communis is able to grow in dry and hot environments where other crops would not grow and still produce good yield (Vijaya Kumar et al., 1997). This species is, therefore, considered to be tolerant to a diverse range of environmental stresses, including drought, heat, and salt (Silva César and Otávio Batalha, 2010). For example, *R. communis* plants are able to partially maintain their photosynthetic functions when experiencing periods of severe drought stress, but as soon as the drought stress is relieved, they can fully recover their photosynthetic machinery within one day (Sausen and Rosa, 2010). Avoidance mechanisms such as early and efficient stomatal control are used by *R. communis* plants in order to minimize water loss by transpiration and demand for soil water (Sausen and Rosa, 2010). Furthermore, *R. communis* can deal with soil contamination by heavy metals such as lead, nickel and zinc (Liu et al., 2008; Romeiro et al., 2006). Since *R. communis* possesses the ability to grow in adverse environments, it makes this species an ideal candidate to provide a better understanding of seed germination, seedling performance and adaptation to high temperature. In general, most model plant species are not able to

germinate at temperatures higher than 30ºC, which makes *R. communis* an important species to investigate the biochemical and molecular aspects of germination and seedling establishment under varying temperatures. A better understanding of the biochemical and physiological aspects of germination and seedling growth is crucial for the breeding of high yielding varieties adapted to various growing environments that could be used for family farmers worldwide.

Thesis motivation and outline

The effects of the temperature on seed germination and seedling performance have been assessed in several species (Booth and Bai, 1999; Karim et al., 2000; Niu et al., 2012; Wheeler et al., 2000), but growth and development of *R. communis* is still poorly understood, as compared with other major crops. For example, metabolite profiling studies in *R. communis* are limited to two studies describing the use of LC-MS, HPLC-UV and ^{1}H NMR-based methodologies to reveal differences in the seed metabolome that could be used to characterize both provenance and cultivar (Ovenden et al., 2010; Pigott et al., 2012). There is, however, a lack of studies that correlate physiological events, such as germination or seedling establishment, with metabolic and molecular changes under different environmental conditions. In this thesis, I present a metabolome and transcriptome profiling study of *R. communis* germination and seedling establishment, which adds new insights to the understanding of *R. communis* plasticity in response to different temperatures.

In Chapter 2 I analyzed the potential use of 17 candidate reference genes across a diverse set of samples, including several tissues, different developmental stages and environmental conditions encompassing seed germination and seedling growth in *R. communis*. These genes were tested by RT-qPCR and ranked according to the stability of their expression using two different approaches: GeNorm and NormFinder. The optimal combination of reference genes for a subset of root, endosperm and cotyledons samples is presented, as well as a validation procedure of the selected reference genes by normalizing the expression levels of three target genes involved in energy metabolism.

In Chapter 3 I studied *R. communis* seed imbibition and germination under different temperatures. A thermo-sensitive window was observed during seed germination in which high temperatures compromise seedling development by down-regulating metabolic processes which are crucial to support successful seedling establishment. Cloning and transient expression of malate synthase and glycerol kinase provided important insights into their role in seedling establishment under different temperatures. I also provided new insights into the role of GABA during seed germination and into the molecular requirements for vigorous seedling growth of *R. communis* under different temperatures.

In Chapter 4, I undertook a functional genomics approach using microarray analysis to determine transcriptome changes during seed germination at 20, 25 and 35ºC that could explain this thermo-sensitive

window. I showed that most of the differences in the *R. communis* transcriptome occurred between 6 hours of imbibition and the commence of germination, i.e. radicle protrusion. This coincides with the thermo-sensitive window identified during seed germination in which high temperatures compromise seedling development.

In Chapter 5 I analysed the effect of temperature on growth of young *R. communis* seedlings and I measured primary and secondary metabolites in roots and cotyledons. The biochemical changes observed in response to the increasing temperature provide leads into understanding plant adaptation to harsh environmental conditions, which would be very helpful in developing strategies for *R. communis* crop improvement research.

In Chapter 6 the effect of temperature on biomass allocation in two *R. communis* genotypes by measuring dry weights of roots, stems, and cotyledons of seedlings grown at three different temperatures was assessed. Primary metabolites were measured in roots of seedlings grown at 20ºC, 25ºC, and 35ºC to assess metabolic changes associated with temperature that could provide further insights into the mechanisms underlying root biomass allocation. Additionally, transcript levels of genes encoding for antioxidant enzymes were assessed in an additional attempt to identify possible underlying mechanisms that could explain *R. communis'* differential ability to sustain biomass production at high temperatures.

In Chapter 7 I raise the question whether carbohydrate accumulation in *R. communis* leaves, roots, and seeds, grown at relative low temperatures, as compared to higher temperatures, results from up-regulation of biosynthetic pathways, from down-regulation of catabolic pathways, or both. Therefore, we measured transcript levels of genes encoding for key enzymes involved in starch biosynthesis, starch catabolism, and gluconeogenesis.

In Chapter 8 I discuss the main findings of this thesis and present future perspectives.

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Chapter 2

Identification of reference genes for gene expression studies during seed germination and seedling establishment in *Ricinus communis* L.

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A**bstract**

RT-qPCR is an important technology to analyze gene expression levels during plant development or in response to different treatments. An important requirement to accurately measure gene expression levels is a properly validated set of reference genes. In this context, we analyzed the potential use of 17 candidate reference genes across a diverse set of samples, including several tissues, different stages and environmental conditions encompassing seed germination and seedling growth in *Ricinus communis* L. These genes were tested by RT-qPCR and ranked according to the stability of their expression using two different approaches: GeNorm and NormFinder. GeNorm and Normfinder indicated that *ACT*, *POB* and *PP2AA1* is the optimal combination for normalization of gene expression data in inter-tissue (heterogeneous sample panel) studies. We also describe the optimal combination of reference genes for a subset of root, endosperm and cotyledons samples. In general, the most stable genes suggested by GeNorm are very consistent with those indicated by NormFinder, which highlights the strength of the selection of reference genes in our study. We also validated the selected reference genes by normalizing the expression levels of three target genes involved in energy metabolism with the reference genes suggested by GeNorm and NormFinder. The approach used in this study to identify stably expressed genes, and thus potential reference genes, was successfully applied for *R. communis* and it provides important guidelines for RT-qPCR studies in seeds and seedlings for other species (especially in these cases where extensive microarray data is not available).

Keywords: Castor bean, gene expression, GeNorm, normalization, NormFinder, quantitative real-time PCR

1 Introduction

Ricinus communis L. (castor bean) is a drought resistant species which is well adapted to hot climates. It is an important oilseed crop because of its unique oil composition consisting of up to 94% of the fatty acid ricinoleic acid (12-hydroxy-cis-9-octadecenoic acid) (Gong et al., 2005). This fatty acid confers unique properties to the oil and the biodiesel produced from it (Anjani, 2012; Conceicao et al., 2007; Salimon et al., 2010). *R. communis* oil is used in the chemical industry worldwide, but its production is limited by insufficient supply of feedstock (Severino et al., 2012). India, Brazil, and China are the major oil crop producers in the world and, recently, biodiesel production has received wide attention in Brazil, where the Program for Production and Use of Biodiesel (PNPB) has identified castor bean as the ideal species for oil production which, in addition, could help to promote social development in the semi-arid regions of Brazil (Cesar and Batalha, 2010). Its agronomic importance spurred molecular research in *R. communis* related to seed maturation (Cagliari et al., 2010; Loss-Morais et al., 2013; Sánchez-García et al., 2010), plant development (O'Leary et al., 2011), and responses to biotic and abiotic stresses (Maciel et al., 2011; Wei et al., 2010). Characterization and expression profiles of several genes from *R. communis* have been reported (Cagliari et al., 2010; Chileh et al., 2010; Maciel et al., 2011; Wei et al., 2010). In this study we focus on seed germination and seedling growth, since a better understanding of the biochemical and molecular aspects of these processes in *R. communis* is crucial for the breeding of high yielding varieties adapted to various growing environments (Severino and Auld, 2013).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is the method of choice for gene expression profiling studies due to its high sensitivity, accuracy, and speed. Accurate gene expression measurements involve normalization to correct for differences in sample input due to errors in measurements, pipetting and differences in enzymatic efficiencies (Vandesompele et al., 2002). Internal control genes or ‘reference genes’ are used to normalize for such differences. Reference genes are presumed to be stably expressed across different tissues and at various developmental stages and growth conditions. However, the stability of used reference genes needs to be properly validated for accurate and reliable normalization of gene expression data, rather than to trust their presumed expression stability. This is an important requirement for a reliable RT-qPCR experiment (Vandesompele et al., 2002). In the last decade several tools have become available to determine which candidate reference genes are stably expressed across a set of samples e.g. GeNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004). These tools helped to identify stable reference genes in a range of plant species (Artico et al., 2010; Dekkers et al., 2012; Jarosová and Kundu, 2010; Pettengill et al., 2012). However, so far, no validated reference genes for RT-qPCR studies during germination and early seedling establishment in *R. communis* are available.

In this study, the expression of 17 candidate reference genes was analyzed across a set of 14 samples, using three biological replicates per sample, that include several tissues at different stages under different environmental conditions including light/dark, water/nutrient solution and temperature treatments. Furthermore, to illustrate the usefulness and to validate the candidate reference genes for analysis of gene expression purposes, we analyzed the expression levels of three *R. communis* genes, oil body lipase 2 (*OBL2*), chlorophyll A/B binding protein (light-harvesting complex - *LHCB3*) and *α*-amylase (*AMY3*), which are involved in primary energy metabolism. This is the first systematic report on the selection of reference genes for *R. communis*, providing useful guidelines for future accurate gene expression profiling experiments by RT-qPCR.

2 Materials and methods

2.1 Plant material

Ricinus communis L. seeds (genotype MPA11) used in this work were kindly supplied by Empresa Baiana de Desenvolvimento Agrícola S.A (EBDA), Salvador-Bahia, Brazil. After seed coat removal, seeds were allowed to imbibe and germinate using paper rolls as substrate at 25ºC in the dark. In the first part of the experimental design, germinated seeds were allowed to grow on paper rolls at 25°C, in the dark, for 9 days. Roots, endosperm and cotyledons of 3, 6 and 9-day-old germinated seeds were collected, immediately frozen in liquid nitrogen, freeze-dried, ground and stored at -80ºC prior to analysis. Three biological replicates of 30 seeds each were used. In the second part of the experimental design germinated seeds were transferred from paper rolls to moist vermiculite and were allowed to grow at 20ºC in continuous light for 10 days. Half of the 10-day-old seedlings were then transferred to an incubator at 35ºC with continuous light, while the other half was kept at the original condition. After 4 days, roots and green cotyledons (three biological replicates of 15-18 seedlings each) were collected, immediately frozen in liquid nitrogen, freeze-dried, ground and stored at -80ºC prior to analysis. Details of the different samples are shown in Supplementary Table 1.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from 20 mg of dry material using the RNeasy Plant Mini Kit (Qiagen) including the DNase digestion of genomic DNA performed using the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. RNA quantification and quality control was performed spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Samples showing A260/A280 and A260/A230 ratios of 1.8–2.2 were used for subsequent analysis. RNA quality was further inspected by the integrity of ribosomal RNA bands on a 1% agarose gel. Sharp and

intense 18S and 28S ribosomal RNA bands, without visible degradation, confirmed the suitability of the isolation method. The first strand cDNA was synthesized from 1 µg of total RNA using the iScriptTM cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Reverse transcription was performed at 37°C for 40 min followed by 85°C for 5 min. The cDNA was diluted 20 times for the use in RT-qPCR reactions. All cDNAs were stored at -20°C prior to the analysis.

2.3 Reference gene selection, primer design and measurement of amplification efficiency

Potential reference genes were selected based on available literature data of commonly used reference genes in RT-qPCR studies in Arabidopsis, tomato, *Jatropha curcas* and *R. communis*. We started our selection for candidate reference genes by a literature search for reference genes commonly used in *R. communis*, which includes: *18S*, protein Pob (*POB*), NADH-ubiquinone oxidoreductase (*NADH_OXI*) and elongation factor 1-beta (*EF1B*) (Arroyo-Caro et al., 2013; Cagliari et al., 2010; Chen et al., 2007; Eastmond, 2004; Li et al., 2012a; Loss-Morais et al., 2013). In addition to these candidates, 40S ribosomal protein (*40S*), 60S acidic ribosomal protein (*60S*), β-tubulin (*β-TUB*), translation elongation factor G (*EFG*) and structural constituent of ribosome (*SCR*) genes were also selected because of their usage as reference genes for other species (Figure 1, Supplementary Table 2) (Le et al., 2012; Li et al., 2012b; Rapacz et al., 2012). However, only few of these studies have validated the expression stability of these putative reference genes (Cagliari et al., 2010). Since the number of candidate reference genes was very low we extended our search and looked for putative reference genes that were described as stably expressed in seeds of the model plant *Arabidopsis thaliana*, tomato and also for putative reference genes in the closely related species *Jatropha curcas* L.. For Arabidopsis, tomato and *J. curcas* candidate genes, the sequences of possible orthologs were identified through a TBLASTX against the *R. communis* translated nucleotide database (NCBI, http://www.ncbi.nlm.nih.gov/). Only sequences that showed high similarity (E-value < 1e-70) were considered putative orthologs in *R. communis* and were selected for primer design. This selection consisted of genes for ubiquitin-conjugating enzyme E2 (*UBI_E2*), serine/threonine protein phosphatase 2a regulatory subunit A (*PP2AA1*), Type 2A phosphatase activator TIP41 (*TIP41*) and GTP-binding protein Sar1 (*SAR1*) used for Arabidopsis and tomato seeds (Dekkers et al., 2012) and actin (*ACT*), ubiquitin (*UBI*), α-tubulin (*α-TUB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) used for developing *J. curcas* seeds (Figure 1, Supplementary Table 2) (Fan et al., 2013; Gu et al., 2012; Rocha et al., 2013). After an initial quality assessment primer pairs for 17 putative reference genes remained (Supplementary Table 2). The gene identifier (GI) number, gene name and description, amplicon length, primer efficiency and primer sequences (reverse and forward) are listed in Supplementary Table 2. Primers for the 17 potential reference genes were designed using CLCbio software (CLC bio, Aarhus, Denmark) with melting temperatures (Tm) of 58–62°C, primer lengths of 18–

22 bp and amplicon lengths of 80-200 bp. The information of all primers used in this study is summarized in Supplementary Table 2. The PCR amplification efficiency was evaluated based on a standard curve generated by two-fold serial dilutions of a pooled cDNA sample. The specificity of the primers was verified by loading the amplicons on a 2.5% agarose gel and melting curve analysis.

2.4 RT–qPCR conditions

For RT–qPCR experiments, reactions were prepared in a total volume of 10 µL containing 2.5 µL of cDNA, 0.5 µL of primer-mix (from a 10 µM working solution), 5 µL of iQ SYBR Green Supermix (Bio-Rad) and 2 µL of water. The RT–qPCRs were run on a CFX (Bio-Rad). The following qPCR program was used for all PCR reactions: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melting curves were recorded after cycle 40 by heating from 65 to 95°C, increasing the temperature stepwise every 5 s by 0.5°C.

2.5 Evaluation of reference gene expression stability: GeNorm and NormFinder

The expression levels of seventeen candidate reference genes were determined by the number of cycles (Cq) required to reach fluorescence above a specific threshold level. GeNorm (Hellemans et al., 2007; Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) were used for expression stability evaluation of the candidate reference genes across all tested samples. GeNorm ranks the candidate reference genes according to the expression stability index, M. The most stable genes show the lowest M values. The pairwise variation (Vn/Vn+1) was calculated to determine the optimal number of reference genes (Vandesompele et al., 2002). Alternatively, Normfinder (Andersen et al., 2004) was used to identify the optimal reference genes. Normfinder ranks the putative reference genes according to their stability value (Andersen et al., 2004).

2.6 Reference gene validation: Normalization of three target genes

Three genes involved in primary energy metabolism were used to demonstrate the usefulness of the candidate reference genes and to validate them. Oil body lipase 2 (*OBL2*), chlorophyll A/B binding protein (light-harvesting complex - *LHCB3*) and *α*-amylase (*AMY3*) expression levels were normalized using the most stable candidate reference genes across all tested samples. qBase (Biogazelle, Ghent, Belgium) was used to normalize the expression levels using these reference genes and the most stable candidate reference genes across three sample subsets: root, cotyledons and endosperm samples. qBase allows gene expression levels to be normalized by using up to five reference genes (Hellemans et al., 2007). Statistical analysis were conducted using one-way ANOVA and the Tukey post-test with IBM

SPSS Statistics® (Windows version 19, SPSS Inc., Chicago) and Microsoft Excel 2010®. Values of $p < 0.05$ were considered to be statistically significant.

3 Results and Discussion

3.1 Primer specificity, efficiency and expression profile of the seventeen candidate reference genes

Gene expression measurements by RT-qPCR rely on normalization by stably expressed reference genes. A set of validated reference genes is of utmost importance to ensure reliable and accurate data normalization. However, such a set is not available for *R. communis*. Therefore we selected a range of putative reference genes and tested their expression stability over a diverse set of samples encompassing seed germination and seedling establishment in *R. communis*. A successful approach to identify stably expressed genes, and thus potential reference genes, in a given sample panel is to mine microarray data (Czechowski et al., 2005; De Oliveira et al., 2012; Dekkers et al., 2012; Popovici et al., 2009). To our best knowledge microarray data for seed germination and seedling establishment in *R. communis* is not yet available. Therefore, we selected 17 putative reference genes from a literature search. These include four candidate reference genes commonly used in *R. communis* and 13 candidate reference genes used in other species (Figure 1).

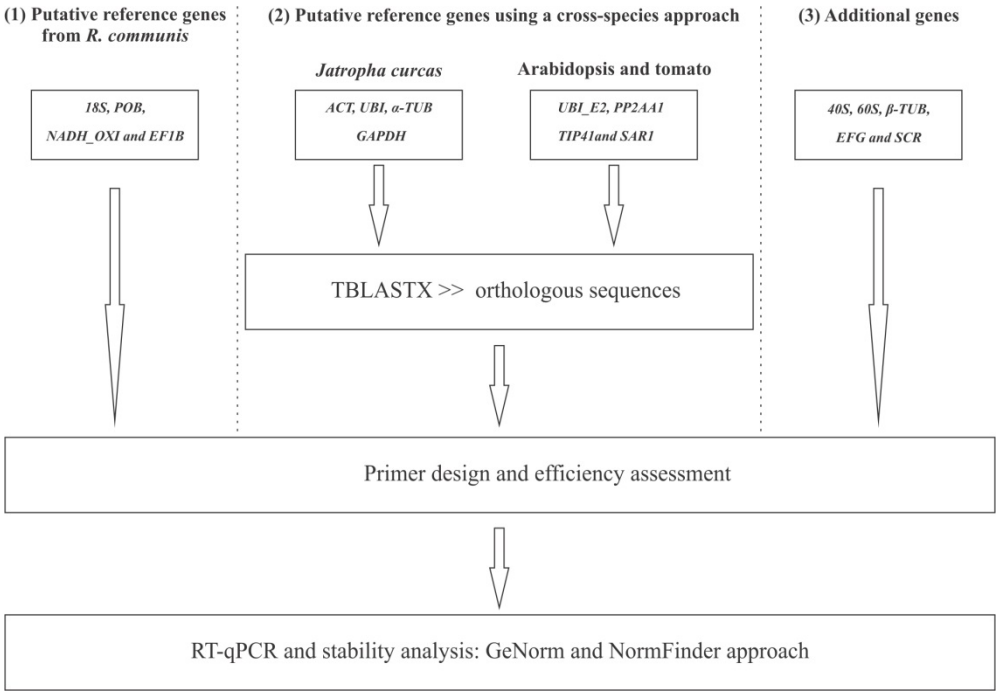

Figure 1 Flowchart showing the steps followed in this study from the selection of putative reference genes to gene expression stability analysis.

To evaluate the primer amplification specificity of these putative reference genes, electrophoresis was performed with the product of an RT-qPCR on a pooled cDNA template with the primers for each candidate reference gene. Additionally, analysis of the melting curves confirmed that all primers generated only a single amplicon (Supplementary Figure 1) and that neither primer dimers nor unexpected products were found. The absence of genomic DNA was confirmed by comparing RT-qPCR with the cDNA template with that of RNA samples which were not reverse transcribed (minus RT control). Most of the minus RT controls did not show any amplification. In some samples, low signals were detected in the minus RT control, but these were at least 7 Cq (quantification cycles) higher than with the cDNA samples. This is clearly above the limit of 5 Cq as proposed by Nolan et al. (2006). Amplification efficiency ranged from 88.77% for *GAPDH* to 111.6% for *β-TUB*, and the coefficient of determination (R^2) varied from 0.9709 to 0.9997 (Supplementary Table 2). This indicates that the reaction conditions are optimal and that the results obtained are highly repeatable.

The expression levels of the seventeen candidate reference genes across all samples are presented as Cq values (Figure 2) which represent the relative abundance of a particular transcript. The mean values of most candidate reference genes were between a Cq of 21 (*EF1B*) and 29 (*SCR*). This difference represents a 150-fold higher abundance of *EF1B* over *SCR*. The mean Cq value of *18S* was 8.41. *SAR1* showed the lowest variation in expression across all tested samples ($Cq_{max} - Cq_{min} = 6.22$), whereas *β-TUB* showed the highest variation ($Cq_{max} - Cq_{min} = 13.34$).

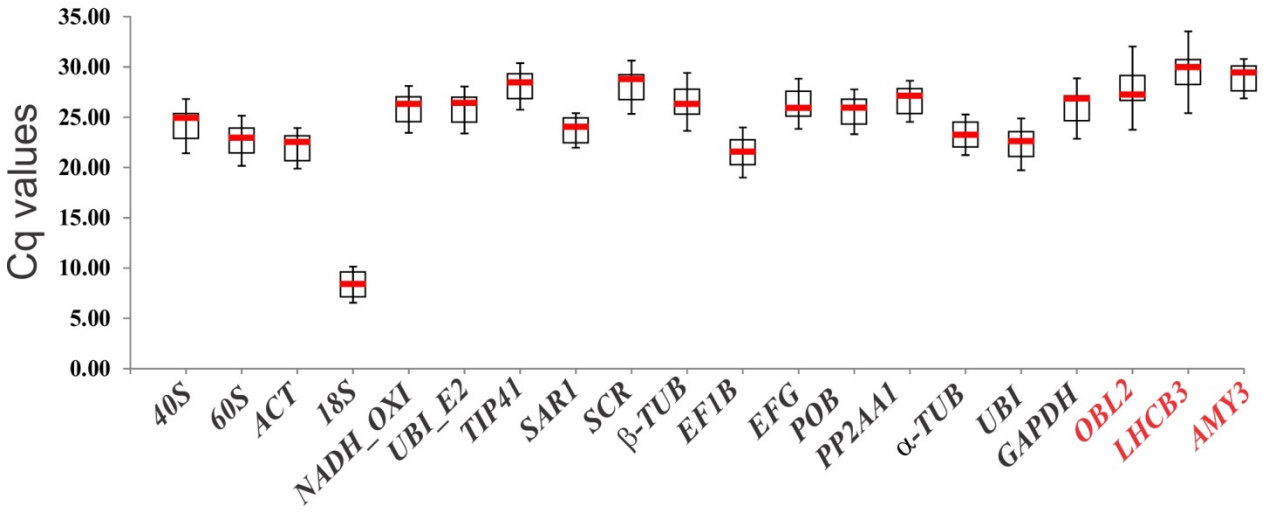

Figure 2 Quantification cycle values (Cq) of all seventeen candidate reference and three target genes across all samples. The red horizontal line across the boxes depicts the median Cq value.

3.2 Gene expression stability analysis

First, gene expression stability was analyzed across all samples aiming at selecting reference genes that could be used to normalize between tissues. Then the samples were subdivided in three tissue-specific subsets i.e. root, cotyledons and endosperm samples (Supplementary Table 1). These four sample panels were analyzed using two methods to assess the gene expression stability: GeNorm and NormFinder. GeNorm calculates the average expression stability (M) based on the average pairwise variation between all tested genes. A lower value of M indicates more stable gene expression and vice versa (Vandesompele et al., 2002). The ranking order according to the M value is shown in Figure 3 and Table 1. For more heterogeneous sets of samples (all tested samples), M values lower than 1 were used (Hellemans et al., 2007). M values lower than 0.5 were used to identify stably expressed reference genes in relatively homogeneous sets of samples (tissue-specific).

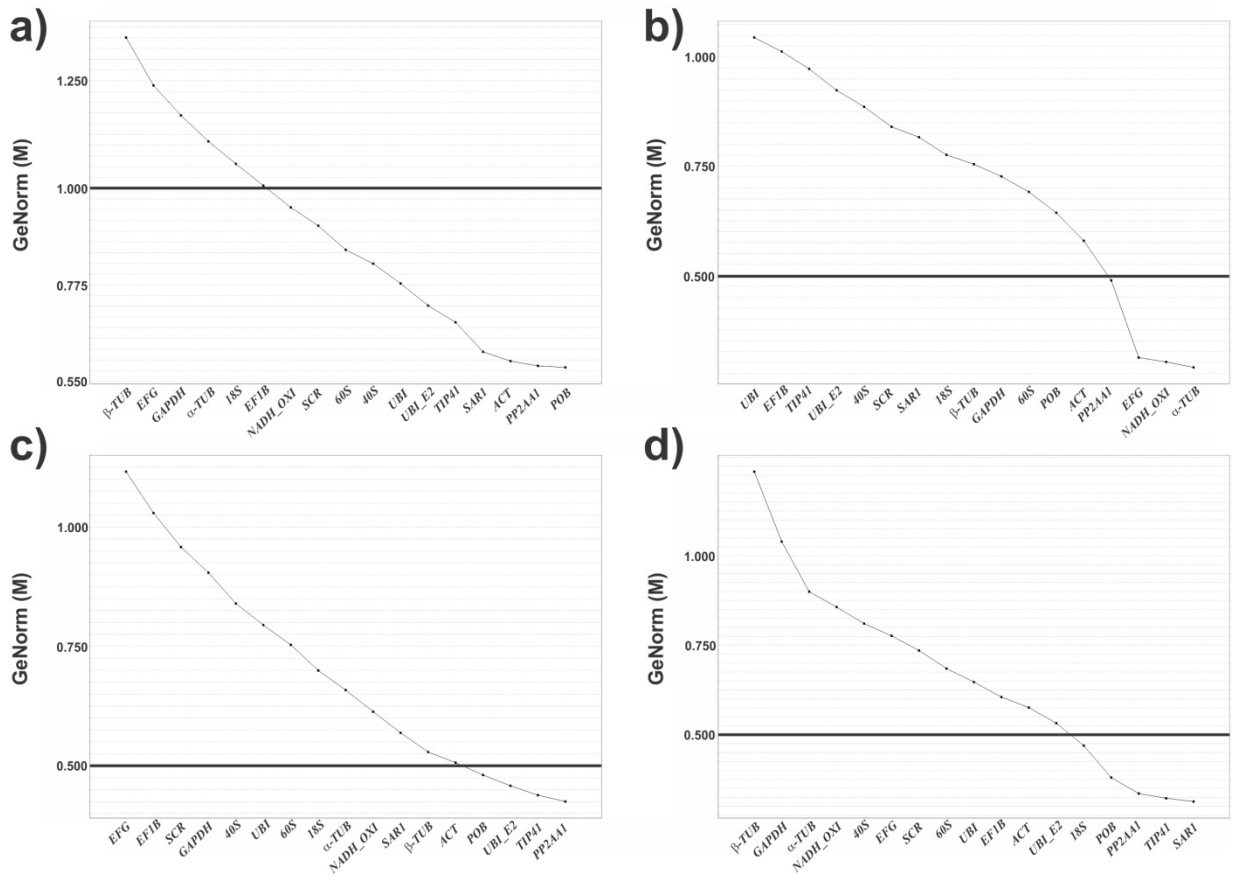

Figure 3 Expression stability values (M) of 17 candidate reference genes. (a) All *R. communis* samples, (b) root samples in different developing stages and temperatures, (c) cotyledon samples in different developing stages and temperatures, (d) endosperm samples in different developing stages. A lower M value indicates more stable expression.

Table 1. Expression stability analysis of seventeen candidate reference genes by GeNorm.

Experimental sets	Most stable genes	Optimal combination (V < 0.15)	Least stable genes
total	*ACT*, *TIP41*, *SAR1*, *POB* and *PP2AA1* (M < 1.0)	*ACT*, *POB* and *PP2AA1*	*β-TUB*, *EFG*, *α-TUB* and *GAPDH*
root	*NADH_OXI*, *EFG*, *PP2AA1* and *α-TUB* (M < 0.5)	*NADH_OXI* and *α-TUB*	*UBI_E2*, *TIP41*, *EF1B* and *UBI*
cotyledons	*UBI_E2*, *TIP41*, *POB* and *PP2AA1* (M < 0.5)	*UBI_E2*, *TIP41* and *PP2AA1*	*SCR*, *EF1B*, *EFG* and *GAPDH*
endosperm	*18S*, *TIP41*, *SAR1*, *POB* and *PP2AA1* (M < 0.5)	*TIP41* and *SAR1*	*NADH_OXI*, *β-TUB*, *α-TUB* and *GAPDH*

POB, *PP2AA1*, *ACT* and *SAR1* were defined as the four most stable reference genes (0.575 < M < 0.625) for the entire set of samples (inter-tissue) (Figure 3a). GeNorm also determines the optimal number of reference genes (GeNorm V < 0.15) required for calculating an accurate normalization factor, which is based on pairwise variation (V_n/V_{n+1}). In order to accurately normalize gene expression data for the entire set of samples, the use of the three most stable genes is suggested (V_3/V_4 < 0.15) (Figure 4a; Table 1).

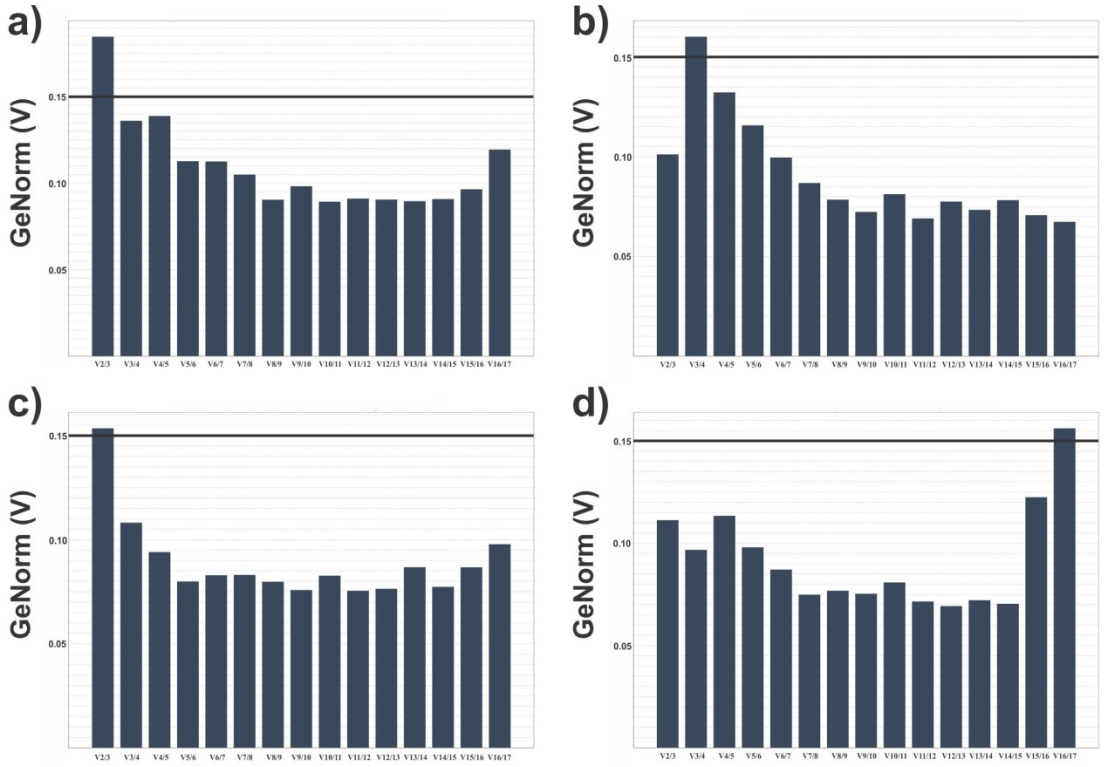

Figure 4 Determination of optimal number of reference genes based on pairwise variation (V) analysis of normalization factors of the candidate reference genes. (a) All *R. communis* samples, (b) root samples in different developing stages and temperatures, (c) cotyledon samples in different developing stages and temperatures, (d) endosperm samples in different developing stages. The Vn/n+1 value was calculated based on every comparison between two of the seventeen consecutive candidate reference genes and used to determine the optimal number of reference genes for use in RT-qPCR data normalization

For accurate normalization of root and endosperm subset samples two reference genes should be used (Figure 4b and 4d; Table 2), while for cotyledons the optimal number is three (Figure 4c; Table 2). *NADH_OXI* and *α-TUB* were the most stable genes across all root samples, *UBI_E2*, *TIP41* and *PP2AA1* for cotyledons and *TIP41* and *SAR1* for endosperm samples (Figure 3; Table 2). As expected, the most stable genes showed the lowest variation in expression across the entire sample panel ($Cq_{max} - Cq_{min} \approx 6$-7) while the least stable genes showed the highest variation ($Cq_{max} - Cq_{min} \approx 9$-13) (Figure 2, Table 1, Supplementary Table 2).

NormFinder is a freely available Visual Basic application for Microsoft Excel, which automatically calculates the stability value for all candidate reference genes tested on a sample set containing any number of samples organized in any given number of groups (Andersen et al., 2004). NormFinder ranks all the candidate reference genes according to their stability value in a robust manner which shows low sensitivity toward co-regulation of the candidate reference genes (Andersen et al., 2004). The ranking of reference genes based on the NormFinder algorithm for the entire set of samples indicated *ACT*, *PP2AA1* and *POB* as the three most stable genes (Table 2), which is very consistent with the output obtained from GeNorm (Figure 3a; Table 1). Furthermore, NormFinder indicated *ACT*, *PP2AA1* and *POB* as the three most stable genes across all root samples, *UBI_E2*, *PP2AA1* and *POB* for cotyledons and *ACT*, *EFG* and *18S* for endosperm samples (Table 2).

Table 2. Expression stability analysis of seventeen candidate reference genes by NormFinder

Total		**Roots**		**Cotyledons**		**Endosperm**	
Best gene	**Stability value**	**Best gene**	**Stability value**	**Best gene**	**Stability value**	**Best gene**	**Stability value**
ACT	0.277	*ACT*	0.014	*UBI_E2*	0.012	*ACT*	0.064
PP2AA1	0.278	*POB*	0.023	*PP2AA1*	0.015	*EFG*	0.074
POB	0.289	*PP2AA1*	0.074	*POB*	0.028	*18S*	0.091
NADH_OXI	0.327	*60S*	0.221	*TIP41*	0.049	*POB*	0.198
60S	0.345	*40S*	0.230	*β-TUB*	0.066	*NADH_OXI*	0.200

Additionally, genes encoding the ribosomal units *18S*, *40S* and *60S* were suggested by NormFinder as suitable reference genes for normalization of RT-qPCR data in *R. communis* (Table 2). In order to rule out a potential sensitivity of this algorithm to co-regulation of these ribosomal genes, we re-calculated the stability value of all candidate reference genes without the gene expression data of *40S*. However, the removal of *40S* did hardly affect the candidate reference gene output of NormFinder (Table 3).

Table 3. Expression stability analysis of seventeen candidate reference genes by NormFinder (excluding *40S*)

Total		Roots		Cotyledons		Endosperm	
Best gene	**Stability value**	**Best gene**	**Stability value**	**Best gene**	**Stability value**	**Best gene**	**Stability value**
ACT	0.270	*ACT*	0.014	*PP2AA1*	0.004	*ACT*	0.054
PP2AA1	0.278	*POB*	0.033	*UBI_E2*	0.008	*EFG*	0.084
POB	0.308	*PP2AA1*	0.060	*POB*	0.023	*18S*	0.084
NADH_OXI	0.312	*EFG*	0.224	*β-TUB*	0.048	*NADH_OXI*	0.157
60S	0.352	*60S*	0.228	*TIP41*	0.064	*UBI_E2*	0.202

3.3 Expression profile of three functional genes

To evaluate our sets of reference genes we measured and normalized the expression of three target genes involved in energy metabolism. *OBL2* is an oil body-associated lipase from *R. communis* endosperm that is induced upon seed germination and is responsible for the hydrolysis of a range of triacylglycerols (Eastmond, 2004). *OBL*-like proteins are present in many species and they probably play an important role in regulating lipolysis during germination (Eastmond, 2004). The *LHCB3* (light-harvesting chlorophyll A/B) gene is responsible for the conversion of free energy from absorbed light into ATP, reduced ferredoxin (Fd) and NADPH that can be used to drive metabolism in the chloroplast and cytosol (Foyer et al., 2012). Down-regulation or disruption of any member of the LHC family reduces responsiveness of stomata movement to ABA and therefore results in a decrease in plant tolerance to drought stress in *Arabidopsis thaliana* (Xu et al., 2012). *α*-Amylase is involved in the degradation of starch and it has been demonstrated that in Arabidopsis leaves it is induced by biotic and abiotic stress and secreted (Doyle et al., 2007). The expression levels of these three target genes were measured and normalized by (a) using *ACT*, *POB* and *PP2AA1* as reference genes and by (b) using tissue-specific reference genes for which the expression stability value M was below the recommended value of 0.5 for each subset. The results show that *OBL2*, *LHCB3* and *AMY3* genes were expressed in all samples (Figure 5) and the mean Cq values varied between 27.23 (*OBL2*) and 29.96 (*LHCB3*) (Figure 2, Supplementary Table 2). *LHCB3* showed the highest variation in expression across all tested samples ($Cq_{max} - Cq_{min}$ = 13.3), whereas *AMY3* showed the lowest variation ($Cq_{max} - Cq_{min}$ = 6.81). The observed wide range of Cq values for *LHCB3* is due to the fact that photosynthetic tissues, such as 14-day-old greening cotyledons, showed relatively higher expression as compared to the non-photosynthetic endosperm tissues (data not shown). Expression levels of *OBL2* were more than a 1000-fold higher in 9-day-old endosperm as compared to the expression in dry seeds (Figure 5g). This is consistent with the function of the gene, which regulates lipolysis during germination (Eastmond, 2004). To illustrate the effect of reference gene set selection on the normalization and the accuracy of the RT-qPCR data, we compared the normalized expression data of the target genes obtained by using *ACT*, *POB* and *PP2AA1* as reference genes with the

values obtained by using tissue-specific reference genes. For most of the genes, a high correlation was found between the normalized data obtained by using *ACT*, *POB* and *PP2AA1* as reference genes and the data normalized by the respective tissue-specific reference genes (Figure 5). The correlation coefficient (R^2) varied from 0.4348 (*AMY3*, roots) to 0.9986 (*OBL2*, endosperm)(Figure 5a-i). We only found a weak correlation for *OBL2* (Figure 5a) and *AMY3* (Figure 5c) in the root sample panel. However, it is important to highlight that the combination of genes suggested for the entire dataset by GeNorm (as well as Normfinder) is the same as that suggested by NormFinder for the root subset and thus similar! This difference is likely caused by the different algorithms used in both methods.

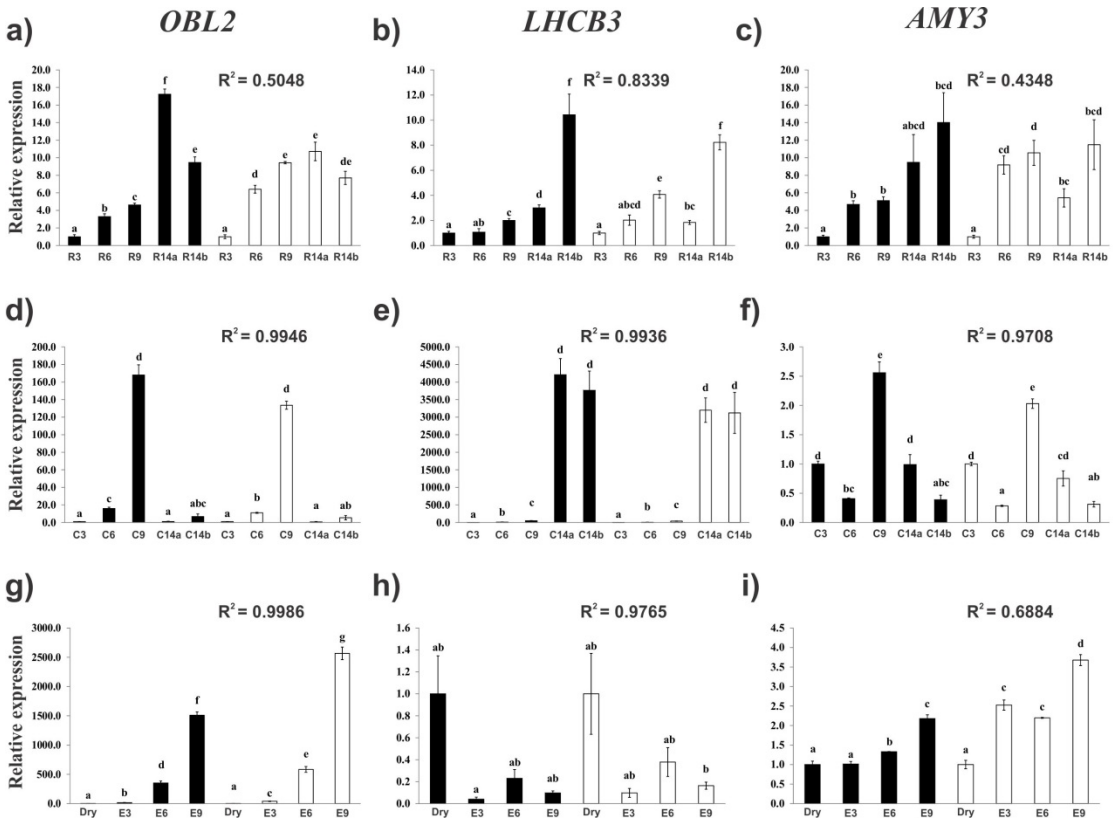

Figure 5 Relative expression of *OBL2* (a, d and g), *LHCB3* (b, e and h) and *AMY3* (c, f and i) in root (a-c), cotyledons (d-f) and endosperm (g-i) samples. Black bars represent the expression levels normalized by using *ACT*, *POB* and *PP2AA1* reference genes as the optimal combination selected by GeNorm to normalize an inter-tissue (heterogeneous) sample panel. White bars represent the expression levels normalized by using reference genes found as the optimal combination selected by GeNorm for the sample specific sets: *NADH_OXI* and *α-TUB* in root samples (a-c), *UBI_E2*, *TIP41* and *PP2AA1* in cotyledons samples (d-f) and *TIP41* and *SAR1* in endosperm samples (g-i). Averages and standard errors of three biological and two technical replicates are shown. Expression levels were normalized towards the first column (R3, C3 and Dry) for roots, cotyledons and endosperm sample panels, respectively. Results are expressed as mean ± SD. Letters above the bars indicate significant differences between different samples by Tukey's HSD ($p<0.05$).

The fact that most stable genes suggested by GeNorm are very consistent with those indicated by NormFinder for the entire sample panel and for the cotyledon subset, highlights the strength of the selection of the reference genes in our study. However, little overlap was observed for endosperm samples (Table 1 and 2). Therefore, we also normalized the expression of the three studied target genes by using the most stable genes suggested by NormFinder (*ACT* and *EFG*) for endosperm samples (Figure 6). The expression pattern of *OBL2* and *LHCB3* were extremely consistent between all three sets of reference genes used to normalize the expression levels (Figure 6a-b), however the relative expression levels of *OBL2* were slightly higher for the samples normalized by using *TIP41* and *SAR1* reference genes (white bars) as compared to the samples normalized by using *ACT*, *POB* and *PP2AA1* (black bars) and *ACT* and *EFG* (red bars) as reference genes for the subset of endosperm samples (Figure 6a). The expression pattern and expression levels of *AMY3* for the samples normalized by using the optimal combination selected by GeNorm to normalize the complete inter-tissue sample panel (*ACT*, *POB* and *PP2AA1*) and the optimal combination selected by NormFinder for the subset of endosperm samples (*ACT* and *EFG*) were more similar to each other than to the values obtained by using *TIP41* and *SAR1* as reference genes (Figure 6c). Taking together, these results suggest that the approach used to select reference genes for *R. communis* is robust and provides useful guidelines for future accurate gene expression profiling experiments by RT-qPCR.

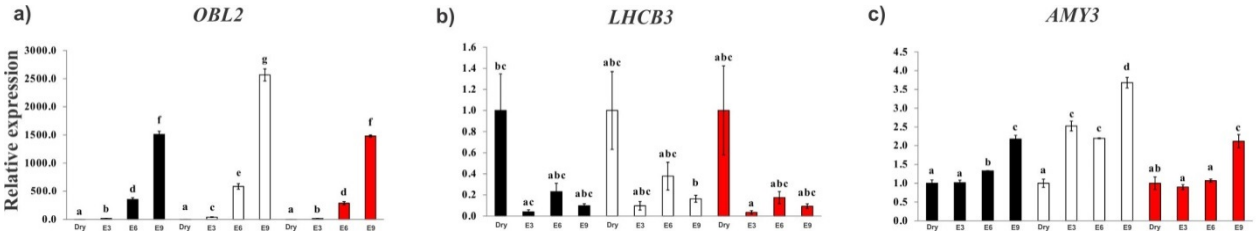

Figure 6 Relative expression of *OBL2* (a), *LHCB3* (b) and *AMY3* (c) in endosperm samples. Black bars represent the expression levels normalized by using *ACT*, *POB* and *PP2AA1* reference genes as the optimal combination selected by GeNorm to normalize an inter-tissue (heterogeneous) sample panel. White bars represent the expression levels normalized by using *TIP41* and *SAR1* reference genes found as the optimal combination selected by GeNorm for the subset of endosperm samples. Red bars represent the expression levels normalized by using *ACT* and *EFG* reference genes found as the optimal combination selected by NormFinder for the subset of endosperm samples. Averages and standard errors of three biological and two technical replicates are shown. Expression levels were normalized towards the first column (R3, C3 and Dry) for roots, cotyledons and endosperm samples, respectively. Results are expressed as mean ± SD. Letters above the bars indicate significant differences between different samples by Tukey's HSD ($p<0.05$).

4 Conclusions

This study reports a systematic analysis aimed at determining the optimal combination of reference genes for the normalization of gene expression data for *R. communis* seed germination and seedling establishment, providing useful guidelines for future accurate gene expression profiling experiments by RT-qPCR. Genes described in the literature as stably expressed in *J. curcas* (*ACT* and *EF1B*), Arabidopsis and tomato (*TIP41, SAR1* and *PP2AA1*) were proven to be stable for *R. communis* seed and seedling samples (Figure 1 and Table 1 and 2). Therefor these genes are likely candidates for successful gene expression studies in seed and seedlings of a broader range of species. More importantly, our data showed that some of the widely used reference genes were not suitable reference genes for our samples (Table 1), highlighting the importance of a proper validation of candidate reference genes for each study. Normalization of expression data of three target genes involved in energy metabolism highlights the reliability of the selected reference genes in our study. The approach used in this study to identify stably expressed genes, and thus potential reference genes, was successfully applied for *R. communis* and it provides important guidelines for finding suitable reference genes for RT-qPCR studies in seeds and seedlings of other species (especially in the case that extensive microarray data is not available).

5 Authors' contribution

PRR carried out the physiological and molecular experiments, data processing, statistical analysis and draft of the manuscript. BJWD, LGF, RDC, WL and HWMH participated in the design of the study, coordination and critical reading of the manuscript. All authors read and approved the manuscript.

6 Conflict of interest

The authors declare no conflict of interest.

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Supplementary Tables

Supplementary Table 1 Description of tissue samples used for reference gene validation.

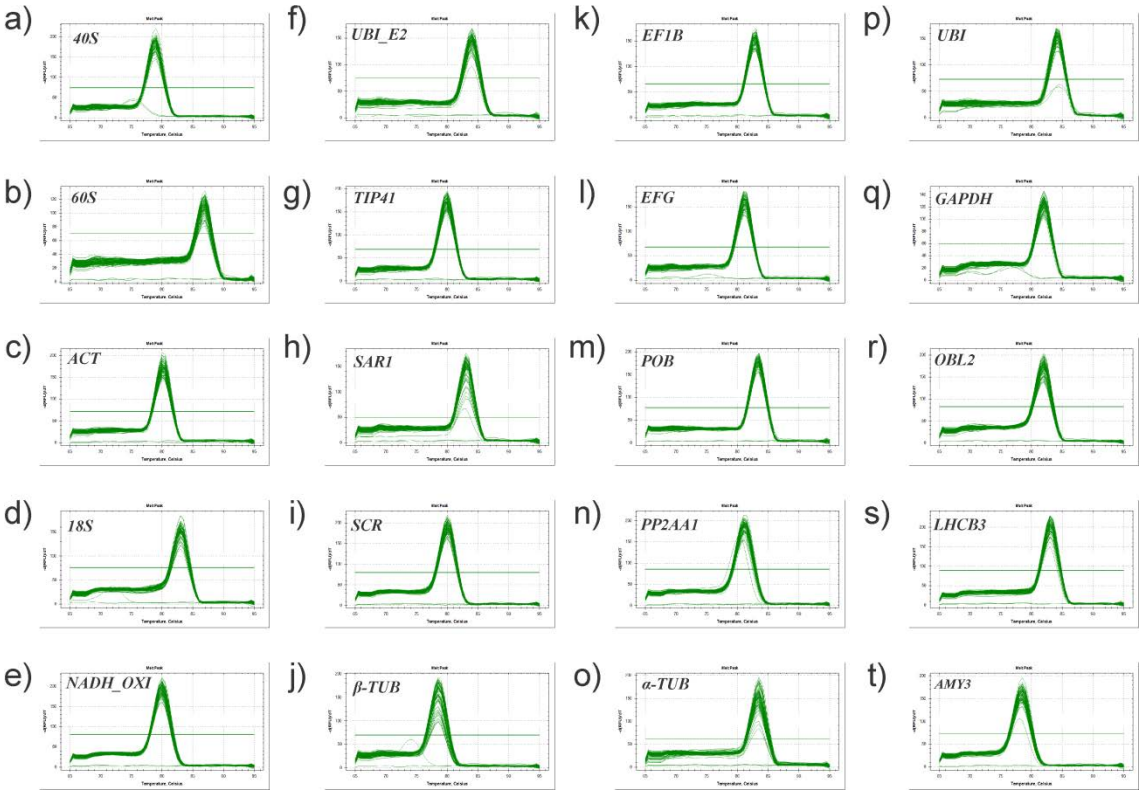
Sample code	Sample type	Age (days)	Growth condition and treatment
C3	cotyledons[a]	3	paper roll, imbibed in water, dark, 25°C
C6	cotyledons[a]	6	paper roll, imbibed in water, dark, 25°C
C9	cotyledons[a]	9	paper roll, imbibed in water, dark, 25°C
C14a	green cotyledons + first real leaves[b]	14	vermiculite, full light, nutrient solution, 20°C
C14b	green cotyledons + first real leaves[b]	14	vermiculite, full light, nutrient solution, 35°C
E3	endosperm[a]	3	paper roll, imbibed in water, dark, 25°C
E6	endosperm[a]	6	paper roll, imbibed in water, dark, 25°C
E9	endosperm[a]	9	paper roll, imbibed in water, dark, 25°C
R3	root[a]	3	paper roll, imbibed in water, dark, 25°C
R6	root[a]	6	paper roll, imbibed in water, dark, 25°C
R9	root[a]	9	paper roll, imbibed in water, dark, 25°C
R14a	root[b]	14	vermiculite, full light, nutrient solution, 20°C
R14b	root[b]	14	vermiculite, full light, nutrient solution, 35°C
Dry	dry seeds[a]	-	-

[a] Three biological replicates of 30 seeds. [b] Three biological replicates of 15-18 seeds.

Supplementary Table 2. Description of seventeen *R. communis* candidate reference genes and three target genes used for RT-qPCR.

Gene	Gene identifier (GI)	Gene description	Amplicon lenght	Primer efficiency (%)	Regression coefficient	Forward	Reverse	Cq min	Cq max
40S	255587655	40S ribosomal protein S8	158	93.1	0.9903	AAGGAAGAAGGGCAAGGG	GTGCGGAATGAGGATCGT	18.97	28.31
60S	255571860	60S acidic ribosomal protein P0	126	101.97	0.9844	AAGGAAGTGAGTGAAGAGGT	GAAAGAGGTCTGAGAGGGG	17.55	27.19
ACT	38259661	actin (ACT)	198	97.02	0.9806	TCCCTCAGTACGTTCCAGCA	CACCTCCATACTCCTCCCT	18.11	25.17
18S	625161	18S ribosomal RNA	150	91.85	0.9893	TTGGTGGAGCGATTTGTC	CCCAGAACATCTAAGGGCAT	4.6	13.64
NADH_OXI	255582279	NADH-ubiquinone oxidoreductase	170	96.23	0.9877	CCTCTTTCTCCTCCGTTCC	CCACAAATACTTCAATCGCATC	21.16	29.76
UBI_E2	255585521	ubiquitin-conjugating enzyme E2	131	97.98	0.9813	CCTCTGCAACCTCCACAA	CAAACAGACTGAAGCGTCCAA	21.94	29.41
TIP41	255573082	type 2A phosphatase activator	155	96.74	0.9923	GAGGACAGCGAGCAGAAAA	CGCAAAAGAAGAAACCAACAAC	23.5	33.23
SAR1	255548480	GTP-binding protein sar1	129	102.27	0.9887	TTTTGGCTTCTCTCGGTTTGT	ATTGGGTAGGCTGATGCTG	20.44	26.66
SCR	255579193	structural constituent of ribosome	159	96.71	0.9916	AAACAACAACAGCAAGACCCA	AGGAGGAGGAACAGAGGA	22.4	31.47
β-TUB	255564501	tubulin beta chain	200	111.6	0.9709	CGTTTTGTTCCTCGTGCT	GCTTCCTTCCTCACAACATCT	21.33	34.67
EF1B	255564427	elongation factor 1-beta,	144	91.08	0.9954	GAAAAAGTTGGAGGAGGCAG	AGGGAGTCCACAGAAACAAG	17.3	26.37
EFG	255537028	elongation factor G	152	98.23	0.9925	CCGCTTCTTCTATCTCTTCTTC	TATCGCACGCTTTGCCTC	21.92	31.12
POB	255559069	protein pob	177	90.74	0.9914	TCCTCCGCTACTTCGTCT	ACAATCCATCTTCCTCGTTACT	21.68	29.44
PP2AA1	255576492	ser/thr phosphatase	119	97.41	0.9897	GTTGGGTGTTGGGTTCTTTG	ATTCTTCAGCAAGGCGTTT	22.13	29.8
α-TUB	255562191	tubulin alpha chain	87	91.43	0.9997	ACAAGACAGTAGGCGGAG	CAAAAACAGCACGAGGAACA	19.21	26.77
UBI	255578905	ubiquitin	153	89.51	0.9945	CGCAAATACAACCAAGACAAGA	CCAGCAAGCACTCTCCATCA	17.55	27.12
GAPDH	255537010	G3P dehydrogenase	97	88.77	0.9969	CTGTCTTGCTCCTTTGGCT	GGTCTTTTGGGTGGCAGT	20.31	31.27
OBL2	55831355	lipase	93	98.1	0.9925	CTACCACTTTCCAAACTCACCT	CTTCTTTGCCCATTCCGTC	20.66	36.05
LHCB3	255569584	chlorophyll A/B binding protein	151	90.22	0.9977	GGAGTTGGAGAGGGCAATGA	GAACAAAGAAGCCAAACATGGA	20.01	37.09
AMY3	255570319	alpha-amylase	81	89.79	0.9962	ATTCTCCACTCTGCTCTGC	CCACCATCCCATCACTCCT	24.36	31.91

Supplementary Figure



Supplementary Figure 1 Dissociation curves of seventeen candidate reference genes (a-q) and three target genes (r-t).

Chapter 3

Metabolite profiling of *Ricinus communis* germination at different temperatures provides new insights into thermo-mediated requirements for successful seedling establishment

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Abstract

Ricinus communis seeds germinate to a high percentage and faster at 35ºC than at lower temperatures, but with compromised seedling establishment. However, seedlings are able to cope with high temperatures at later stages of seedling establishment if germination occurred at lower temperatures. Our objective was to assess the biochemical and molecular requirements of *R. communis* germination for successful seedling establishment at varying temperatures. For that, we performed metabolite profiling (GC-TOF-MS) and measured transcript levels of key genes involved in several energy-generating pathways such as storage oil mobilization, β-oxidation and gluconeogenesis of seeds germinated at three different temperatures. We identified a thermo-sensitive window during seed germination in which high temperatures compromise seedling development, most likely by down-regulating some energy-generating pathways. Overexpression of genes encoding for malate synthase (*MLS*) and glycerol kinase (*GK*) resulted in higher starch levels in *N. benthamiana* leaves, which highlights the importance of these genes in energy-generating pathways for seedling establishment. Additionally, we showed that GABA, which is a stress-responsive metabolite, accumulated in response to the water content of the seeds during the initial phase of imbibition. Herewith, we provide new insights into the molecular requirements for vigorous seedling growth of *R. communis* under different environmental conditions.

Keywords: carbon-nitrogen balance, GABA, gluconeogenesis, glyoxylate cycle, seedling establishment, stress responsive metabolites

1 Introduction

Seed germination is a crucial process in the life cycle of higher plants and determines whether seedling establishment will be successful or not. Germination begins with the uptake of water by the seed (imbibition), is followed by reactivation of metabolism, and ends with visible protrusion of the radicle through the surrounding layers (Bewley et al., 2013). In higher plants, seed germination is controlled by a combination of genetic and environmental factors. Temperature is one of the main environmental factors influencing seed germination and plant growth (Kendall and Penfield, 2012). In general, a progressive increase in temperature shortens the time required for germination until it reaches an optimum temperature at which the maximum germination percentage is achieved. This optimum temperature may vary between species (Cochrane et al., 2014; Da Mota and Garcia, 2013). Following imbibition, cells of the embryo change to a metabolically active state in which several physiological, molecular, and biochemical events occur such as cell elongation, cell cycle activation, transcription, translation, cellular respiration, repair mechanisms, and organelle reassembly (Graham, 2008; Miransari and Smith, 2014; Rajjou et al., 2012; Rosental et al., 2014; Weitbrecht et al., 2011). These processes are generally supported by the initiation of central metabolism for energy generation and the production of building blocks for cellular structures (Rosental et al., 2014). Therefore, seed germination also determines whether seedling establishment will be successful or not.

Seed reserves that are mobilized upon germination are utilized as energy resources and new building blocks to support the development of the seedling until it becomes photo-autotrophic (Bewley et al., 2013; Rosental et al., 2014). Fatty acid β-oxidation and gluconeogenesis are essential for the catabolism of storage lipid reserves in oilseed species, providing metabolic energy and carbon skeletons to fuel germination and early post-germination growth (Andre and Benning, 2007; Runquist and Kruger, 1999). Although these biochemical pathways are well characterized, the effect of temperature on these pathways and the consequences for seed germination and seedling establishment are still unclear.

Ricinus communis L. is a member of the Euphorbiaceae and is also known as castor bean. It is widespread throughout tropical, sub-tropical and warm temperate regions (Santiago et al., 2010; Scarpa and Guerci, 1982). The oil extracted from its seeds is mainly used for pharmaceutical and industrial applications due to its unique chemical composition (Severino et al., 2012). *R. communis* can be grown in dry and hot environments, where most other crops would not grow, and still display good yield (Vijaya Kumar et al., 1997). It has been shown that *R. communis* seedlings have a specific metabolic signature associated with adjusted growth and a likely role in maintaining cellular homeostasis at higher temperatures. A shift in their carbon-nitrogen metabolism is the main biochemical response to high temperatures (Ribeiro et al., 2014b). However, there is a lack of studies that assess the effect of

temperature on *R. communis* seed germination and seedling growth. Therefore, we addressed the question whether different temperatures during seed germination have an effect on important biochemical and molecular mechanisms required for seedling establishment. Our results provide leads for the understanding of the underlying mechanisms that are not only required to support vigorous seedling growth, but also for adaptation to harsh environmental conditions in semi-arid areas worldwide.

2 Materials and Methods

2.1 Plant material and germination conditions

R. communis (*cv.* MPB01) used in this study was developed by the breeding program of the *Empresa Baiana de Desenvolvimento Agrícola S.A* (EBDA-Brazil). This genotype is 1m high, has a short flowering time and has high seed yield, making it an attractive cash crop alternative for poor family farmers in the semi-arid regions of Brazil. These farmers have limited resources (land, labor, inputs and capital) and therefore face a number of setbacks which hamper their engagement in oil crop production (Leite et al., 2013).

After seed coat removal, seeds were allowed to imbibe using paper rolls as substrate at three different temperatures (20, 25 and 35ºC) in the dark. Four different stages were defined and sampled for follow-up experiments: dry seeds (Dry), 6-hour-imbibed seeds (6hIS), seeds at radicle protrusion (RP) and seeds with a radicle of 2 cm (R2) (Supplementary Fig. S1). Germination percentage was scored on daily basis for 9 days. For germination under water restricted conditions, seeds were sown in trays with filter paper as substrate and imbibed in water (control) or PEG 8000 solution (-2.0 MPa) at 35°C for 6 hours. Water content of the seeds was measured every hour during the first 6 hours of imbibition. Additionally, seeds with water content of 24.4% (fresh weight basis) were sampled for follow-up experiments. For this, seeds were imbibed for 6 hours at 35ºC, for 7 hours at 25ºC, and for 8 hours at 20°C.

2.2 Primary metabolite profiling

Primary metabolites were analyzed by gas chromatography coupled to a quadrupole time of flight mass spectrometry system (GC-TOF-MS) as TMS derivatives as described previously (Ribeiro et al., 2014b; Ribeiro et al., 2015). Approximately 20 mg of freeze-dried seeds were used. Ribitol (1 mg/mL) was used as internal standard.

Fatty acids were extracted in methanol:chloroform (1:1) and injected in an Agilent 7809A gas chromatograph (Agilent Technologies) coupled to a Triple-Axis detector (Agilent 5975C), using a ZB-5 (Phenomenex; 30 m x 0.25 mm) capillary column (0.25 mm film thickness) using helium as carrier gas as described previously (Ribeiro et al., 2014b). Approximately 10 mg of freeze-dried seeds were used and a

mixture of hexadecane and heptadecane (1:1) was used as internal standard. GABA levels were additionally measured by GC-TOF-MS in seeds after they had taken up water to 24.4% of their dry weight. All analyses were performed on three biological replicates of 20 seeds each.

2.3 Soluble carbohydrate analysis

Soluble carbohydrates were determined as described previously (Ribeiro et al., 2014b). Approximately 20 mg of freeze-dried seeds were used. Melezitose (40 µg/mL) was used as internal standard. Samples were injected into a Dionex HPLC system (Dionex, Sunnyvale, CA) using a CarboPac PA100 4- x 250-mm column (Dionex) preceded by a guard column (CarboPac PA100, 4 x 50 mm), a gradient pump module (model GP40, Dionex) and followed by an ED40-pulsed electrochemical detector (Dionex). Peaks were identified by co-elution of standards. Three biological replicates of 20 seeds each were used for this analysis.

2.4 Starch analysis

The pellets remaining from the previous carbohydrate analysis were used to quantify starch. Starch was determined as glucose produced by enzymatic digestion with α-amylase, as described previously (Ribeiro et al., 2014b). Approximately 10 mg of dried material was used for the analysis. A standard curve ranging from 1 to 40 µg/mL starch was used to calculate the absolute concentration in the samples. Lactose (10 µg/mL) was used as internal standard. Three biological replicates of 20 seeds each were used for this analysis.

2.5 GC-MS Data processing and compound identification

Data processing and compound identification were performed as described previously (Ribeiro et al., 2014b). Raw data was processed by ChromaTOF software 2.0 (Leco Instruments) followed by alignment of the chromatograms using the MetAlign software. MSClust was used to remove metabolite signal redundancy in aligned mass peak tables and to retrieve mass spectral information of metabolites using mass peak clustering. The mass spectra of the representative masses were used for tentative identification by matching to spectral libraries (National Institute of Standards and Technology [NIST08] and Golm metabolome database [http://gmd.mpimp-golm.mpg.de/]) and by comparison with the retention index calculated by using a series of alkanes. Authentic reference standards were used to confirm the identity of the metabolites. Levels of identification according to Sumner (2007) are presented in Supplementary Table S1.

2.6 Multivariate statistical analysis

Normalized data were uploaded at MetaboAnalyst 2.0; a web-based analytical pipeline for high-throughput metabolomics studies as described previously (Ribeiro et al., 2014b; Ribeiro et al., 2015). Before data analysis, a data integrity check was performed to ensure that all the necessary information was present. Row-wise normalization was performed to allow general-purpose adjustment for differences among samples. Log transformation and auto-scaling were performed to allow comparison of features. Uni- and multivariate analysis were performed using log transformed and auto-scaled data. ANOVA was performed to assess the overall variation in metabolite levels, followed by post-hoc analyses (Bonferroni correction, FDR < 0.05).

2.7 RNA extraction, DNase digestion, RNA quality control and cDNA synthesis

Total RNA was extracted from 20 mg of dry material using the RNeasy Plant Mini Kit (Qiagen) including the DNase digestion of genomic DNA performed using the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. RNA quantification and quality control were performed spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Samples showing A260/A280 and A260/A230 ratios of 1.8–2.2 were used for subsequent analysis. RNA quality was further inspected by the integrity of ribosomal RNA bands on a 1% agarose gel. Sharp and intense 18S and 28S ribosomal RNA bands without visible degradation, confirmed the suitability of the isolation method. The first strand cDNA was synthesized with 1 µg of total RNA using the iScriptTM cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Reverse transcription was performed at 37°C for 40 minutes followed by 5 min at 85°C. The cDNA was diluted 20 times and stored at -20°C prior to further analysis by RT-qPCR.

2.8 Target gene selection, primer design and analysis of amplification efficiency

Orthologs of selected Arabidopsis genes were identified through a TBLASTX of the sequence against the *R. communis* translated nucleotide database (NCBI, http://www.ncbi.nlm.nih.gov/). The selected genes were genes encoding for oil body-associated lipase (*OBL*), peroxisomal long-chain acyl-CoA synthetase (*LACS6/7*), glycerol kinase (*GK*), acyl-CoA oxidase (*ACX*), multifunctional protein (*MFP*), 3-keto-acyl-CoA thiolase (*KAT*), malate synthase (*MLS*), isocitrate lyase (*ICL*), phosphoenolpyruvate carboxykinase (*PCK*), ascorbate peroxidase (*APX*), hydroascorbate reductase (*MDAR*), two small heat shock proteins (*HSP1* and *HSP2*), glutamate decarboxylase (*GAD*), 4-aminobutyrate transaminase (*GABA-T*) and succinic semialdehyde dehydrogenase (*SSADH*). Primers for these genes were designed using CLCbio software (CLC bio, Aarhus, Denmark) with melting

temperatures (Tm) of 58–62°C, primer lengths of 18–22 bp and amplicon lengths of 80-200 bp. The PCR amplification efficiency was evaluated based on a standard curve generated by a two-fold serial dilution series of a pooled cDNA sample. The specificity of the primers was verified by separating the products on a 2.5% agarose gel and melting curve analysis. Genes for actin (*ACT*) and Pob (*POB*) were used as reference genes (Ribeiro et al., 2014a). The gene identifier number (GI), gene name and description, amplicon length, primer efficiency and primer sequences (reverse and forward) are listed in Supplementary Table S2.

2.9 RT-qPCR conditions

RT-qPCR was performed in a total volume of 10 µL containing 2.5 µL of cDNA (20x diluted), 0.5 µL of primer (10 µM), 5 µL of iQ SYBR Green Supermix (Bio-Rad) and 2 µL of milliQ. RT-qPCR experiments were run on a CFX (Bio-Rad). The following qPCR program was used for all PCR reactions: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melting curves were obtained after this program by heating from 65 to 95°C, increasing the temperature stepwise by 0.5°C every 5 s (Ribeiro et al., 2014a).

2.10 Cloning

Cloning of *GK* and *MLS* genes was performed using the Gateway® technology according to the manufacturer's instructions. Full-length coding regions were amplified from *R. communis* seed cDNA. Primers used and additional information about the cloned genes are presented in Supplementary Table S3. Amplified products were recombined in the donor vector pDONr207, to produce pDONr207-*MLS* and pDONr207-*GK*. These plasmids were transferred to *Escherichia coli* (DH5α) cells by electroporation. Transformed single colonies were inoculated in 5 mL LB (lysogeny broth) medium containing gentamycin (25 µg/mL) for 24 hours. pDONr207-*MLS* and pDONr207-*GK* were isolated using the QIAprep Spin Miniprep High-Yield kit (Qiagen) according to the manufacturer's instructions. Thereafter, the plasmids were recombined by an LR reaction (Invitrogen) in the gateway binary vector pGD625 carrying CaMV35S (CaMV - cauliflower mosaic virus). Expression vectors pGD625-*GK* and pGD625-*MLS* were transformed to *Agrobacterium tumefaciens* (Agl0) cells by electroporation. Transformants were selected on LB broth supplemented with kanamycin (100 µg/mL) and rifampicin (25 µg/mL). Colony PCR, digestion with restriction enzymes and sequencing were used to confirm the presence of the desired gene sequences in the isolated plasmids.

2.11 Transient expression

Transformed Agl0 single colonies were inoculated in 5 mL LB containing kanamycin (100 µg/mL) and rifampicin (25 µg/mL) for 24 hours. This pre-culture was used to inoculate 50 mL LB which was grown for another 20 hours at 28°C. *Agrobacterium tumefaciens* cells were harvested by centrifugation at 5000g and 4°C for 15 min and re-suspended in a buffer (500 mM MgCl2, 500 mM MES-KOH and 100 mM acetosyringone) to obtain an OD600 of 0.5. Leaves of 4-week old *Nicotiana benthamiana* plants were infiltrated with the cell suspension by pressing a 1 mL syringe against the abaxial side of the leaf. An *A. tumefaciens* strain co-expressing the p19 protein was co-infiltrated to improve transgene expression by suppressing post-transcriptional gene silencing (Qu and Morris, 2002). After 7 days, infiltrated leaves were collected, immediately frozen and stored at -80°C prior to further analysis.

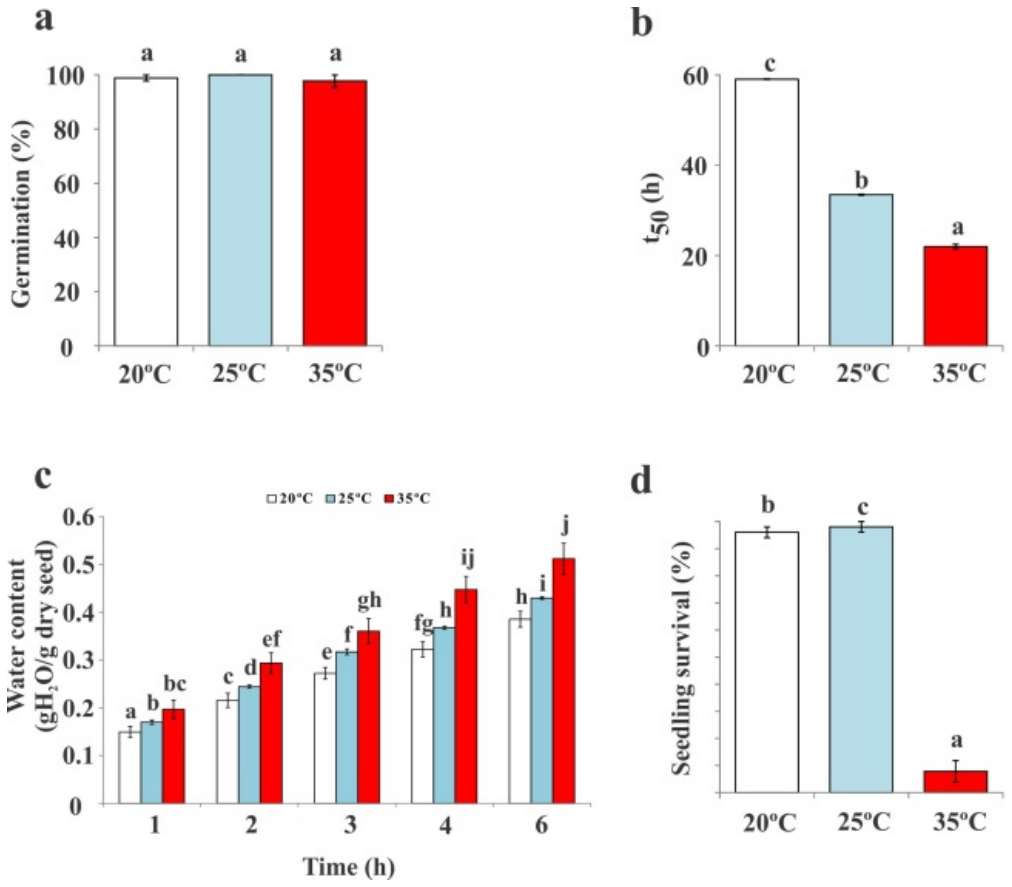

Figure 1. *Ricinus communis* germination behavior, water content and seedling survival at different temperatures. (**a**) Final germination percentage after 9 days, (**b**) time to reach 50% germination (t_{50}), (**c**) water content of seeds imbibed at 20, 25 and 35ºC and (**d**) seedling survival percentage after 9 days of germination. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p<0.05$).

3 Results

3.1 *R. communis* seed germination and seedling establishment at different temperatures

Temperature significantly influenced germination speed ($p<0.05$) of *R. communis* seeds as measured by t_{50} (time to reach 50% germination), but did not affect final germination percentage (Fig. 1a,b). Radicle protrusion commenced within 18 hours of imbibition at 35ºC, whereas at 20ºC and 25ºC seeds began to germinate within 55 and 30 hours, respectively. Thus, the t_{50} values decreased with increasing temperature (Fig. 1b). Despite the marked differences in germination speed, nearly 100% of germination was reached at all tested temperatures (Fig. 1a). Since the increased germination speed might be the result of faster imbibition, we measured the water content of seeds imbibed at the different temperatures. Already after one hour of imbibition, water content was higher in seeds imbibed at 35ºC than at 20ºC (Fig. 1c) and water content continued to increase with increasing temperature. After 6 hours of imbibition, the water content was 0.385 gH_2O/g dry seed at 20ºC, 0.430 gH_2O/g dry seed at 25ºC, and 0.512 gH_2O/g dry seed at 35ºC.

To obtain a better understanding of how different temperatures during seed germination might affect seedling performance, seeds were allowed to germinate and to develop at 20, 25 and 35ºC for 9 days after which seedling survival was scored. Seedling survival was dramatically reduced at 35ºC as compared with 20ºC and 25ºC (Fig. 1d). Nearly 100% of seedling survival was observed at 20 and 25ºC, whereas only 8% of the seeds survived at 35ºC. We have previously demonstrated that no abnormalities could be observed when seeds are germinated at 25°C for 72 hours (R2 stage) and, subsequently, grown at 35ºC (Ribeiro et al., 2014b). In fact, these seedlings showed higher growth rates, as evidenced by the faster appearance of the first true leaves (Ribeiro et al., 2014b). Thus, we identified a thermo-sensitive window during germination in which high temperatures compromise subsequent seedling development. To better understand the biochemical and/or molecular mechanisms underlying this thermo-sensitive window, we performed a metabolite profiling analysis (GC-TOF-MS) of seeds germinated at the three different temperatures. Additionally, we measured transcript levels of key genes involved in several energy-generating pathways such as storage oil mobilization, β-oxidation and gluconeogenesis.

3.2 Biochemical and molecular changes in *R. communis* seeds during germination

3.2.1 Overall variation in metabolite composition

Primary metabolites were measured by GC-TOF-MS in seeds that germinated at 20ºC, 25ºC and 35ºC. Our aim was to assess metabolic changes associated with temperature that could provide further insights into the mechanism underlying the thermo-sensitive window that affects seed germination and seedling development. More than one hundred peaks were detected of which forty-one metabolites were

annotated. Twenty amino acids, four carbohydrates and four TCA cycle intermediates were identified, along with 13 other metabolites (Table 1). Principal component analyses (PCA) was applied to the whole data set, using both identified and unidentified metabolites (Table 1 and Supplementary Table S4), aiming at finding the directions that best explain the variance in the data set. Principal component 1 (PC1) explained 57.0% of total variance, whereas principal component 2 (PC2) explained 12.6% of total variance (Fig. 2). Four clusters of samples were observed in the PCA: dry seeds, 6hIS, RP and R2. This indicates that most of the variation in the metabolite composition of the seeds can be attributed to differences in the developmental stage rather than to temperature.

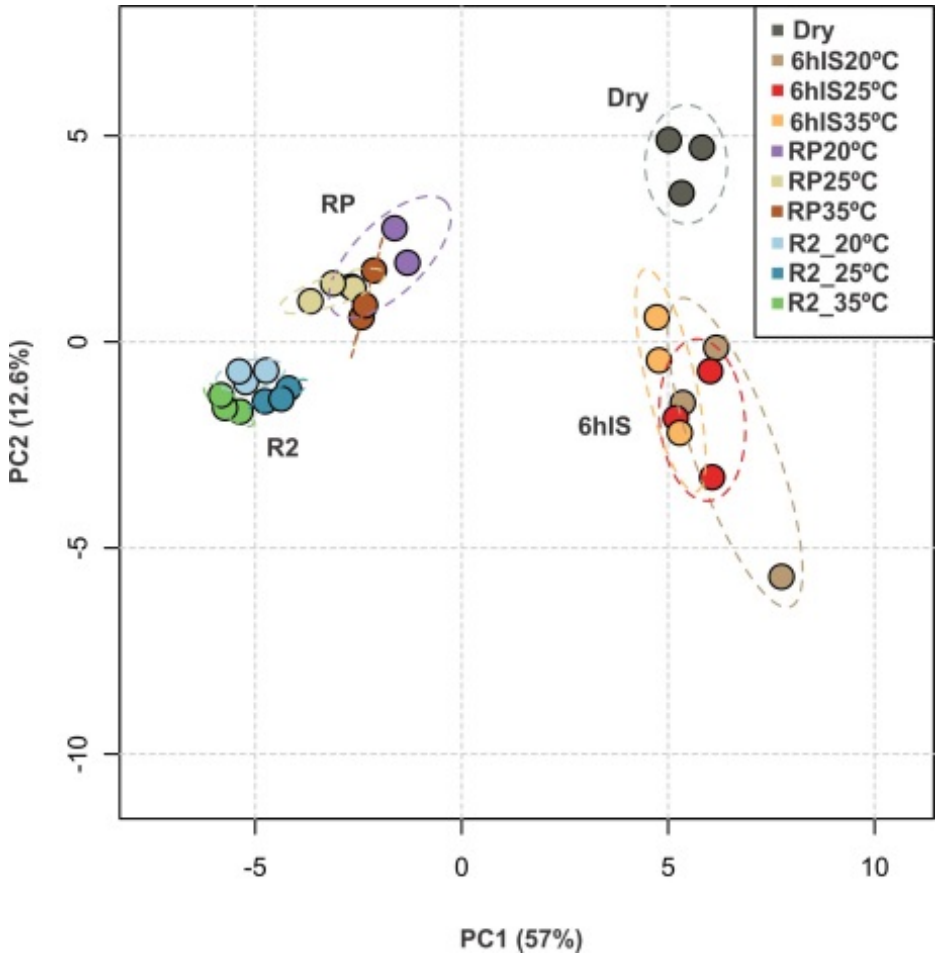

Figure 2. Principal component analysis (PCA) based on polar metabolite levels (identified and non-identified metabolites) during seed germination in response to different temperatures. Four different stages were analysed: dry seeds (Dry), 6-hour imbibed seeds (6hIS), seeds at radicle protrusion (RP) and with a radicle length of 2 cm (R2). Seed germination was performed at three different temperatures: 20, 25 and 35ºC.

In order to assess whether the temperature had an effect on the metabolite composition of a given developmental stage, principal component analysis was performed for 6hIS, RP and R2 samples,

separately (Supplementary Fig. S2). PC1 explained 50.3% of the total variance for 6hIS, 43.7% of the total variance for RP, and 53.1% of the total variance for R2, whereas PC2 explained 23.6% of the total variance for 6hIS, 23.6% of the total variance for RP, and 25.4% of the total variance for R2. Although 6h-imbibed seeds at 20ºC and 25ºC were partially overlapping in the PCA plot (Supplementary Fig. S2a), they were clearly differentiated at the RP and R2 developmental stages (Supplementary Fig. S2b,c).

Five free fatty acids were detected in germinating seeds (Table 1). Ricinoleate levels were up to 44-fold greater in 6hIS samples than in dry seeds. Similarly, ricinoleate levels were up to 244-fold greater in R2 samples germinated at 25ºC and 35ºC than in dry seeds (Table 1). Linoleate and oleate were quickly mobilized after 6 hours of imbibition at 25ºC and 35ºC, reaching their highest levels at the R2 stage. Stearate and palmitate levels hardly varied during imbibition and germination as compared with those found in dry seeds, except for a 6- to 10-fold increase at the R2 stage at 25ºC and 35ºC (Table 1).

During germination and seedling development an increase in the levels of nearly all identified metabolites was observed at all three temperatures (Table 1). Galactinol was a remarkable exception, since galactinol levels were up to 100-fold greater in dry seeds than in RP and R2 samples. Myo-inositol, a product of galactinol breakdown, increased at all temperatures during seedling growth (Table 1). Glucose and fructose levels sharply increased in 6hIS samples compared with dry seeds, whereas sucrose levels hardly varied. Xylose levels did not vary after 6 hours of imbibition at 20ºC and 25ºC, but they were 3-fold higher in 6hIS at 35ºC as compared with dry seeds (Table 1). A continuous increase in fructose, glucose and xylose levels was also observed during development (in RP and R2), whereas sucrose levels between these stages showed little variation (Table 1).

Low levels of starch were found in dry seeds and hardly any significant difference was found among 6hIS samples. In contrast, starch levels showed a remarkable increase in RP and R2 samples at all three temperatures as compared with dry seeds and 6hIS, except for RP samples at 35ºC in which starch levels were not significant different than those found in 6hIS samples at 35ºC and dry seeds. Interestingly, starch levels were greater in RP and R2 seeds imbibed at 20ºC than at 35ºC (Fig. 3).

Four TCA cycle intermediates were detected: citrate, fumarate, malate, and succinate (Table 1). Fumarate and succinate levels were higher in 6hIS than in dry seeds, whereas malate and citrate hardly showed any variation. At RP stage, fumarate levels were 3-fold greater in seeds imbibed at 35ºC than at 20ºC and 25ºC. Succinate reached the highest levels in RP seeds imbibed at 25 and 35ºC. Once again, malate and citrate hardly showed any variation. At R2 stage, fumarate levels were up to 5.7-fold greater in seeds imbibed at 25ºC and 35ºC than at 20ºC. Malate showed a slight increase at 35ºC as compared with 20ºC and 25ºC, whereas succinate levels were up to 1.7-fold greater in seeds imbibed at 20ºC than at 25ºC and 35ºC (Table 1).

Table 1. Normalized data (relative to dry seeds) of the metabolite profiles of *R. communis* seeds in response to an increase in temperature during germination.

Name	6 hours imbibed seeds			radicle protrusion			root 2 cm length		
	Dry	20°C	25°C	35°C	20°C	25°C	35°C	20°C	25°C	35°C
2-hydroxy-pyridine	1 (ab)	0.99 (a)	1.14 (ab)	1.35 (b)	1.27 (ab)	1.06 (ab)	1.02 (ab)	0.81 (a)	0.94 (a)	0.90 (a)
5-aminovalerate	1 (abc)	0.92 (a)	1.02 (abc)	1.27 (c)	1.17 (abc)	1.04 (abc)	1.00 (abc)	0.85 (ab)	1.11 (bc)	1.06 (bc)
ascorbate	1 (ab)	0.88 (a)	1.90 (b)	6.31 (ab)	122.27 (e)	85.513 (d)	64.933 (c)	174.99 (f)	174.49 (f)	105.45 (de)
butylamine	1 (a)	0.57 (a)	0.79 (a)	0.99 (a)	0.87 (a)	0.69 (a)	0.74 (a)	0.48 (a)	0.50 (a)	0.52 (a)
citrate	1 (a)	0.97 (a)	0.93 (a)	0.96 (ab)	1.37 (c)	1.14 (ab)	1.20 (b)	1.69 (d)	1.46 (cd)	1.39 (c)
ethanolamine	1 (a)	1.40 (b)	1.57 (abcd)	1.62 (bc)	1.88 (cd)	1.54 (bc)	1.63 (c)	1.86 (bcd)	3.11 (e)	2.23 (d)
fructose	1 (a)	6.66 (b)	8.97 (b)	10.60 (b)	61.55 (de)	48.32 (d)	26.09 (c)	240.99 (g)	161.87 (f)	111.14 (ef)
fumarate	1 (a)	6.53 (c)	8.20 (c)	8.69 (c)	2.44 (b)	2.43 (ab)	7.35 (c)	6.50 (c)	26.70 (d)	37.08 (e)
GABA	1 (ab)	5.75 (d)	4.93 (d)	2.02 (c)	0.58 (a)	1.24 (b)	1.02 (ab)	1.11 (b)	2.50 (c)	5.76 (d)
galactinol	1 (d)	1.04 (cd)	1.00 (cd)	0.82 (c)	0.02 (ab)	0.01 (a)	0.04 (ab)	0.01 (a)	0.02 (ab)	0.02 (b)
glucose	1 (a)	6.38 (bc)	7.69 (bcd)	9.38 (bcd)	11.39 (d)	10.43 (cd)	6.67 (b)	110.95 (f)	67.82 (e)	62.08 (e)
glycerate	1 (a)	1.77 (b)	1.79 (b)	2.23 (b)	3.44 (c)	3.58 (c)	2.15 (b)	3.63 (c)	6.72 (e)	4.34 (d)
glycerol	1 (a)	7.39 (bc)	10.18 (bc)	5.30 (bc)	3.76 (b)	14.84 (c)	14.65 (c)	7.21 (bc)	71.66 (e)	60.54 (d)
lactate	1 (abc)	1.00 (abc)	1.20 (abc)	1.23 (b)	1.27 (bc)	1.00 (abc)	0.85 (ac)	0.79 (a)	0.63 (a)	0.74 (abc)
L-alanine	1 (a)	0.91 (a)	1.10 (a)	2.28 (b)	5.43 (c)	10.94 (e)	6.85 (d)	6.82 (d)	9.20 (e)	24.14 (f)
L-asparagine	1 (a)	0.89 (a)	0.90 (a)	1.04 (a)	0.92 (a)	2.00 (b)	1.04 (a)	3.04 (c)	3.46 (c)	5.59 (d)
L-aspartate	1 (b)	0.22 (a)	0.35 (a)	0.55 (ab)	6.23 (d)	7.13 (e)	3.40 (c)	8.46 (f)	7.92 (ef)	8.42 (f)
L-cysteine	1 (a)	0.98 (a)	1.06 (a)	1.40 (a)	8.98 (c)	11.12 (c)	7.95 (b)	21.21 (d)	23.04 (d)	23.07 (d)
L-glutamate	1 (c)	0.32 (a)	0.46 (b)	0.86 (c)	2.29 (d)	3.10 (e)	3.53 (f)	4.01 (f)	6.32 (g)	5.55 (g)
L-glutamine	1 (a)	0.43 (a)	0.65 (a)	0.74 (a)	5.69 (b)	7.93 (b)	5.69 (b)	14.61 (c)	13.56 (c)	21.17 (d)
L-glycine	1 (a)	1.14 (a)	1.31 (a)	1.43 (a)	4.01 (b)	6.07 (c)	4.79 (b)	9.43 (d)	10.82 (d)	15.24 (e)
L-histidine	1 (a)	0.88 (a)	1.15 (a)	1.15 (a)	4.60 (b)	13.21 (bc)	9.35 (c)	48.60 (de)	37.32 (d)	64.44 (e)
L-isoleucine	1 (a)	2.01 (ab)	3.59 (b)	4.63 (b)	20.01 (c)	33.96 (d)	23.17 (c)	79.25 (e)	91.26 (f)	161.56 (g)

L-lysine	1 (ab)	0.45 (a)	0.74 (ab)	0.82 (ab)	1.11 (abc)	1.43 (bc)	1.16 (abc)	1.21 (abc)	1.60 (bc)	2.59 (c)
L-methionine	1 (a)	0.69 (a)	1.62 (a)	1.92 (a)	12.13 (ab)	34.15 (b)	22.62 (b)	32.81 (b)	49.65 (c)	93.36 (d)
L-phenylalanine	1 (a)	0.81 (a)	1.03 (a)	1.13 (a)	3.57 (b)	4.81 (c)	3.18 (b)	10.09 (d)	11.00 (d)	16.25 (e)
L-proline	1 (a)	1.78 (b)	2.45 (bc)	3.70 (c)	18.07 (d)	30.81 (ef)	24.25 (e)	38.12 (f)	54.03 (g)	89.77 (h)
L-serine	1 (a)	1.00 (a)	1.02 (a)	1.03 (a)	6.20 (b)	9.70 (c)	6.05 (b)	13.21 (d)	15.61 (d)	21.86 (e)
L-threonine	1 (a)	1.48 (ab)	2.08 (bc)	2.87 (c)	11.46 (d)	19.21 (e)	18.73 (e)	23.19 (f)	31.71 (g)	55.18 (h)
L-tryptophan	1 (a)	0.55 (a)	0.48 (a)	0.73 (a)	2.26 (b)	3.15 (c)	3.29 (c)	7.96 (d)	8.78 (d)	15.38 (e)
L-tyrosine	1 (a)	0.67 (a)	0.92 (a)	1.37 (a)	7.84 (b)	16.60 (c)	18.08 (c)	25.38 (d)	29.04 (d)	42.18 (e)
L-valine	1 (a)	1.69 (b)	2.09 (bc)	2.94 (c)	8.25 (d)	13.58 (f)	10.15 (e)	31.07 (g)	33.28 (g)	60.08 (h)
malate	1 (a)	1.04 (a)	1.01 (a)	1.01 (a)	2.88 (bc)	3.20 (c)	2.64 (b)	4.61 (d)	4.84 (d)	6.31 (e)
myo-inositol	1 (a)	1.30 (b)	1.36 (b)	2.27 (c)	3.68 (d)	5.15 (e)	4.51 (e)	4.70 (e)	4.72 (e)	5.15 (e)
phosphate	1 (ab)	1.03 (a)	1.18 (ab)	1.43 (b)	2.15 (c)	3.02 (cd)	1.98 (c)	4.14 (d)	5.19 (e)	7.60 (f)
putrescine	1 (ab)	0.48 (a)	0.58 (a)	1.49 (b)	7.27 (de)	5.99 (d)	3.47 (c)	9.32 (e)	7.33 (de)	4.29 (c)
pyroglutamate	1 (a)	0.95 (a)	0.99 (a)	0.96 (a)	4.79 (b)	6.60 (c)	4.46 (b)	9.27 (d)	11.46 (cde)	14.57 (e)
pyruvate	1 (a)	0.57 (a)	0.77 (a)	0.93 (a)	0.82 (a)	0.63 (a)	0.73 (a)	0.55 (a)	0.64 (a)	0.66 (a)
succinate	1 (a)	2.21 (b)	2.98 (b)	3.94 (c)	4.46 (c)	6.98 (e)	5.47 (d)	17.08 (g)	10.05 (f)	11.36 (f)
sucrose	1 (abc)	0.91 (ab)	1.01 (abc)	1.05 (bc)	0.93 (abc)	0.83 (a)	0.99 (abc)	0.93 (abc)	1.08 (c)	1.00 (abc)
xylose	1 (a)	1.24 (a)	1.75 (a)	3.03 (b)	25.1 (e)	17.47 (d)	7.96 (c)	47.89 (f)	22.26 (de)	13.43 (cd)
oleate	1 (a)	2.34 (ab)	3.21 (b)	2.38 (b)	2.25 (b)	2.78 (b)	2.99 (b)	2.64 (b)	17.43 (c)	11.64 (c)
linoleate	1 (a)	4.04 (ab)	5.85 (b)	3.22 (b)	2.89 (b)	4.41 (b)	5.44 (b)	4.04 (b)	25.71 (c)	17.75 (c)
ricinoleate	1 (a)	27.41 (bc)	44.16 (c)	18.76 (abc)	13.37 (ab)	35.54 (c)	41.11 (bc)	20.85 (bc)	244.06 (e)	138.39 (d)
stearate	1 (a)	1.36 (ab)	1.44 (ab)	1.71 (ab)	1.17 (ab)	1.35 (ab)	1.34 (ab)	1.78 (b)	10.20 (c)	6.63 (c)
palmitate	1 (a)	1.34 (ab)	1.40 (b)	1.60 (ab)	1.15 (ab)	1.30 (ab)	1.20 (ab)	1.67 (b)	8.72 (c)	6.25 (c)

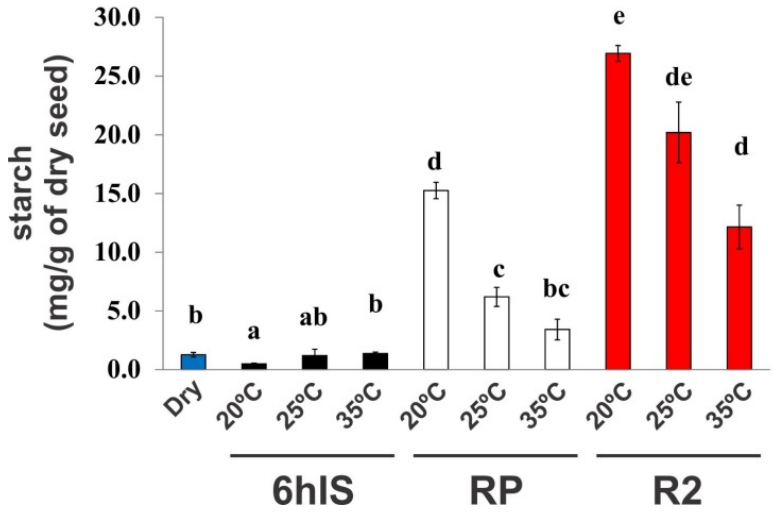

Figure 3. Starch levels during seed germination and early seedling establishment. Four different stages were analyzed: dry seeds (Dry, blue bars), 6-hour imbibed seeds (6hIS, black bars), seeds at radicle protrusion (RP, white bars) and seeds with a radicle length of 2 cm (R2, red bars) at 3 different temperatures as depicted in the graphs. Average and standard errors of three biological replicates containing 15-20 seeds each are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p<0.05$).

3.2.2 Changes in metabolite composition during the initial phase of seed imbibition

ANOVA was used to compare the overall variation in metabolite composition associated with temperature within the first 6 hours of imbibition. Only seven metabolites showed significant differences: ascorbate, γ-aminobutyric acid (GABA), myo-inositol, putrescine, glutamate, alanine and succinate (data not shown). Of these seven metabolites, four are involved in the GABA shunt (Fig. 4a).

GABA levels were higher in seeds imbibed at 20ºC than at 35ºC (Table 1). In contrast, glutamate levels were higher in seeds imbibed at 35ºC than at 20ºC and 25ºC. Moreover, increased levels of alanine and succinate, which are products of GABA catabolism, were detected in seeds imbibed at 35ºC (Table 1). These results indicate that GABA catabolism is enhanced at 35ºC and/or GABA biosynthesis is enhanced at 20ºC. It is important to highlight that higher levels of GABA would be expected in seeds imbibed at 35ºC as compared to 20ºC if GABA levels would have changed in relation to heat stress. For this reason, heat stress is not the likely explanation of GABA accumulation during early imbibition.

In order to provide further insight into the observed GABA accumulation at lower temperatures, we measured the transcript levels of genes encoding enzymes involved in the GABA shunt: glutamate decarboxylase (*GAD*), GABA transaminase (*GABA-T*) and succinic semialdehyde dehydrogenase (*SSADH*) (Fig. 4b).

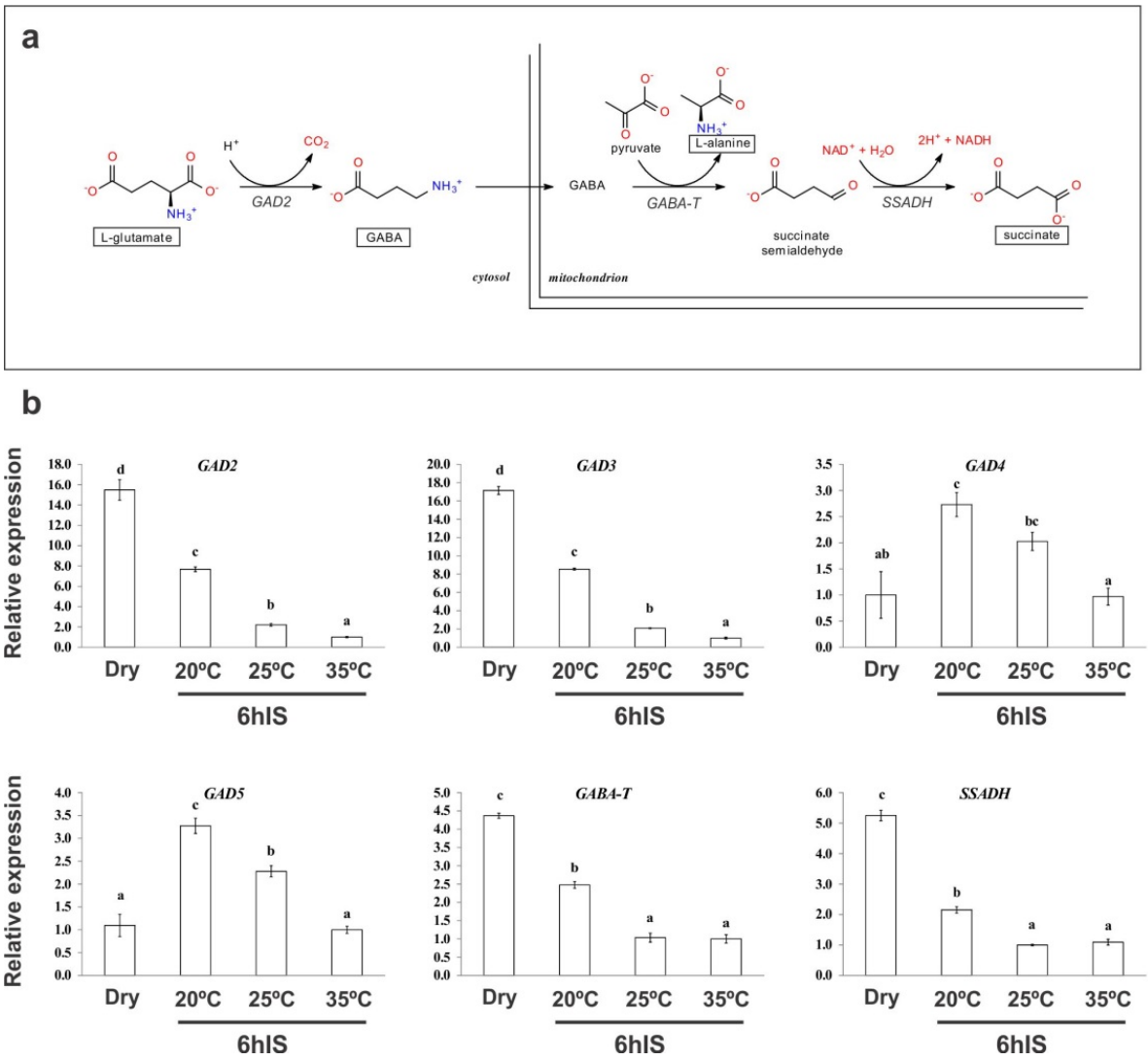

Figure 4. Effect of temperature on the GABA shunt during early imbibition (6hIS). (a) Schematic representation of the GABA shunt. Metabolites with significant changes after 6 hours of imbibition due to the temperature are depicted in the boxes. (b) Transcript levels of GABA shunt related genes. Means and standard errors of three biological replicates containing 15-20 seeds each are shown. Temperatures are depicted in the graphs. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p<0.05$).

The *R. communis* genome contains five sequences annotated as glutamate decarboxylases (*GAD1*, *GAD2*, *GAD3*, *GAD4*, and *GAD5*) (Supplementary Table S2; http://www.ncbi.nlm.nih.gov/). The transcript levels of *GAD1* could not be measured reliably due to its very low expression. Transcript levels of each of the other four *GAD* genes were greater in seeds imbibed at 20ºC than at 35ºC, but to different extents. For example, an increase of 7.7-fold was observed for *GAD2*, 8.5-fold for *GAD3*, 2.8-fold for *GAD4*, and 3.3-fold for *GAD5* in seeds imbibed at 20ºC as compared with seeds imbibed at 35ºC.

Transcript levels of *GABA-T* and *SSADH* were higher in seeds imbibed at 20ºC than at 35ºC (up to 2.5-fold) (Fig. 4b). Thus, there is a good correlation between transcript levels of four *GAD* genes and GABA levels and therefore, the higher expression levels of *GAD* are likely responsible for accumulation of GABA in 6h-imbibed seeds at 20ºC. All germination experiments were performed under controlled conditions, with temperature as the only variable. Based on the water content of the seeds, we can infer that seeds imbibed at 35ºC are more hydrated than seeds imbibed at 20ºC, although none of the 6hIS samples are fully hydrated (Fig. 1c). We raised the question whether GABA is higher accumulated at 20ºC in response to differences in water content of the seeds. To address this question, we measured GABA levels in seeds that contained the same water content, but that imbibed at 20ºC, 25ºC, and 35ºC. This resulted in a reduced difference in GABA levels between seeds imbibed at 20ºC and 35ºC (Fig. 5a). In line with our hypothesis, hardly any difference in GABA levels was found for the fully imbibed RP seeds (Table 1). Finally, we measured GABA levels in seeds that imbibed for 6 hours in water (control) and under water restricted conditions using -2.0 MPa PEG8000, both at 35ºC. GABA levels were 2.4-fold higher in seeds that imbibed under water restricting conditions than in control seeds (Fig. 5b).

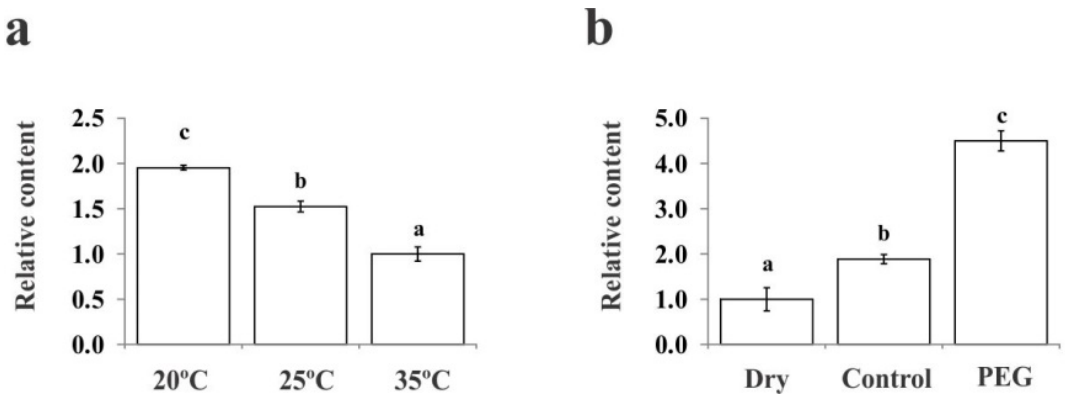

Figure 5. Relative GABA levels. GABA levels in (**a**) seeds after they had taken up water to 24.4% of their dry weight, (**b**) in seeds imbibed in water and under water restricted conditions at 35ºC. Average and standard errors of three biological replicates of 15-20 seeds each are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p<0.05$).

3.2.3 *R. communis* seeds perceive 35ºC as a heat-stress condition

GABA levels were up to 5.2-fold greater in R2 samples imbibed at 35ºC than at 20ºC and 25ºC (Table 1). This result positively correlates with transcript levels of the four *GAD* isoforms. Thus, the higher expression levels of *GAD* is likely responsible for accumulation of GABA in R2 samples imbibed at 35ºC. These results suggest that seeds might be subjected to heat stress at 35ºC. In line with this hypothesis, higher transcript of the genes encoding for ascorbate peroxidase (APX) and

monodehydroascorbate reductase (MDAR) was observed in R2 seeds imbibed at 35ºC as compared with R2 seeds imbibed at 20ºC (Supplementary Table S5). Additionally, the transcript levels of two genes encoding small heat-shock proteins (*HSP1* and *HSP2*) were assessed. Both genes are homologs of Arabidopsis genes that are known to be expressed in seeds and to be highly induced upon heat stress in seedlings. Levels of both transcripts were higher in seeds imbibed at 35ºC than at 20ºC and 25ºC for all developmental stages. For example, transcript levels of *HSP1* and *HSP2* were up to 8.2-fold greater in 6hIS at 35ºC than at 20ºC and 25ºC. The highest transcript levels of *HSP1* was observed in R2 seeds imbibed at 35ºC, whereas *HSP2* reached the highest transcript levels in RP seeds imbibed at 35ºC (Supplementary Table S5).

3.2.4 High temperature during seed germination leads to down-regulation of important metabolic processes which are crucial for successful seedling establishment

At the RP stage, levels of most amino acids were higher in seeds imbibed at 25ºC than at 20ºC or 35ºC. Glutamate was the only amino acid that showed higher levels at 35ºC. Levels of proline, threonine, tryptophan and tyrosine did not vary between RP samples imbibed at 25ºC and 35ºC, but they were higher than in RP samples imbibed at 20ºC (Table 1). Levels of glucose, fructose and xylose were higher in seeds imbibed at 20ºC and 25ºC than at 35ºC (Table 1). At the R2 stage, levels of the majority of the identified amino acids were higher in seeds imbibed at 35ºC than at 20ºC and 25ºC. In contrast, levels of the majority of the identified carbohydrates were higher in seeds imbibed at 20ºC than at 35ºC (Table 1). Starch levels decreased with increasing temperature in RP and R2 seeds (Fig. 3).

Storage oil mobilization has a pivotal role in providing carbon and energy during seed germination to fuel seedling establishment (Andre and Benning, 2007; Graham, 2008; Pinfield-Wells et al., 2005). To illustrate the role of this process in our system, we measured the transcript levels of key genes involved in several energy-generating pathways such as storage oil mobilization, β-oxidation and gluconeogenesis (Fig. 6a). Transcript levels of the oil body-associated lipase (*OBL*) were strongly affected by temperature and two contrasting patterns were observed: levels of *OBL* was higher in 6hIS samples imbibed at 20ºC than at 35ºC, whereas levels of *OBL* was higher in RP and R2 seeds imbibed at 35ºC than at 20ºC and 25ºC (Fig. 6b and Supplementary Table S5). Levels of glycerol and free fatty acids (FFAs) were higher in 6hIS samples than in dry seeds (Table 1), suggesting that triacylglycerol (TAG) hydrolysis is initiated shortly upon imbibition. Transcript levels of the peroxisomal long-chain acyl-CoA synthetase (*LACS6/7*) decreased with increasing temperature for 6hIS samples. In contrast, transcript levels of acyl-CoA oxidase (*ACX*), multifunctional protein (*MFP*) and 3-keto-acyl-CoA thiolase (*KAT*) were higher in 6hIS samples imbibed at 20ºC and 35ºC than at 25ºC (Fig. 6b). Although transcript levels of *LACS6/7* were higher in R2 seeds imbibed at 25ºC and 35ºC than at 25ºC, transcript levels of *ACX* suggest that the

initial step of β-oxidation is up-regulated at 20ºC and 25ºC. Little variation in *MFP* and *KAT* transcript levels in response to temperature suggests that activation of FFAs by LACS6/7 and subsequent initial oxidation by ACX, control initial triacylglycerol (TAG) hydrolysis and oxidation of FFAs (Fig. 6b). These results suggest that the initial steps of storage oil mobilization are enhanced at 20ºC as compared to higher temperatures. This is in agreement with the lower levels of FFAs in R2 samples at 20ºC. Additionally, lower levels of FFAs in R2 samples at 20ºC could be explained by the lower transcript levels of *OBL2* in these samples.

Glycerol kinase (*GK*) encodes an enzyme that transfers a phosphate group from ATP to glycerol to produce glycerol-3-phosphate (G3P). G3P is then converted to dihydroxyacetone phosphate and fed into gluconeogenesis (Fig. 6a). Transcript levels of *GK* were higher in RP seeds imbibed at 20ºC and 25°C than at 35ºC. However, a late upregulation of *GK* was observed in R2 seeds imbibed at 35°C compared with R2 seeds imbibed at 20ºC and 25ºC (Fig. 6b).

Transcript levels of malate synthase (*MLS*) were higher in R2 seeds imbibed at 20ºC than at 25ºC and 35ºC, whereas no variation in transcript levels of isocitrate lyase (*ICL*) was observed in response to temperature (Fig. 6b). The enzymes encoded by these genes belong to the glyoxylate cycle, which enables the cell to use acetyl-CoA to generate increased levels of TCA cycle intermediates for biosynthetic pathways such as gluconeogenesis.

Despite higher transcript levels of phosphoenolpyruvate carboxykinase (*PCK*) in 6hIS samples imbibed at 35ºC than at 20 and 25ºC (Fig. 6b), no differences in levels of fructose, glucose, sucrose and starch were observed (Table 1 and Fig. 3). In contrast, transcript levels of *PCK* were 4.6-fold higher in R2 seeds imbibed at 20ºC than at 35ºC (Fig. 6b). Based on the transcript levels of these genes, it can be suggested that the biosynthesis of soluble carbohydrates and starch are enhanced in RP and R2 seeds imbibed at lower temperatures (Fig. 6b). Overexpression of genes encoding for malate synthase (*MLS*) and glycerol kinase (*GK*) resulted in higher starch levels in *N. benthamiana* leaves, which highlights the importance of these genes in energy-generating pathways necessary for seedling establishment (Fig. 7).

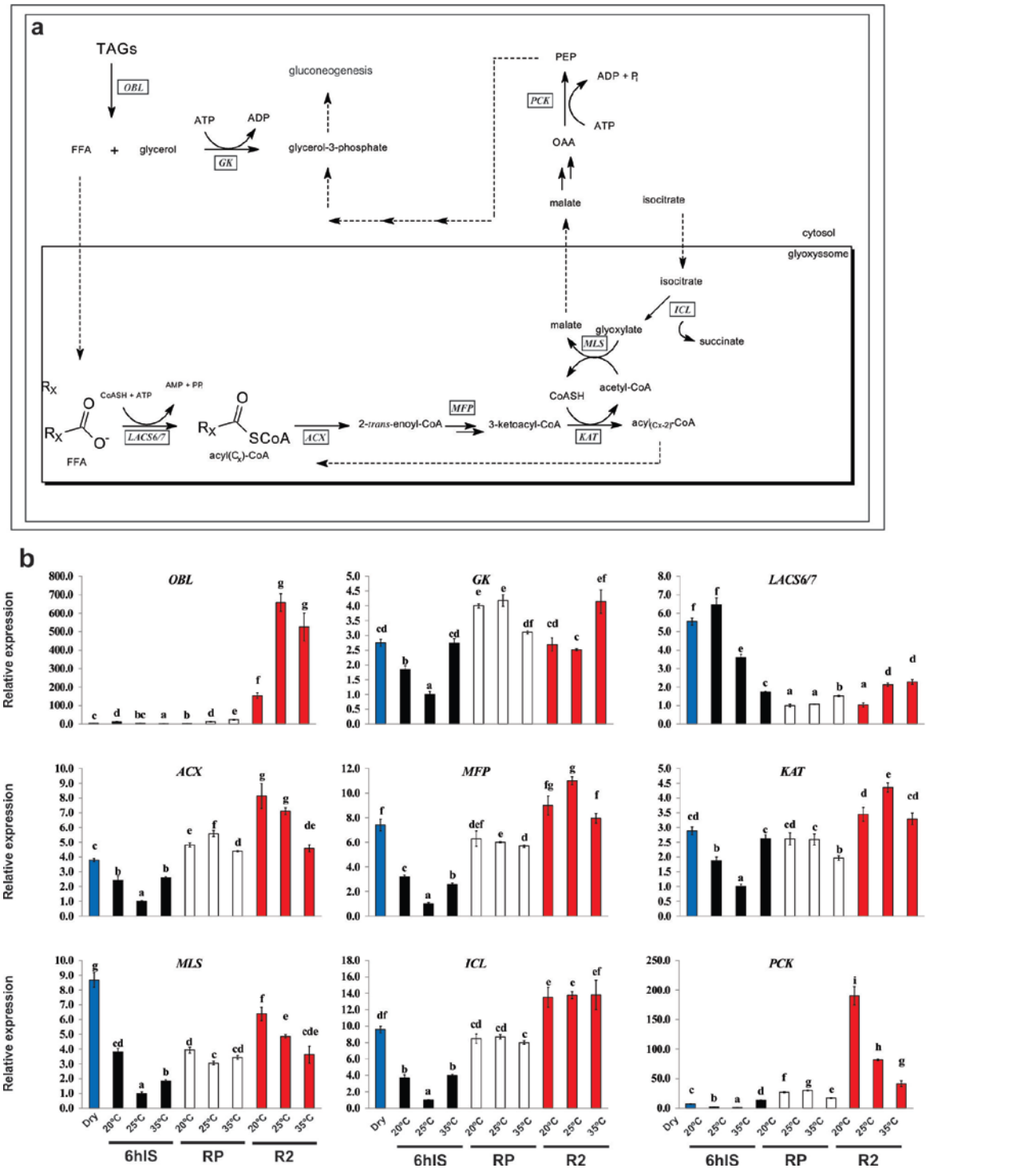

Figure 6. Effect of temperature on the mobilization of storage oil and carbohydrate metabolism during *R. communis* germination. (**a**) Schematic representation of the storage oil mobilization, *β*-oxidation and gluconeogenesis pathways and (**b**) transcript levels of some key genes involved in lipid mobilization. Average and standard errors of three biological replicates containing 15-20 seeds each are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p<0.05$).

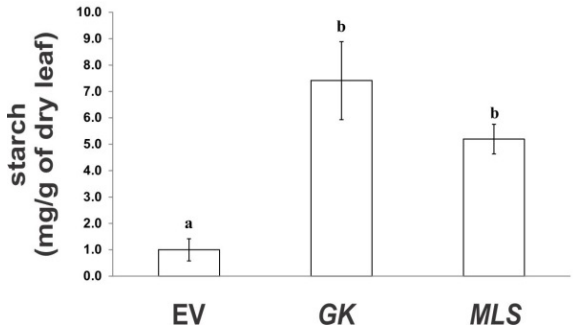

Figure 7. Starch levels of agro-infiltrated *N. benthamiana* leaves. EV, empty vector; *GK*, glycerol kinase; *MLS*, malate synthase. Average and standard errors of three biological replicates containing 4 infiltrated leaves from different plants are shown. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p<0.05$).

4 Discussion

4.1 *R. communis* germination at different temperatures

Temperature is a key factor in the seed germination process, in which a difference of only a few degrees may already lead to a notable change in germination parameters, such as maximum germination percentage and speed of germination. *R. communis* seeds displayed high percentage of germination across the tested range of temperatures (20ºC to 35°C). This indicates that this species possesses a high degree of thermo-plasticity with faster germination at higher temperatures, which is very suitable for a species that grows in harsh environmental conditions, such as the arid and semi-arid zones. Faster germination at 35ºC might be attributed to faster imbibition of seeds at this temperature. However, faster germination does not necessarily imply a better performance during seedling establishment. During germination, seeds prepare their molecular and biochemical machinery to support successful seedling establishment. The results of this study showed that *R. communis* seeds are not able to cope with high temperatures during early seed germination, but they are resistant to high temperature at later stages of seedling establishment and plant growth (Ribeiro et al., 2014b). Additionally, these results suggest that there is a thermo-sensitive time window during seed germination in which high temperatures compromise subsequent seedling development.

The integration of transcript and metabolite data has been used to pinpoint relevant metabolic pathways for a certain phenotype, as an indication for causal relationship (Ibáñez et al., 2014; Kostyn et al., 2012; Lu et al., 2015; Rizhsky et al., 2004). Here we investigated which biochemical and/or molecular

mechanisms occur during seed germination that may be required for proper seedling establishment and are negatively affected by a high temperature.

4.2 GABA as a stress-responsive molecule

GABA is a non-protein amino acid that has been associated with abiotic stresses, signaling and nitrogen storage in plants (Kinnersley and Lin, 2000; Kinnersley and Turano, 2000). GABA may act as a signaling molecule in nitrogen metabolism, as a regulator of the cytosolic pH and as osmoprotectant to prevent oxidative damage in response to various abiotic stresses (Renault et al., 2010). GABA can be produced from glutamate by glutamate decarboxylase (GAD). GAD catalyzes the decarboxylation of glutamate to CO_2 and GABA and its activity is restricted to the cytosol and is specific for glutamate (Renault et al., 2010; Vicente-Carbajosa and Carbonero, 2005). GABA is further transported to the mitochondria where it is converted to succinic semi aldehyde (SSA) and succinate, by aminobutyrate transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH), respectively (Shelp et al., 2012). GABA usually accumulates in response to heat and drought stress (Bor et al., 2009). In this study, GABA levels were greater in 6hIS samples imbibed at 20ºC than at 35ºC. Therefore, heat stress is not the likely explanation of GABA accumulation during early imbibition, since an accumulation of GABA in seeds imbibed at 35ºC instead of 20ºC would be expected in that case. Due to the very negative matrix potential of imbibing seeds, initially little or no water is left for uptake by the cells of the embryo. Seeds imbibed at 35ºC have higher water content that seeds imbibed at 20ºC. Because of their lower water content, it is reasonable to assume that seeds imbibed at 20ºC have a lower matrix potential than seeds imbibed at 35ºC resulting in water stress. This hypothesis is in agreement with the fact that at 2 days of imbibition, the embryo water potential of coffee seeds was -7.7 MPa and it increased to -4.3 MPa at day 4 of imbibition [33]. Based on these results, we suggest that the lower matrix potential of seeds imbibed at 20ºC might be causal for GABA accumulation. Additional studies are, however, required to examine proof this hypothesis possibility. In line with this hypothesis, we showed that at radicle protrusion (RP stage), when seeds are fully imbibed, hardly any differences in GABA levels were found.

GABA levels increased in R2 seeds in response to the increasing temperature, which correlates with higher levels of *GAD* in seeds imbibed at 35ºC as compared to 20ºC and 25ºC. Therefore, we suggest that during early seedling establishment, high temperatures lead to GABA accumulation. We cannot, however, rule out that post-translational regulation of GAD activity may also provide further explanation for the observed phenotypes. The catalytic activity of GAD is regulated by its ability to bind Ca^{2+}/calmodulin (Baum et al., 1993; Baum et al., 1996) and since abiotic stresses are known to induce intracellular transient Ca^{2+} signals (Pandey et al., 2002; Sheen, 1996), it is possible that differences in GABA levels may result from post-translational regulation of GAD activity.

Since GABA has also been pointed out as a signaling molecule for seed and pollen germination (Ling et al., 2013; Weitbrecht et al., 2011), we raise the hypothesis that GABA accumulation during early imbibition is an important signal for subsequent metabolic processes necessary for successful seedling establishment. This may also explain the higher seedling survival at 20ºC and 25ºC. Our results indicate a role of GABA during seed germination and seedling establishment, although more research is needed to fully understand its functional significance.

4.3 Biochemical and molecular aspects of *R. communis* germination required for successful seedling establishment

An efficient reactivation of metabolism, e.g., storage oil breakdown and further reserve mobilization, is essential for successful seedling establishment (Andre and Benning, 2007; Graham, 2008). During seed maturation, storage biomolecules are accumulated in order to support germination and further seedling growth (Vicente-Carbajosa and Carbonero, 2005). Initial imbibition is often accompanied by large metabolic changes which set the course for subsequent radicle protrusion and are essential for successful seedling establishment (Bewley et al., 2013; Vicente-Carbajosa and Carbonero, 2005). Within minutes after the start of imbibition, reactivation of enzymes that were stored during maturation can be observed, followed by an increase in respiratory metabolism and gene expression (Botha et al., 1992; Sánchez-Linares et al., 2012). Our results show that temperature affects the metabolite composition of seeds already at the beginning of imbibition. This effect persists until germination and during post-germination seedling growth.

In germinating seeds, monosaccharides can be produced from the breakdown of stored starch, sucrose hydrolysis and through gluconeogenesis (Kobr and Beevers, 1971; Sánchez-Linares et al., 2012; Weitbrecht et al., 2011). *R. communis* seeds contain low levels of starch and sucrose levels showed little variation during seed imbibition and germination. Gluconeogenesis is, therefore, the most likely source of monosaccharides during *R. communis* germination.

Many oil crop species use TAGs as the major seed storage reserve, which are mobilized during imbibition and germination to support subsequent seedling growth (Graham, 2008; Reale et al., 2012). TAGs undergo a complex pathway for their conversion to carbohydrates, which is required for seedling development and to support the start of photoautotrophic metabolism (Chapman and Galleschi, 1985; Jordy and Favre, 2003). TAG hydrolysis is catalyzed by lipases which act on the ester-carboxylate bonds resulting in the release of free FFAs and glycerol (Barros et al., 2010). OBL is an oil body-associated lipase from *R. communis* that is induced upon seed germination and is responsible for the hydrolysis of a range of TAGs. OBL-like proteins are present in many species and it is likely that they play an important role in regulating lipolysis during germination (Eastmond, 2004).

FFAs released from TAG lipolysis are usually transported across the glyoxysome by the comatose ABC transporter (CTS) and activated by LACS in the glyoxysome lumen where *β*-oxidation and part of the glyoxylate cycle occurs (Graham, 2008). Nevertheless, higher transcript levels of *OBL2* and *LACS6/7* in 6hIS samples at 20ºC did not lead to an increase of glycerol and FFA levels as compared with 25 and 35ºC, suggesting that after being released from TAG, FFAs might quickly be metabolized into the *β*-oxidation pathway. Evidently, post-transcriptional, translational and post-translational control of these genes also cannot be ruled out. The first step in the oxidation of acyl-CoA to 2-*trans*-enoyl-CoA is catalyzed by acyl-CoA oxidases (ACX). Then, 2-*trans*-enoyl-CoA undergoes two of the core *β*-oxidation pathway reactions catalyzed by the multifunctional protein complex (MFP) to yield 3-ketoacyl-CoA. The enzyme 3-ketoacyl-CoA thiolase (KAT) catalyzes the last step of FFA *β*-oxidation, which involves the thiolytic cleavage of 3-ketoacyl-CoA to acyl-CoA (Graham, 2008).

Acetyl-CoA and glyoxylate (produced by peroxisomal isocitrate lyase) are converted to malate by malate synthase (MLS). Cornah and colleagues (2004) suggested that *MLS* is partially dispensable for lipid utilization and gluconeogenesis in *Arabidopsis* seedlings under normal growth conditions. Studies based on mutants lacking the key enzyme isocitrate lyase showed that *ICL* is not essential for germination, but it is required for seedling establishment (Eastmond et al., 2000). However, here we hypothesize that during *R. communis* germination *MLS* might be a key responsive element in lipid mobilization and gluconeogenesis to sustain successful seedling growth. This is in agreement with the fact that transcript levels of *MLS* were affected by temperature, whereas no variation in transcript levels of *ICL* was found. In *R. communis*, the transcript levels of *MLS* were higher in 6hIS and R2 seeds imbibed at 20ºC as compared with higher temperatures, supporting the hypothesis that gluconeogenesis might be enhanced at lower temperatures. Low transcript levels of *GK* and *MLS* at 35°C in RP and R2 stage, respectively, might be an indication that downstream processes such as gluconeogenesis and starch biosynthesis are down-regulated at higher temperatures.

Overexpression of *GK* and *MLS* in *N. benthamiana* leaves resulted in higher starch levels in those leaves and these results support our hypothesis that higher transcript levels of *GK* and *MLS* are associated with starch accumulation at low temperatures during germination of *R. communis* seeds. Additionally, this also might be an explanation for the negative effect of high temperature on seedling performance. Although the contribution of post-transcriptional, translational and post-translational regulation of the involved genes cannot be ruled out, the observed correlations between gene expression and metabolite levels are certainly an indication of possible involvement of the specific genes in the observed phenotypes.

In conclusion, seeds imbibing at higher temperatures showed a faster germination with a high germination percentage, but with compromised seedling establishment. It seems that high temperature has a negative effect on important metabolic processes, such as storage oil breakdown, fatty acid oxidation

and gluconeogenesis. Although these biochemical pathways are well characterized, there is still a lack of information about the correlation between physiological events, such as germination and seedling establishment under different environmental conditions, and associated metabolic changes. From increased levels of GABA in R2 seeds imbibed at 35ºC together with the increase in the transcript levels of *APX*, *MDAR* and genes encoding for two heat-shock proteins, it can be concluded that seeds perceive 35ºC during germination as a heat-stress condition. Our results provide a better understanding of the underlying mechanisms required to support vigorous seedling growth, which will be helpful to develop germination and seedling production protocols for the sustainable exploitation of *R. communis*.

5 Authors' contribution

P.R. Ribeiro carried out the physiological and gene expression experiments, starch and carbohydrate measurements, statistical analysis and wrote the manuscript. P.R. Ribeiro and L.A.J. Willems performed the metabolite profiling analysis, data processing and compound identification. P.R. Ribeiro and M. Mutimawurugo performed the cloning and transient expression experiments. W. Ligterink and H.W.M. Hilhorst participated in the design of the study, coordination and critical reading of the manuscript. L.G. Fernandez and R.D. de Castro participated in the critical reading and discussion of the manuscript. All authors read and approved the final manuscript.

6 Conflict of interest

The authors declare no conflict of interest.

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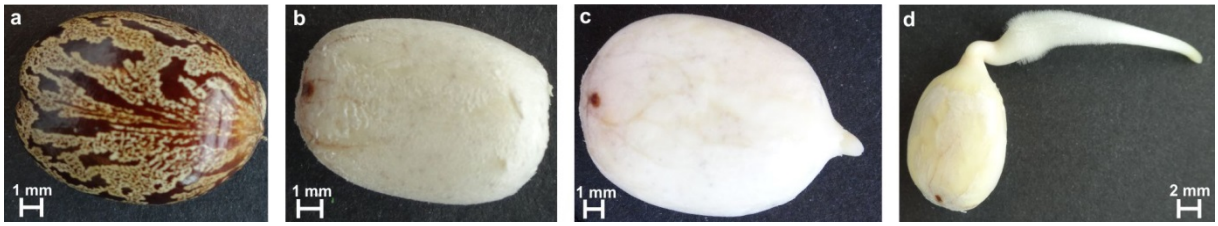
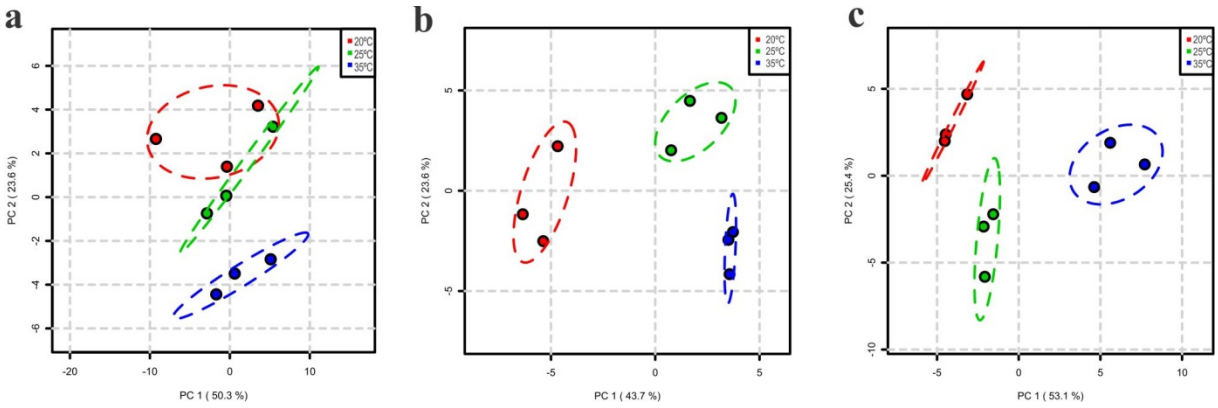
8 Supplementary Figure



Supplementary Figure 1. *Ricinus communis* seeds at different developmental stages. Three different stages are presented: (**a**) dry seeds with seed coat, (**b**) dry seeds without seed coat, (**c**) seeds at radicle protrusion and (**d**) seeds with a radicle length of 2 cm.



Supplementary Figure 2. Principal component analysis (PCA) of polar metabolite profiles during seed germination and early seedling establishment in response to different temperatures. Three different stages were analysed separately: (**a**) 6-hour imbibed seeds, (**b**) seeds at radicle protrusion and (**c**) seeds with a radicle length of 2 cm. The different temperatures are indicated in the graphics.

9 Supplementary Table Captions

Supplementary Table S1. Retention index and levels of identification according to Sumner, et al. (2007). (1) Identified compounds (chemical reference standards). (2) Putatively annotated compounds (e.g. without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries.

Supplementary Table S2. Description of target genes used for RT-qPCR.

Supplementary Table S3. Description of genes used for cloning and transient expression experiments.

Supplementary Table S4. Normalized data of the unidentified metabolites detected in *R. communis* seeds in response to an increase in temperature during germination.

Supplementary Table S5. Transcript levels of some key genes.

Supplementary tables can be found at http://www.wageningenseedlab.nl/thesis/prribeiro/SI/

Chapter 4

Transcriptome analysis of *Ricinus communis* germination at different temperatures

Paulo R. Ribeiro, Anderson Tadeu Silva, Leo A.J. Willems, Johan Bucher, Basten Snoek, Wilco Ligterink and Henk W.M. Hilhorst

Abstract

A thermo-sensitive window during seed germination in which high temperatures compromise seedling development has been identified previously in *R. communis*. We studied the molecular mechanisms that could explain this thermo-sensitive window with a genomics approach using microarray analysis to determine transcriptome changes during seed germination at 20, 25 and 35ºC. Although temperature had a strong effect on the *R. communis* transcriptome, most of these differences occurred between 6 hours of imbibition and the commencement of germination, i.e. radicle protrusion. This coincided with the identified thermo-sensitive window. We identified several heat stress responsive genes that might be involved in the thermotolerance of *R. communis*. For example, temperature had a major effect on genes involved in energy generating pathways, such as the Calvin-Benson-Bassham cycle, gluconeogenesis, and starch- and triacylglycerol degradation. Transcripts of ATP binding proteins, DNA binding proteins, RNA binding proteins, DNA-directed RNA polymerases I, II, and III, heat shock factor proteins, multiprotein-bridging factor proteins, and zinc finger protein were also affected by temperature suggesting that transcriptional reprogramming mechanisms were disturbed. Among the downregulated transcripts, only three were shared by all three stages: one oxidation-related zinc finger 2, one F-box and wd40 domain protein, and one DNA binding protein/ MYB-like transcription factor. Among the upregulated transcripts, nine were shared by all three stages: one BET1P/SFT1P-like protein 14BB, one low-molecular-weight cysteine-rich protein LCR78, one WD-repeat protein, one GAST1 protein, one adenylate kinase 1/P-loop containing nucleoside triphosphate hydrolases superfamily protein, and four conserved hypothetical proteins These genes constitute good candidate genes for further characterization of temperature-responsive molecular mechanisms in *R. communis*.

Keywords: cytochrome P450s, heat shock proteins, heat stress, microarray analysis, transcriptional regulation

1 Introduction

Temperature is one of the main environmental factors influencing seed germination and plant growth (Kendall and Penfield, 2012). Temperatures above the optimum are perceived as heat stress (HS) by all living organisms and this optimum temperature may vary between species (Cochrane et al., 2014; Da Mota and Garcia, 2013). HS damage consists of various sets of complex perturbations of the homeostasis of the plant, which depend on developmental stage, as well as types of plant tissues and organs (Jagadish et al., 2014; Kotak et al., 2007; Liu et al., 2008; Rienth et al., 2014; Sun et al., 2002). For example, HS affects the stability of various proteins, membranes, RNA species and cytoskeleton structures, it alters the efficiency of enzymatic reactions thereby obstructing the major physiological processes, and creates metabolic imbalance (Hasanuzzaman et al., 2013; Pagamas and Nawata, 2008; Suzuki et al., 2012). In the course of evolution, plants have developed a variety of responses to elevated temperatures aiming at minimizing damage and ensuring protection of cellular homeostasis (Hasanuzzaman et al., 2013; Kotak et al., 2007; Wolfe and Tonsor, 2014). Some of the mechanisms to cope with high temperature involve changing leaf orientation, transpirational cooling, alteration of membrane composition, closure of stomata, increased stomatal and trichomatous densities, and larger xylem vessels (Hasanuzzaman et al., 2013; Iba, 2002; Schulze et al., 1973; Wolfe and Tonsor, 2014; Zheng et al., 2011).

Transcriptome analysis of several plant species in response to temperature has revealed the involvement of heat stress responsive genes in thermotolerance (Kaplan et al., 2004; Krasensky and Jonak, 2012; Li et al., 2013; Liao et al., 2015). For example, Liao et al. (2015) identify potential candidate genes involved in the high night temperature response in thermo-tolerant and -sensitive rice lines. Transcripts involved in electron transfer in the mitochondrial respiratory chain, mainly cytochrome P450s, were found to be differentially expressed when comparing the heat-tolerant and -sensitive lines after high temperature exposure (Liao et al., 2015). Mitochondria play an important role in adjusting and maintaining cellular homeostasis. Therefore, disturbances in the electron transfer may prevent the proper functioning of mitochondria in response to different abiotic stresses, since the energy cost for biosynthetic processes cannot be fulfilled under these circumstances (Naydenov et al., 2010; Rachmilevitch et al., 2007). Heat stress factors (Hsfs) and Heat shock proteins (Hsps) play a central role in the response to heat stress and acquired thermotolerance in plants (Hasanuzzaman et al., 2013; Kaplan et al., 2004; Qu et al., 2013; Sun et al., 2002). Hsfs serve as the transcriptional activator component of signal transduction and mediate the expression of Hsps (Qu et al., 2013). Aqcuisition of thermotolerance through overexpression of Hsf and Hsp may be achieved by overexpression of single genes or a combination of genes (Kotak et al., 2007). For example, transgenic *Arabidopsis* plants overexpressing a wheat chloroplastic sHSP (HSP26) were

substantially more tolerant to high temperature (35ºC) than wild-type plants, as measured by photosystem II (PSII) activity, higher biomass and seed yield. Additionally, the transgenic plants showed higher germination than the wild-type plants under high temperature (35ºC) (Chauhan et al., 2012).

Ricinus communis seeds germinate to a high percentage, and faster, at 35ºC than at lower temperatures, but with compromised seedling establishment. However, seedlings are able to cope with high temperatures at later stages of seedling development if germination occurs at lower temperatures (Ribeiro et al., 2015b). A thermo-sensitive window during seed germination in which high temperatures compromise seedling development has been identified. Most likely high temperature has a negative effect on important metabolic processes, such as storage oil breakdown, fatty acid oxidation and gluconeogenesis (Ribeiro et al., 2015b). There is still a lack of information about the correlation between physiological events, such as germination and seedling establishment under different temperatures, and associated transcriptome changes. We performed a genomics approach using microarray analysis to determine transcriptome changes during seed germination at 20, 25 and 35ºC that could explain this thermo-sensitive window.

2 Materials and Methods

2.1 Plant material and germination conditions

Seeds of *Ricinus communis* (*cv.* MPB01) were used in this study. After seed coat removal, seeds were allowed to imbibe using paper rolls as substrate at three different temperatures (20, 25 and 35ºC) in the dark. Four different stages were defined and sampled for follow-up experiments: dry seeds (Dry), early imbibition (EI), seeds at radicle protrusion (RP) and seeds with a radicle of 2 cm (R2) (Supplementary Figure S1). EI samples consisted of seeds that had imbibed for 6 hours at 35ºC, for 7 at 25ºC, and for 8 hours at 20°C. Different lengths of imbibition were necessary in order to obtain a uniform seed water content of around 24%. All analyses were performed on three biological replicates of 20 seeds each.

2.2 Microarray probe design

The whole genome sequence of *R. communis* is publicly available (Chan et al., 2010). We designed microarray probes for the Agilent microarray platform based on the predicted gene models of the reference genome sequence v0.1 (as downloaded from Phytozome 10.0, http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Rcommunis). From the 31,225 *R. communis* predicted transcripts, 31,111 could be successfully used to design specific 60-mer probes with the web based Agilent eArray tool (https://earray.chem.agilent.com/earray/). The microarray layout (Design ID

058689) consists of 45,220 probes of which 1417 are standard Agilent control probes and 43,803 are *R. communis* specific probes corresponding to the 31,111 different *R. communis* transcript IDs.

2.3 RNA extraction

Total RNA was extracted according to the hot borate protocol modified by Wan and Wilkins (1994) and Maia et al. (2011). Approximately 20 mg of seeds were homogenized and mixed with 800 µL of extraction buffer (0.2 M Na borate decahydrate (Borax), 30 mM EGTA, 1% SDS, 1% Na deoxycholate (Na-DOC)) containing 1.6 mg DTT and 48 mg PVP40 which had been heated to 80°C. 1 mg proteinase K was added to this suspension and incubated for 15 min at 42°C. After adding 64 µl of 2 M KCL the samples were incubated on ice for 30 min and subsequently centrifuged for 20 min at 12,000 g. Ice-cold 8 M LiCl was added to the supernatant in a final concentration of 2 M and the tubes were incubated overnight on ice. After centrifugation for 20 min at 12,000 g at 4°C, the pellets were washed with 750 µl ice-cold 2 M LiCl. The samples were centrifuged for 10 min at 10,000 g at 4°C and the pellets were re-suspended in 100 µl DEPC treated water. The samples were phenol chloroform extracted, DNAse treated (RQ1 DNase, Promega) and further purified with RNEasy spin columns (Qiagen) following the manufacturer's instructions. RNA quality and concentration were assessed by agarose gel electrophoresis and UV spectrophotometry.

2.4 Experimental design for microarray hybridization

Microarray hybridizations were performed as described by Basnet et al. (2013). Cy3 and Cy5 dyes were incorporated into cRNA samples according to the Agilent two-colour microarray based transcript expression analysis protocol (Low input quick Amp labelling G4140-90050, Agilent Technologies, Inc., Santa Clara, CA, USA) and hybridized on arrays following a specific design (Supplementary Figure S2).

2.5 Microarray data analysis

2.5.1 Normalization

Loess was used for within-array normalization and quantile normalization for between array normalization using the limma package in R (Basnet et al., 2013). The normalized Cy3 and Cy5 intensities were used as measures of transcript abundance. No background correction was performed as recommended by Zahurak et al. (2007) and successfully applied in (Snoek et al., 2014; Volkers et al., 2013). For further investigation log2 of the absolute intensities or the log2 ratio with the mean of each spot over all samples were used.

2.5.2 ANOVA and multiple testing corrections

ANOVA was used to find transcripts significantly affected by stage, temperature and the interaction between stage and temperature (adjusted $p < 0.001$). We used a two-sided t-test, assuming unequal variance to determine if samples collected at 20ºC and 35ºC were significantly different within each stage. The obtained p-values were adjust for multiple testing by the BY method in R (http://www.r-project.org/).

2.5.3 Principal component analysis (PCA)

Normalized data were uploaded in MetaboAnalyst 2.0; a web-based analytical pipeline for high-throughput studies as described previously (Ribeiro et al., 2014; Ribeiro et al., 2015a; Xia et al., 2012). Before data analysis, a data integrity check was performed to ensure that all the necessary information was present. Row-wise normalization was performed to allow general-purpose adjustment for differences among samples. Log transformation and auto-scaling were performed to allow comparison of features. Principal component analysis (PCA) was performed using log transformed and auto-scaled data.

2.5.4 Venn diagram

R. communis identifiers for each subset were used as input for the construction of the Venn diagram in the Venny 2.0 toolkit (http://bioinfogp.cnb.csic.es/tools/venny/).

2.5.5 Singular enrichment analysis

Finding enriched gene ontology (GO) terms corresponds to finding enriched biological facts, and the term enrichment level is judged by comparing the query list to a background population from which the query list is derived (Li et al., 2013). In this study the background query list comprised of 19,659 annotated *R. communis* transcripts (http://castorbean.jcvi.org/index.php). *R. communis* identifiers for each subset were used as input for singular enrichment analysis in the agriGO toolkit (http://bioinfo.cau.edu.cn/agriGO/index.php) (Du et al., 2010). The *R. communis* genome loci (TIGR) were selected as background. Fisher's statistical test and Yekutieli [false discovery rate (FDR) under dependency] for multi-test adjustment were selected to identify enriched GO categories with a significance level of 0.05.

3 Results and discussion

3.1 Genome wide variation in transcript levels during *Ricinus communis* germination in response to temperature

3.1.1 PCA analysis

R. communis seeds germinate faster and to a higher percentage at 35ºC than at lower temperatures, but with compromised seedling establishment (Ribeiro et al., 2015b). We undertook a genomics approach using microarray analysis to determine transcriptome changes during seed germination at 20, 25 and 35ºC that could explain this phenotype. We used four different stages: dry seeds (Dry), seeds at early imbibition (EI), seeds at radicle protrusion (RP) and seeds with a protruded radicle of 2 cm (R2) (Supplementary Figure S1).

Initially, ANOVA was applied to the entire dataset aimed at finding significant changes in transcript levels in response to differences in developmental stage, temperature and interaction between developmental stage and temperature. Most of the observed changes in transcript levels were due to differences in developmental stage (10750), followed by temperature (500) and the interaction between stage and temperature (304).

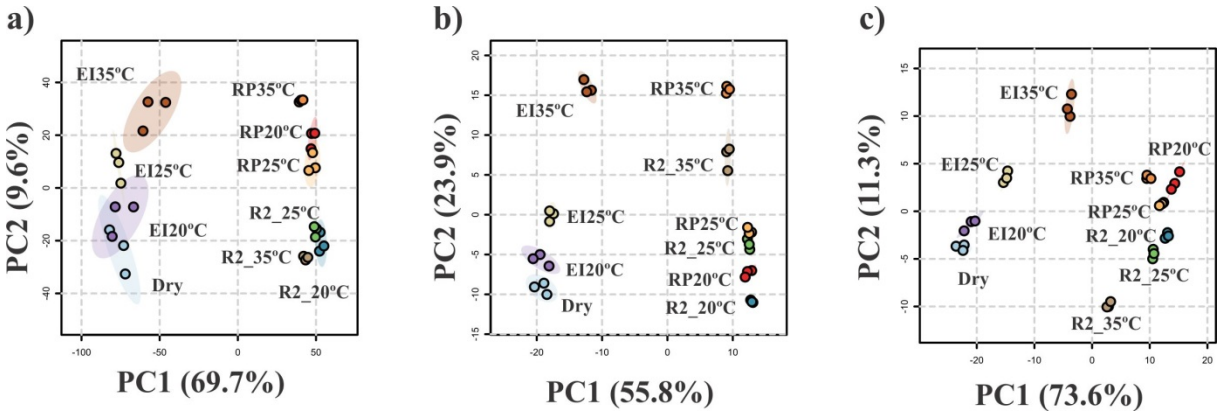


Figure 1. Principal component analysis (PCA) based on transcript levels during germination of *R. communis* seeds in response to different temperatures. (**a**) subset of transcripts significantly affected by developmental stage, (**b**) subset of transcripts significantly affected by temperature, and (**c**) subset of transcripts significantly affected by the interaction between developmental stage and temperature. Four different developmental stages were analysed: dry seeds (Dry), seeds at early imbibition (EI), seeds at radicle protrusion (RP) and with a radicle length of 2 cm (R2). Seed germination was performed at three different temperatures: 20, 25 and 35ºC.

Principal component analysis (PCA) was performed for these three subsets of transcripts, individually (Figure 1). For the subset of transcripts significantly affected by developmental stage, principal component 1 (PC1) explained 69.7% of the total variance, whereas principal component 2 (PC2) explained 9.6% (Figure 1a). PC1 effectively separated dry and EI seeds from RP and R2 seeds. PC2 mainly separated the contribution of temperature, although it also distinguished RP seeds from R2 seeds. For the subset of transcripts significantly affected by temperature, PC1 explained 55.8% of the total variance, whereas PC2 explained 23.9% (Figure 1b). Again, PC1 effectively discriminated dry and EI seeds from RP and R2 seeds, whereas PC2 reflected the contribution of temperature. For the subset of transcripts significantly affected by the interaction between developmental stage and temperature, PC1 explained 73.6% of the total variance, whereas PC2 explained 11.3% (Figure 1c). Although, it seems that PC1 played the major role in separating dry and EI seeds from RP and R2 seeds, PC2 also contributed to this separation. Similarly, PC1 and PC2 seem to act together to separate the contribution of temperature. It is worth to highlight that regardless of the stage, seeds imbibed at 35ºC were clearly differing from seeds imbibed at 20ºC and 25ºC. Therefore, using the concept that proximity means similarity, seeds that had imbibed at 20ºC and 25ºC seem to be closer related in terms of transcriptome, than seeds imbibed at 35ºC.

3.1.2 Singular enrichment analysis

To investigate the biological significance of the transcripts within a given subset it is enlightening to identify the gene ontologies (GO) associated with these transcripts. Therefore, we identified enriched GO terms associated with each subset of transcripts. GO terms are divided in three groups: biological processes, molecular functions, and cellular components. In this study, we focused our attention mainly on GO terms related to biological processes and molecular functions. Additionally, because the main objective of this study is to unravel the changes in the transcriptome in response to temperature, we decided to selected only two subsets for singular enrichment analysis. These subsets consisted of transcripts significantly affected by temperature (ST) and by the interaction between developmental stage and temperature (SST).

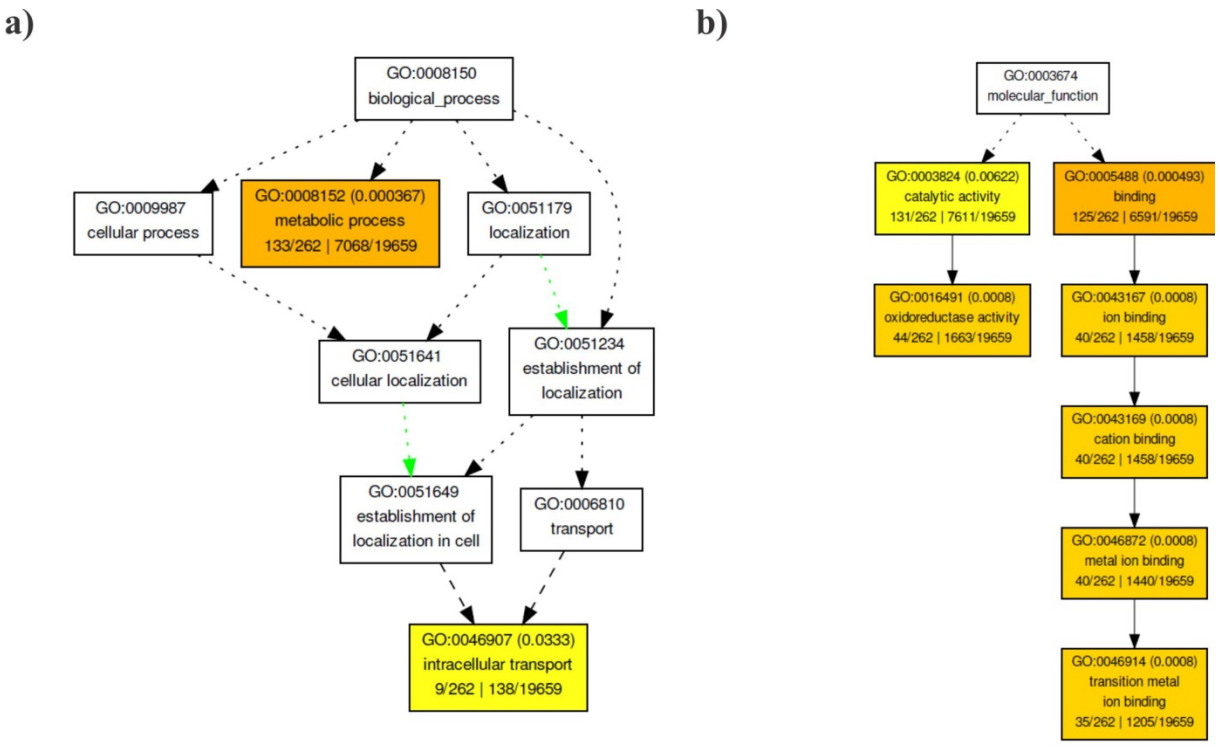


Figure 2. Gene ontology analysis of transcripts significantly affected by temperature in *R. communis* using agriGO. (**a**) Significantly over-represented GOs associated with biological processes and (**b**) Significantly over-represented GOs associated with molecular function. The coloured boxes indicate significantly enriched GOs. The box colours indicates levels of statistical significance with yellow < 0.05; orange < e-05 and red < e-09.

For the transcripts belonging to ST, only one GO term associated with biological processes was enriched: metabolic processes (133 transcripts) (Figure 2a). Transcripts in the GO metabolic processes are associated with important energy generating pathways, such as the Calvin-Benson-Bassham cycle, gluconeogenesis, starch degradation, and triacylglycerol degradation. For example, transcript levels of sedoheptulose-1,7-bisphosphatase (29610.m000407) were up to 5.3-fold higher in RP and R2 imbibed at 20ºC than at 25ºC and 35ºC. This gene is a major regulatory step in the control of the Calvin cycle (Supplementary Table S1). The Calvin cycle is a metabolic pathway responsible for the fixation of carbon and it is located in the stroma of the chloroplasts. Carbon enters this cycle as CO_2, which is then used in the carboxylation of ribulose-1,5-bisphosphate to produce 3-phosphoglycerate by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). For every three molecules of CO_2 that enter the cycle, the net output is one molecule of 3-phosphoglycerate (3PGA) (Raines, 2003). Then, 3PGA can be converted into three forms of hexose phosphate (glucose 1-phosphate, glucose 6-phosphate, and fructose 6-phosphate) in a pathway parallel to that of gluconeogenesis (Berg et al., 2002).

Another important pathway present in this GO term was starch degradation. Transcript levels of a β-amylase (29726.m003986) were up to 3.7-fold higher in RP and R2 imbibed at 20ºC than at 25ºC and 35ºC (Supplementary Table S1). Starch degradation in the endosperm is assumed to differ from that in other organs (Smith et al., 2005). Nevertheless, starch degradation is accompanied by increases in carbohydrates such as maltose and sucrose, but also provides carbon skeletons and energy (Smith et al., 2005).

Finally, transcripts involved in triacylglycerol degradation were also present in the GO term metabolic processes. For example, the transcript 29864.m001449 showed high homology to a GDSL-motif lipase 5 in Arabidopsis. Levels of this transcript were up to 3.5-fold higher in RP and R2 imbibed at 20ºC than at 25ºC and 35ºC (Supplementary Table S1). Triacylglycerol degradation starts with the onset of seed germination and occurs in the glyoxysomes. All these pathways have one thing in common: production of compounds that are converted to soluble sugars which are used presumably to fuel seedling growth (Berg et al., 2002; Graham, 2008; Raines, 2003; Smith et al., 2005). Therefore, it is safe to assume that temperature has a major role in metabolic processes which are crucial to support successful seedling establishment.

Continuing with the analysis of transcripts belonging to ST, we found that two GO terms associated with molecular functions were enriched: catalytic activity (131 transcripts) and binding (125 transcripts). Under the GO category of catalytic activity, only the GO term oxidoreductase activity was enriched, whereas under the GO category of binding, four GO terms related with ion binding were enriched (Figure 2b). Transcripts within GO term oxidoreductase activity included several alcohol- and aldehyde dehydrogenases and cytochrome P450s. Cytochrome P450s are a large and ubiquitously distributed group of heme-containing monooxygenases which catalyze a broad myriad of reactions such as carbon hydroxylation, heteroatom oxygenation, dealkylation, epoxidation, aromatic hydroxylation, reduction, and dehalogenation (Bernhardt, 2006). In particular, cytochrome P450s are involved in the biosynthesis of plant secundary metabolites and hormones, and play important roles in the regulation of plant growth and development (Kim and Tsukaya, 2002). Among the transcripts of cytochrome P450s present in the subset ST, we focus on two of them: *RcP450a* (29982.m000224) and *RcP450b* (30174.m009066).

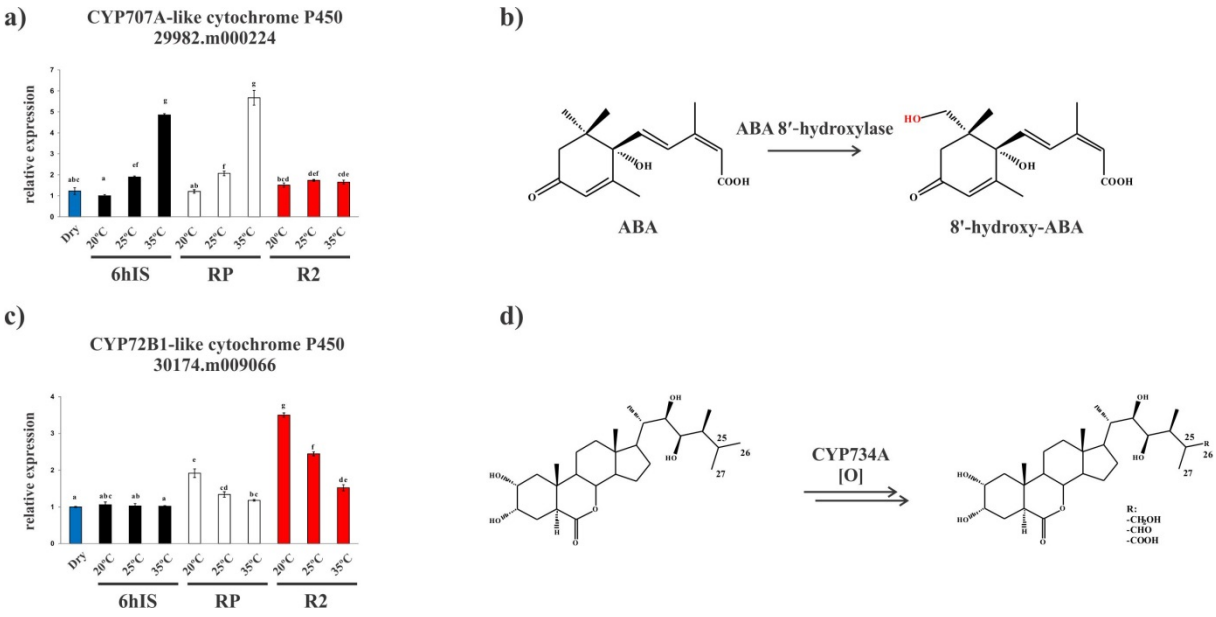


Figure 3. (**a**) Transcript levels of *RcP450a* during germination of *R. communis* seeds in response to different temperatures, (**b**) conversion of ABA to 8'-hydroxy-ABA by ABA 8′-hydroxylase, (**c**) Transcript levels of *RcP450b* during germination of *R. communis* seeds in response to different temperatures, (**d**) C-26 hydroxylation of castasterone and brassinolide by CYP734A. Means and standard errors of three biological replicates containing 15-20 seeds each are shown. Temperatures are depicted in the graphs. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p<0.05$).

Levels of *RcP450a* were higher in EI and RP imbibed at 35ºC than at 20ºC and 25ºC. In R2, levels of *RcP450a* showed little variation in response to temperature (Figure 3a). *RcP450a* showed high homology to four CYP707A-like cytochrome P450s in Arabidopsis: CYP707A1 (AT4G19230), CYP707A2 (AT2G29090), CYP707A3 (AT5G45340), and CYP707A4 (AT3G19270). In Arabidopsis, cytochrome P450 CYP707As encode key enzymes in ABA catabolism: ABA 8′-hydroxylases (Kushiro et al., 2004). ABA 8′-hydroxylase catalyzes the conversion of ABA to 8'-hydroxy-ABA (Figure 3b). Expression analysis revealed that Arabidopsis CYP707A2 is responsible for the rapid decrease in ABA level during seed imbibition (Kushiro et al., 2004). Levels of transcripts involved in ABA catabolism are higher in WT Arabidopsis seeds imbibing at 32ºC than at 21ºC (Chiu et al., 2012). Since ABA is known to inhibit seed germination (Goggin et al., 2009), we raise the hypothesis that faster germination of *R. communis* seeds at higher temperatures might be also regulated by the catabolic inactivation of ABA via hydroxylation at its 8′ position to form 8′-hydroxy-ABA as a means of further regulating ABA concentrations.

Levels of *RcP450b* were higher in RP and R2 imbibed at 20ºC than at 25ºC and 35ºC. In EI, levels of *RcP450b* showed little variation in response to temperature (Figure 3c). *RcP450b* showed high

homology to cytochrome P450 CYP734A1 in Arabidopsis. CYP734A1 encodes a member of the cytochrome P450 family that serves as a control point between multiple photoreceptor systems and brassinosteroid (BR) signal transduction (Sandhu et al., 2012). CYP734A1 also inactivates castasterone and brassinolide by means of C-26 hydroxylation (Figure 3d) (Neff et al., 1999). Brassinosteroids are steroid hormones which regulate plant development, physiology, and responses to biotic and abiotic stresses (Wang et al., 2013). Studies on mutants with defects in BR biosynthesis or signaling demonstrated that BR plays essential roles in nearly all phases of plant development, as these mutants show multiple developmental defects, such as reduced seed germination, extreme dwarfism, photomorphogenesis in the dark, altered distribution of stomata, delayed flowering and male sterility (Divi and Krishna, 2010; Gomes, 2011; Jiang et al., 2013; Kucera et al., 2005; Leubner-Metzger, 2001; Wang et al., 2013; Zeng et al., 2010). Brassinosteroids stimulate seed germination and BR signaling is required to overcome inhibition of germination by ABA (Steber and McCourt, 2001). Higher levels of *RcP450b* in seeds imbibing at 20ºC could explain the slower germination of *R. communis* seeds at lower temperatures. However, further analysis of brassinosteroid metabolism during late imbibition prior to germination is needed since up-regulation of *RcP450b* could not be detected at EI and since *RcP450b* is up-regulated in R2 seeds imbibing at 20ºC compared with R2 seeds imbibed at 25ºC and 35ºC, further analysis of brassinosteroid metabolism after germinatiion is also needed.

Transcripts within GO term binding encompassed several ATP binding proteins, DNA binding proteins, RNA binding proteins, such as splicing factors, ccaat-binding transcription factor subunit A, chaperones, DNA-directed RNA polymerases I, II, and III, heat shock factor proteins, multiprotein-bridging factor proteins, and zinc finger protein. For example, the DNA binding protein ID 29883.m001955 showed high homology with *A. thaliana* heat shock transcription factor A6B (AT3G22830). In general, plants have a flexible and specialized network of heat shock factors (HSFs), which induce the expression of heat shock proteins (Soares-Cavalcanti et al., 2012). The RNA binding protein ID 29709.m001182 showed high homology with the *A. thaliana* RNA-binding (RRM/RBD/RNP motifs) family protein (AT2G44710). These proteins are conserved in plants and play important roles in normal plant growth and development (Peal et al., 2011). DNA-directed RNA polymerases are responsible for the polymerization of ribonucleotides into a sequence complementary to the template DNA. Most RNA polymerases are multimeric enzymes and are composed of a variable number of subunits (Horgen and Key, 1973). Taken together, the set of genes within this GO class indicates that temperature has a large effect on transcriptional reprogramming during germination of R. *communis* seeds.

For the transcripts belonging to SST, also only one GO term associated with biological processes was enriched: metabolic processes (78 transcripts) (Figure 4a). Under the GO category of metabolic processes, primary metabolic process (57 transcripts) was enriched in response to temperature. Transcripts

in this GO are associated with glycolysis IV (plant cytosol), starch biosynthesis, trehalose biosynthesis I, and UDP-glucose biosynthesis (from sucrose). We argued that most transcripts belonging to ST are involved in the production of compounds that are converted to soluble sugars. Not surprisingly, transcripts belonging to SST are involved in pathways that aim at obtaining energy from carbohydrates, such as glycolysis. Hence, these results support our previous statement that temperature plays a major role in metabolic processes which are crucial to support successful seedling establishment.

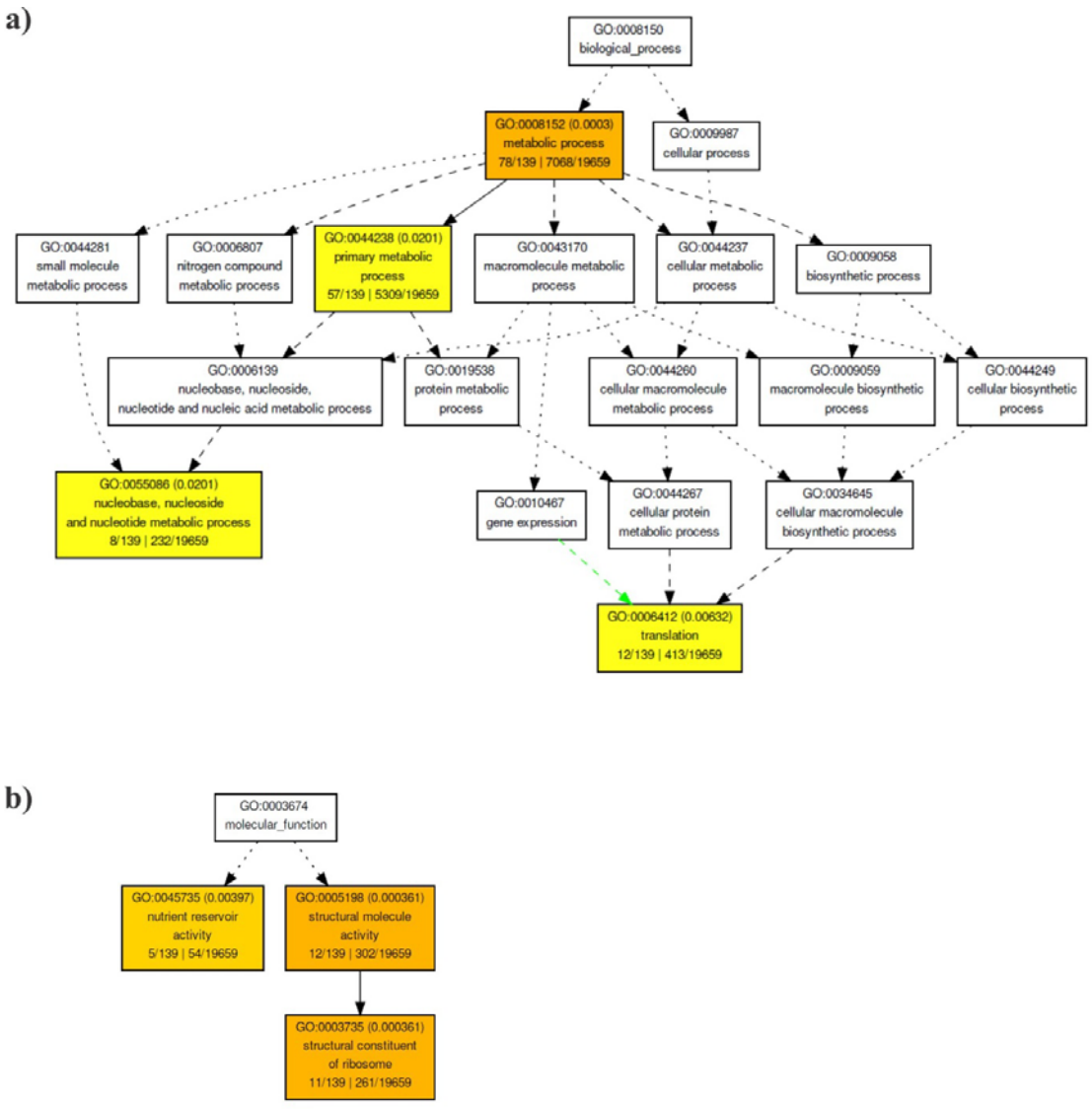


Figure 4. Gene ontology analysis of transcripts significantly affected by the interaction between developmental stage and temperature in *R. communis* using agriGO. (**a**) Significantly over-represented GO terms associated with biological processes and (**b**) Significantly over-represented GO terms associated with molecular function. The coloured boxes indicate significantly enriched GO terms. The box colours indicates levels of statistical significance with yellow < 0.05; orange < e-05 and red < e-09.

Two GO terms associated with molecular functions were enriched in the SST set: nutrient reservoir activity (5 transcripts) and structural molecule activity (12 transcripts) (Figure 4b). Transcripts within GO class reservoir activity encompassed mainly globulins. Globulins are prevalent seed storage proteins of dicotyledonous flowering plants (Borroto and Dure Iii, 1987). In *R. communis*, the subunit polypeptides of the 11S globulin are synthesized as high molecular weight precursors with heterogeneous molecular weights (Fukasawa et al., 1988). The 11S globulin gene family has been characterized in *R. communis*. Their expression profile indicates that they are seed specific and developmentally regulated, with a maximum expression when endosperm reaches its full expansion (Chileh et al., 2010). During imbibition of *R. communis* seeds, the levels of these transcripts decreased with increasing temperature (Figure 5). It seems that this is a simple result of faster mRNA decay at higher temperatures, but the biological significance of these results is still unclear. Under the GO category of structural molecule activity, structural constituent of ribosome (11 transcripts) was enriched in response to temperature (Figure 4b). Transcripts within this GO encompass 40S, 50S, and 60S ribosomal proteins. Transcripts encoding ribosomal proteins are required for new ribosome formation during seed germination, when most of the metabolic machinery is re-activated (Jiménez-López et al., 2011).

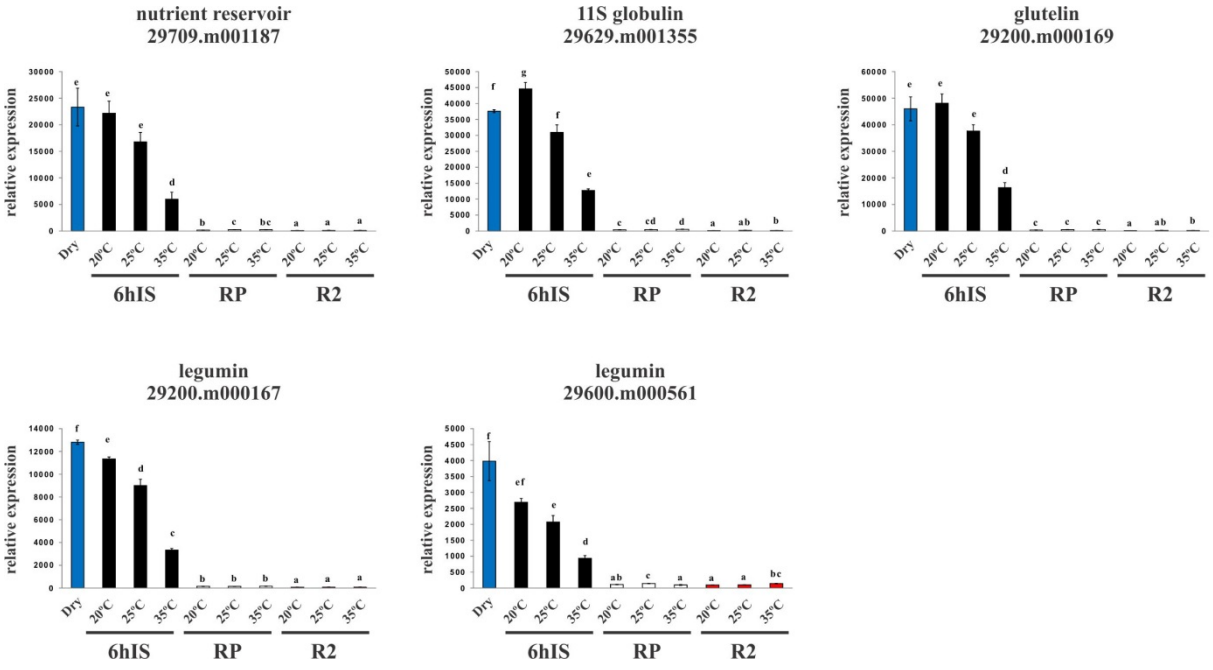


Figure 5. Transcripts within GO term reservoir activity during germination of *R. communis* seeds in response to different temperatures. Means and standard errors of three biological replicates containing 15-20 seeds each are shown. Temperatures are depicted in the graphs. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p<0.05$).

3.2 Stage-specific variation in transcript levels in response to temperature

3.2.1 PCA analysis

In the next step, we analyzed the differential expression of transcripts for each individual stage in response to temperature using a two-sided t-test, assuming unequal variance. We compared samples collected at 20ºC and 35ºC within each individual stage. This resulted in 365 transcripts differentially expressed in response to temperature at EI, 2010 transcripts at RP, and 975 transcripts at R2, indicating temperature has a large effect on transcriptional reprogramming at later stages of germination.

Principal component analysis (PCA) was performed individually for these three subset of transcripts (Supplementary Figure 3). For the subset of transcripts selected for EI seeds, principal component 1 (PC1) explained 66.3% of the total variance, whereas principal component 2 (PC2) explained 16.2% (Supplementary Figure 3a). PC1 effectively separated dry and EI seeds from RP and R2 seeds. PC2 mainly discriminated the contribution of temperature. It is worth to highlight that PC2 is responsible for the great separation of EI and dry seeds. This highlights the robustness of the analysis in discriminating the effect of temperature on EI seeds as compared with RP and R2 seeds. For the subset of transcripts selected for RP seeds, PC1 explained 56.0% of the total variance, whereas PC2 explained 13.4% (Supplementary Figure 3b). Again, PC1 effectively separated dry and EI seeds from RP and R2 seeds. Although RP and R2 seeds imbibed at 35ºC were clearly differing from RP and R2 seeds imbibed at 20ºC and 25ºC, no clear separation between RP seeds imbibed at 20ºC and 25ºC was observed. For the subset of transcripts selected for R2 seeds, PC1 explained 59.3% of the total variance, whereas PC2 explained 13.6% (Supplementary Figure 3c). PC1 separated dry and EI seeds from RP and R2 seeds, whereas PC2 discriminated the contribution of temperature. An overlap was observed among dry seeds and EI seeds imbibed at 20ºC and 25ºC. EI seeds imbibed at 35ºC were, however, clearly differentiated from EI seeds imbibed at 20ºC and 25ºC. Taken together, the closeness of RP and R2 seeds appears to be more related to temperature than to developmental stage.

3.2.2 Singular enrichment analysis

For transcripts differential expressed in EI, two GO terms associated with biological processes were enriched: metabolic processes (100 transcripts) and cellular processes (78 transcripts) (Figure 6a). Transcripts in these GOs are associated with gluconeogenesis I, β-oxidation, Calvin-Benson-Bassham cycle, fatty acid activation, starch degradation II, and tRNA charging. Four GO terms associated with molecular functions were enriched: structural molecule activity (26 transcripts), catalytic activity (93 transcripts), nutrient reservoir activity (six transcripts) and electron carrier activity (14 transcripts) (Figure 6b).

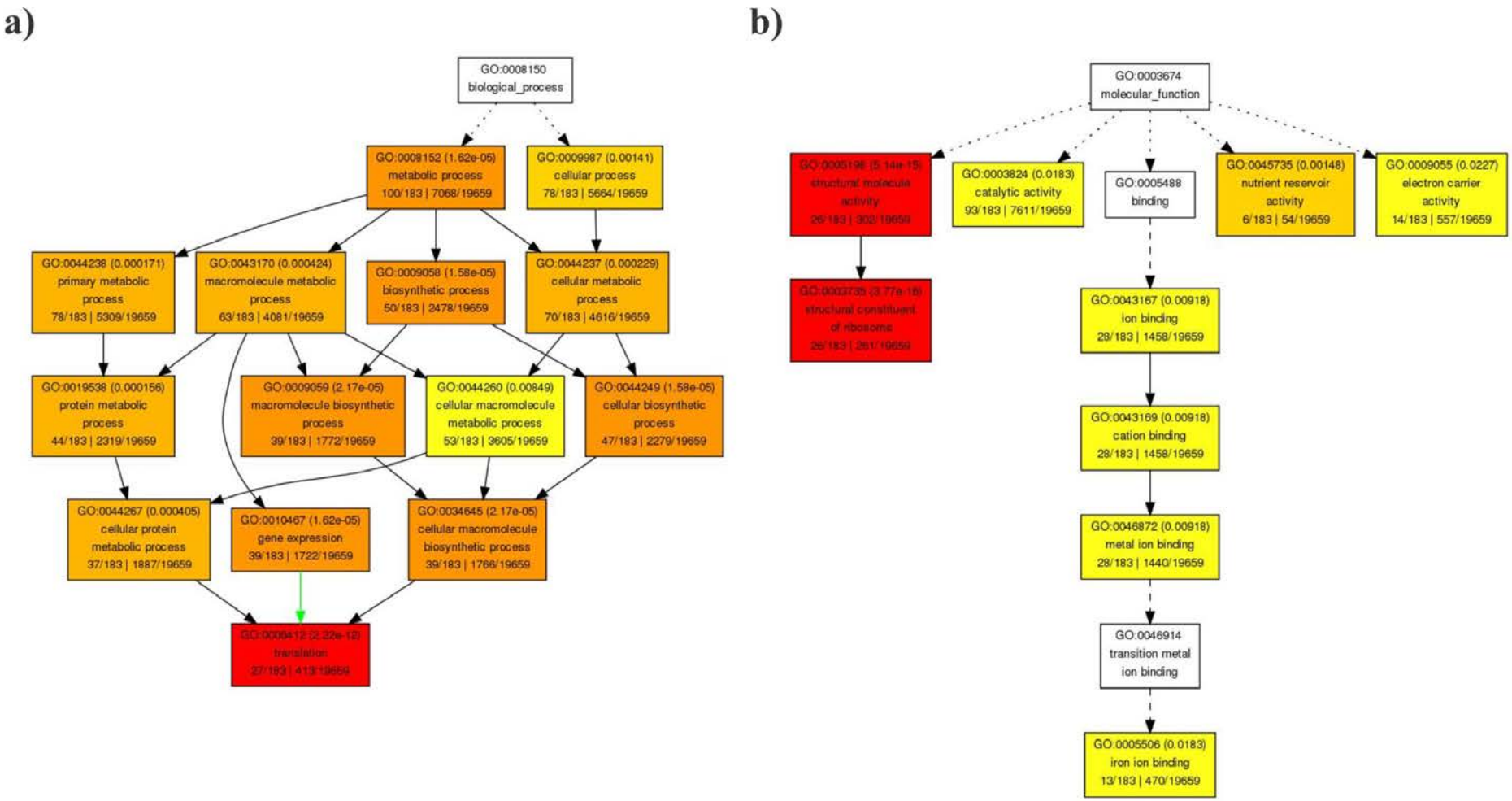


Figure 6. Gene ontology analysis of the subset of transcripts differentially expressed in EI seeds using agriGO. (**a**) Significantly over-represented GO terms associated with biological processes and (**b**) Significantly over-represented GO terms associated with molecular function. The coloured boxes indicate significantly enriched GO terms. The box colours indicates levels of statistical significance with yellow < 0.05; orange < e-05 and red < e-09.

Transcripts within GO electron carrier activity include mainly cytochrome P450 proteins. These results show that the effect of temperature on major energy-generating metabolic processes is initiated very early during imbibition. Nonetheless, in order to obtain further insights to explain the compromised seedling establishment of *R. communis* at high temperatures, we decided to focus on the metabolic changes that occurred at the RP stage. The main reason to focus on the RP stage is because we have demonstrated in chapter 3 that we identified a temperature sensitive window during germination, i.e. before radicle protrusion, in which high temperatures compromise seedling development.

We started our analysis with down-regulated transcripts differential expressed at RP. For this subset, several GO terms associated with biological processes were enriched such as multicellular organismal process (5 transcripts), cellular process (268 transcripts), biological regulation (76 transcripts), metabolic process (323 transcripts), and response to stimulus (34 transcripts) (Figure 7a). Transcripts within GO class multicellular organismal process include two serine-threonine protein kinases. Transcripts within GO class cellular process include several ATP binding proteins, serine-threonine protein kinases, a receptor protein kinase, a calcium-dependent protein kinase, WRKY transcription factors, and several transcripts of the hydrogen production VIII pathway, pentose phosphate pathway (oxidative branch), glycolysis IV (plant cytosol), RuBisCO shunt, and UDP-glucose biosynthesis. Transcripts within the GO class metabolic process include transcripts involved in abscisic acid biosynthesis, the Calvin-Benson-Bassham cycle, hydrogen production VIII, the pentose phosphate pathway (oxidative branch), and starch biosynthesis. For the down-regulated transcripts belonging to RP, three GO terms associated with molecular function were enriched: binding (307 transcripts), catalytic activity (343 transcripts), and electron carrier activity (36 transcripts) (Figure 7b). Thus, based on these results it appears that high temperature compromises seedling development by down-regulating metabolic processes which are crucial to support successful seedling establishment.

For the upregulated transcripts belonging to RP, two GO terms associated with biological processes were enriched: cellular processes (190 transcripts), and metabolic processes (390 transcripts) (Figure 8a). Transcripts within GO cellular processes include chaperones, DNA-directed RNA polymerases, heat shock factor proteins, heat shock proteins, a receptor serine/threonine kinase, and receptor protein kinase CLAVATA1. Transcripts within GO term metabolic processes include transcripts involved in fatty acid activation, pyridoxal 5'-phosphate salvage II (plants) and triacylglycerol degradation.

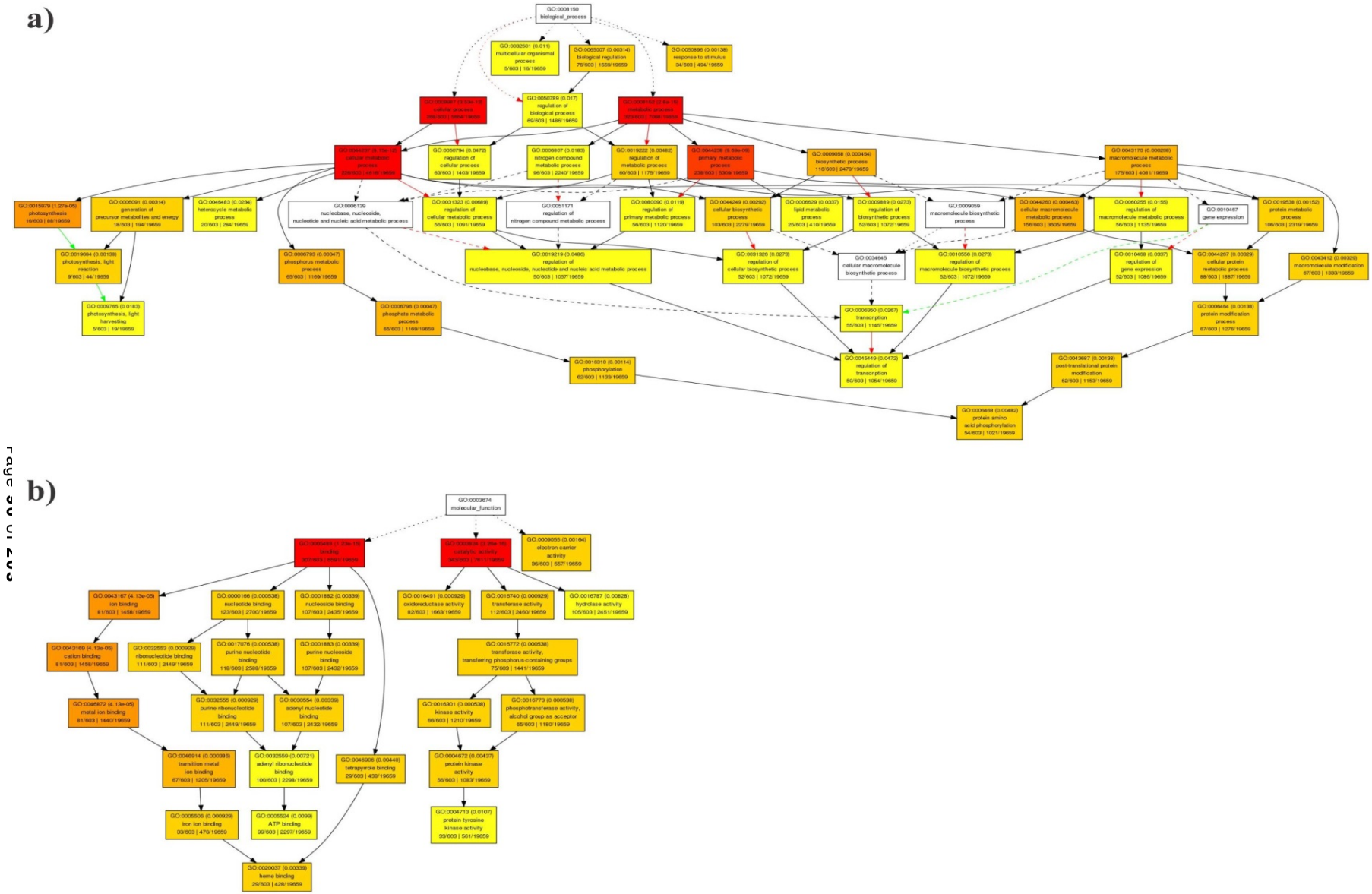


Figure 7. Gene ontology analysis of the down-regulated genes of the subset of transcripts differentially expressed in RP seeds using agriGO. (**a**) Significantly over-represented GO terms associated with biological processes and (**b**) Significantly over-represented GO terms associated with molecular function. The coloured boxes indicate significantly enriched GO terms. The box colours indicates levels of statistical significance with yellow < 0.05; orange < e-05 and red < e-09.

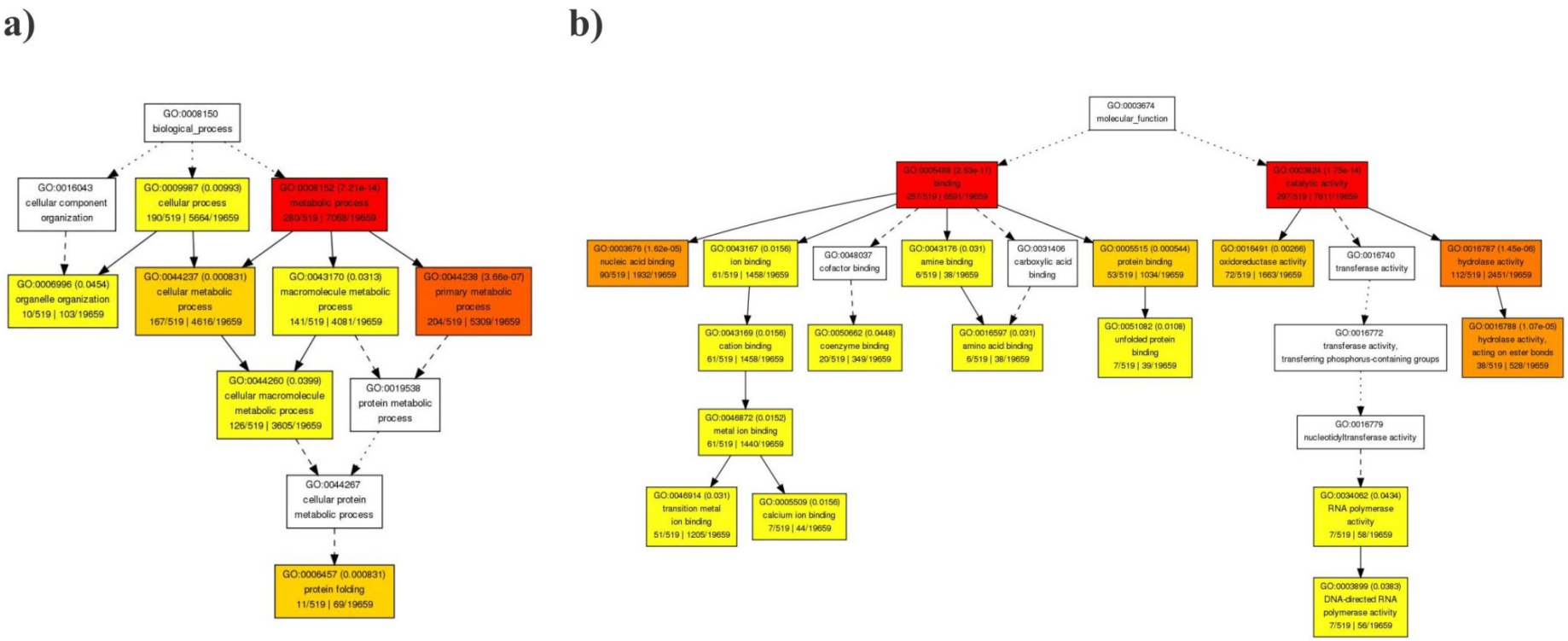


Figure 8. Gene ontology analysis of the upregulated genes of the subset of transcripts differentially expressed in RP seeds using agriGO. (**a**) Significantly over-represented GO terms associated with biological processes and (**b**) Significantly over-represented GO terms associated with molecular function. The coloured boxes indicate significantly enriched GO terms. The box colours indicates levels of statistical significance with yellow < 0.05; orange < e-05 and red < e-09.

Heat stress can cause serious deleterious effects in plants, such as disturbance of cellular homeostasis and destabilization of proteins, membranes, and RNA species, which can lead to severe retardation in growth and development, and even death (Hasanuzzaman et al., 2013; Kotak et al., 2007; Qu et al., 2013). We have demonstrated that seeds which germinated at high temperature reprogrammed their transcriptome and metabolome profiles in order to counterbalance the effect of high temperature. At the transcriptome level, this reprogramming involved the activation of protection mechanisms, mainly through upregulation of transcripts encoding for heat shock proteins and heat shock factor proteins. Heat shock proteins play an important role in protein folding and conformation, stabilizing partially unfolded proteins and preventing undesirable protein aggregation (Dittmar et al., 1997; Lee and Vierling, 2000). Therefore, the enrichment of the GO terms associated with chaperones, DNA-directed RNA polymerases, heat shock factor proteins and heat shock proteins is not surprising, since a large number of proteins can be misfolded under heat stress. Most importantly, these results support the fact that *R. communis* seeds perceive 35^{o}C as a stress condition.

3.2.3 Mining for similar responses in regulation of gene expression to temperature

The number of transcripts differentially expressed in response to temperature was 365 at EI, 2010 at RP, and 975 at R2. In order to find temperature-responsive genes shared by all three stages, we produced a Venn diagram with genes down- and upregulated at EI, RP, and R2 (Figure 9). Regardless of the subset analyzed (transcripts down- or upregulated), RP and R2 shared more transcripts between them than with EI. For example, among the down-regulated RP and R2 shared 154 transcripts, whereas RP shared 16 transcripts with EI. R2 shared even less similarities with EI with only five transcripts. Among the upregulated transcripts, RP and R2 shared 164, whereas RP shared 26 transcripts with EI and R2 shared only three transcripts with EI. Thus, although temperature has a strong effect on the *R. communis* transcriptome, most of these differences occurred between 6 hours of imbibition and the commence of germination, i.e. radicle protrusion. This coincides with the thermo-sensitive window identified during seed germination in which high temperatures compromise seedling development (Ribeiro et al., 2015b).

Only three transcripts were down-regulated at all three stages: one oxidation-related zinc finger 2 (29794.m003367), one F-box and wd40 domain protein (29929.m004787), and one DNA binding protein/ MYB-like transcription factor (29805.m001497). Among the upregulated transcripts, nine were shared by all three stages: one BET1P/SFT1P-like protein 14BB (29603.m000532), one low-molecular-weight cysteine-rich protein LCR78 (27436.m000276), one WD-repeat protein (27436.m000276), one GAST1 protein (30170.m013769), one adenylate kinase 1/P-loop containing nucleoside triphosphate hydrolases superfamily protein (30190.m010959), and four conserved hypothetical proteins (29002.m000150, 30068.m002599, 30170.m014182, and 29667.m000353). One conserved hypothetical protein

(29667.m000353) showed high homology to an *A. thaliana* chaperone binding-ATPase activator (AT5G58110).

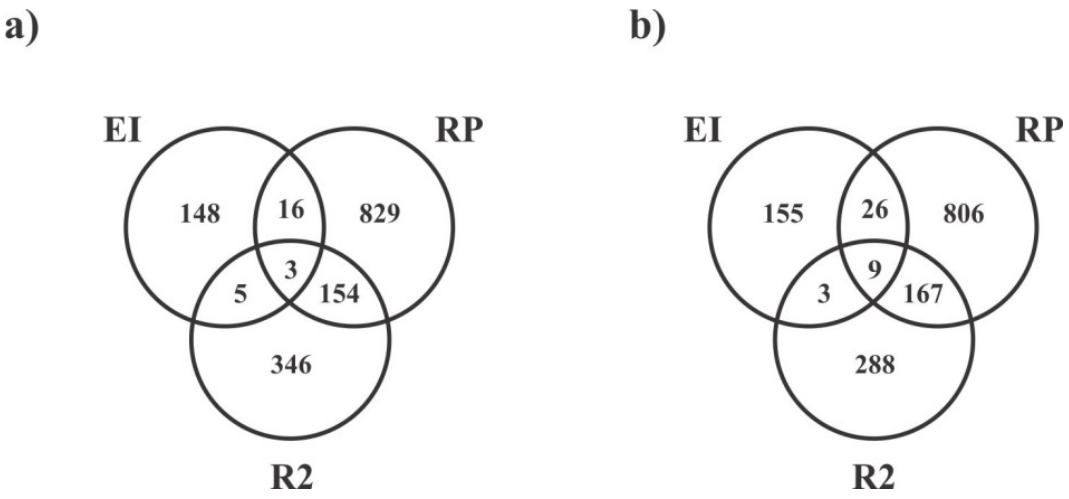


Figure 9. Venn diagram of the differentially expressed genes during germination of *R. communis* seeds in response to different temperatures. (**a**) down-regulated genes and (**b**) upregulated genes.

In Arabidopsis, the oxidation-related zinc finger 2 (*AtOZF2*; AT4G29190) is involved in regulation of transcription and has sequence-specific DNA binding transcription factor activity. *AtOZF2* is involved in ABA and salt stress responses through the ABI2-mediated signaling pathway (Huang et al., 2012). *AtOZF2* is an *AtOZF1* homolog, which confers oxidative stress tolerance to plants (Huang et al., 2011). It was demonstrated that *AtOZF1* and *AtOZF2* are important regulators for plant tolerance to abiotic stress (Huang et al., 2012). WD40 proteins play a crucial role in diverse protein-protein interactions by acting as scaffolding molecules and thus assisting in the proper activity of proteins (Mishra et al., 2014; Mishra et al., 2012). Finally, MYB transcription factors are composed of one, two, or three imperfect helix-turn-helix repeats that recognize the major groove of DNA and which are involved in a variety of plant-specific processes, including cell morphogenesis, secondary metabolism, cell differentiation, and stress responses (Shin et al., 2011; Zhang et al., 2011). These genes constitute good candidates for further characterization of temperature-responsive molecular mechanisms in *R. communis*.

3.3 *Additional evidence to support the hypothesis that during the initial phase of imbibition R. communis seeds perceive partial hydration as a drought stress and therefore accumulate GABA*

We demonstrated that GABA levels were higher in 6h imbibed seeds (6hIS) samples imbibed at 20ºC than at 35ºC. Based on our findings, we suggested that during early imbibition (6hIS), *R. communis* seeds experience a drought stress-like condition as a result of partial hydration, resulting in the

accumulation of GABA (Ribeiro et al., 2015b). To provide additional support for this hypothesis, we mined our transcriptome data of EI samples searching for transcript of genes involved in the GABA shunt. The first enzyme in the GABA shunt is glutamate decarboxylase (GAD) which catalyzes the conversion of glutamate to GABA and CO_2 (Figure 10a) (Renault et al., 2010).

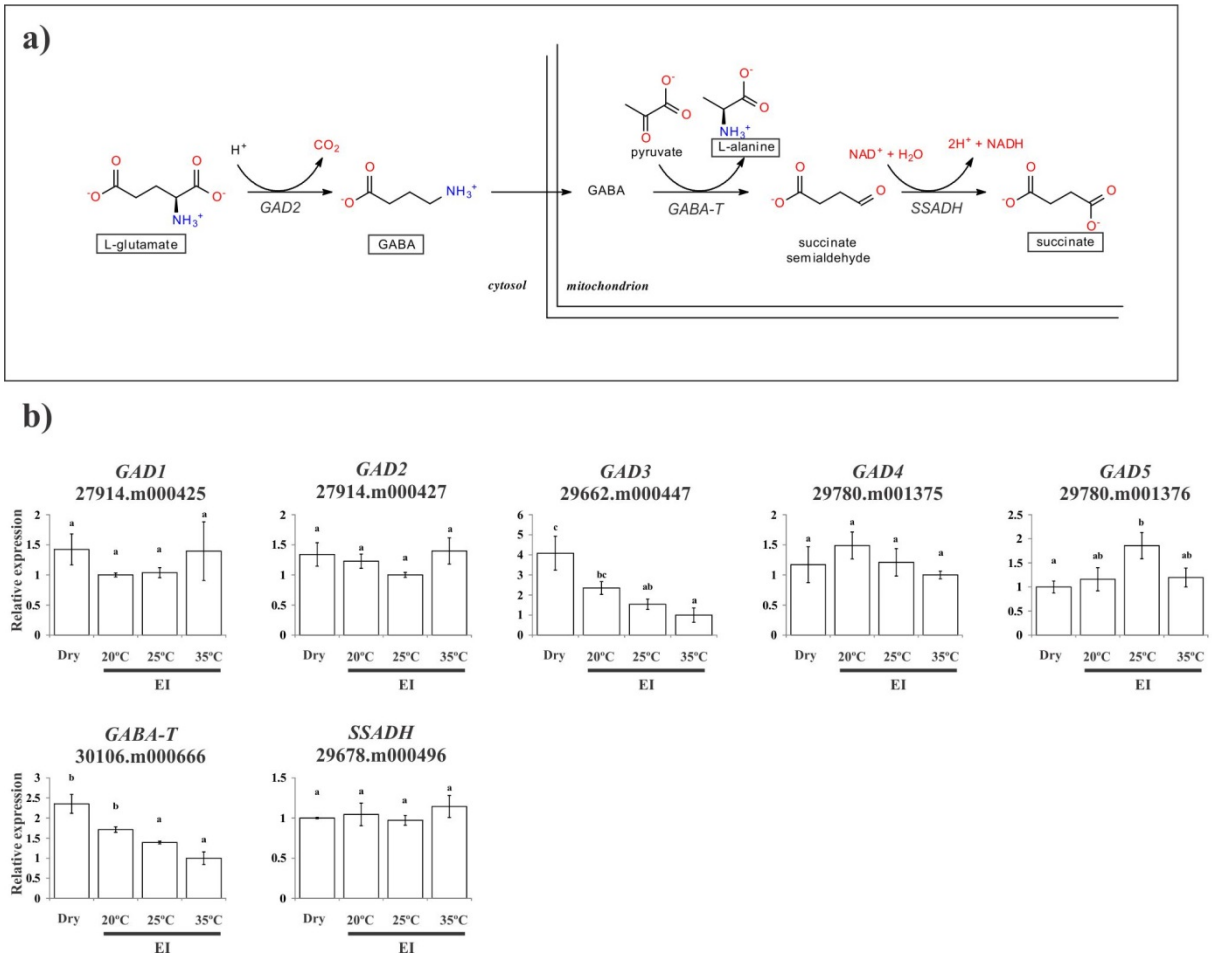


Figure 10. Effect of temperature on the GABA shunt during early imbibition (EI). (a) Schematic representation of the GABA shunt. Metabolites with significant changes after 6 hours of imbibition due to the temperature are depicted in the boxes. (b) Transcript levels of GABA shunt related genes. Means and standard errors of three biological replicates containing 15-20 seeds each are shown. Temperatures are depicted in the graphs. Different letters above the bars indicate significant differences between samples by Tukey's HSD ($p<0.05$).

The *R. communis* genome contains five sequences putatively annotated as glutamate decarboxylase (*RcGAD1*, *RcGAD2*, *RcGAD3*, *RcGAD4*, and *RcGAD5*). Transcript levels of 4 glutamate decarboxylases (*RcGAD1*, *RcGAD2*, *RcGAD4*, and *RcGAD5*) showed none or little variation among EI samples. Transcript levels of *RcGAD3* were 2.34-fold greater in EI seeds imbibed at 20ºC than at 35ºC (Figure 10b). This is, however, much lower than what was observed for 6hIS in which transcript levels of

RcGAD3 were 8.54-fold greater in seeds imbibed at 20ºC than at 35ºC. Another important remark is that in 6hIS transcript levels of *RcGAD2* were 7.68-fold greater in seeds imbibed at 20ºC than at 35ºC, whereas in EI samples no difference was found (Figure 10b). A similar reduction in the difference of the transcript levels of aminobutyrate transaminase (*GABA-T*) was also observed among EI samples. Transcript levels of *GABA-T* were 1.71-fold greater in EI seeds imbibed at 20ºC than at 35ºC, whereas in 6hIS transcript levels of *GABA-T* were 2.47-fold greater in seeds imbibed at 20ºC than at 35ºC (Figure 10b). Finally, transcript levels of succinic semialdehyde dehydrogenase (*SSADH*) showed no variation among EI samples, whereas in 6hIS transcript levels of *SSADH* were 1.95-fold greater in seeds imbibed at 20ºC than at 35ºC (Figure 10b). Taken together, these results support our initial hypothesis that during the initial phase of imbibition *R. communis* seeds perceive partial hydration as a drought stress and therefore accumulate GABA.

5 Acknowledgments

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6 Conflict of interest

The authors declare no conflict of interest.

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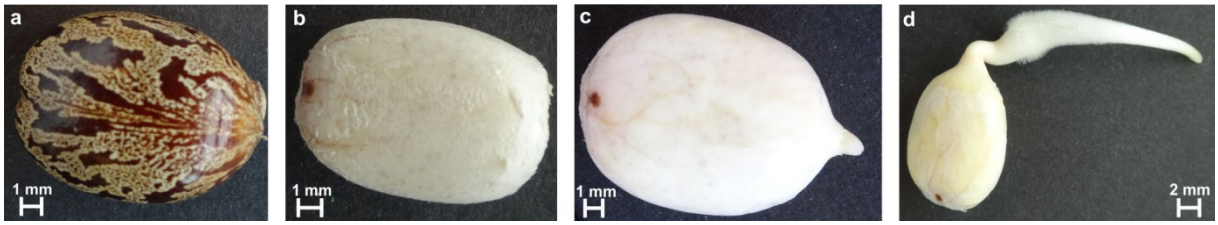
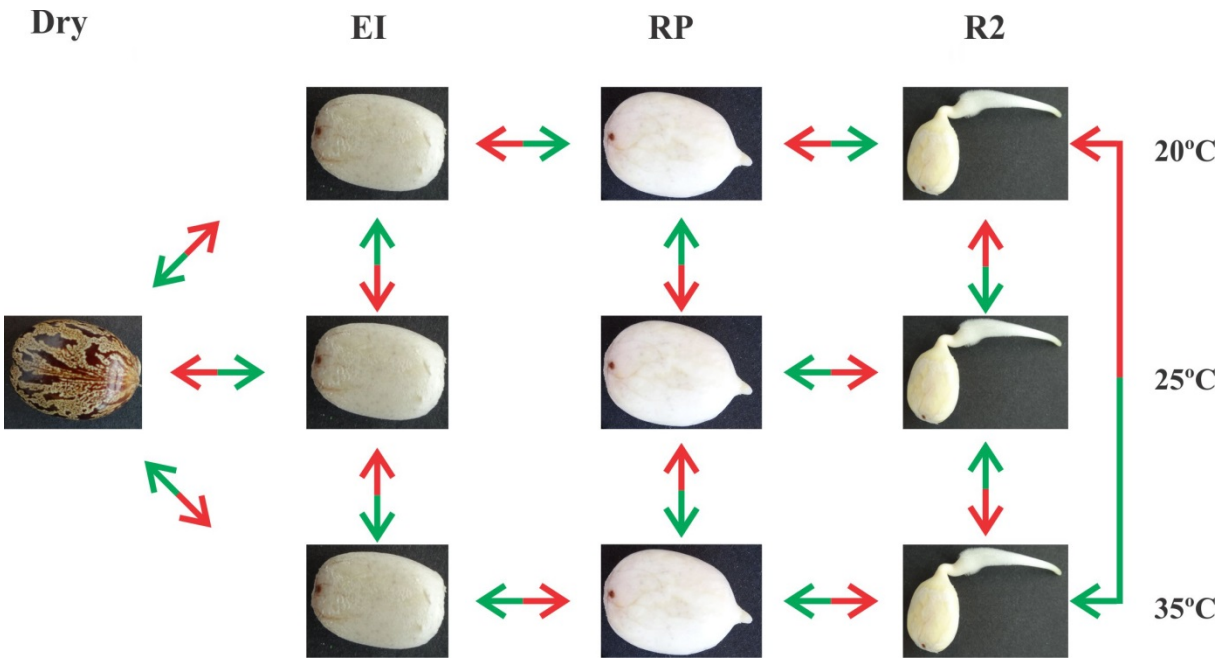
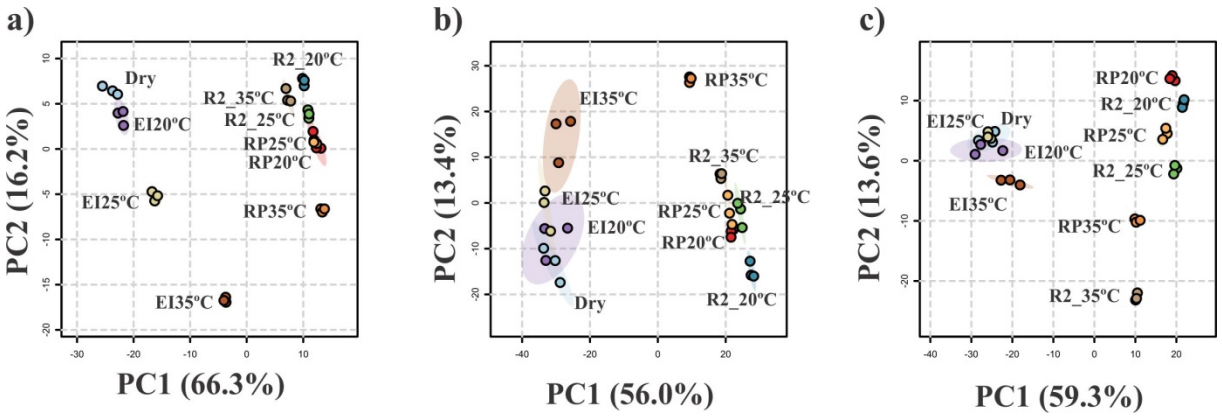
8 Supplementary Figures



Supplementary Figure 1. *R. communis* seeds at different developmental stages. Three different stages are presented: (**a**) dry seeds with seed coat, (**b**) dry seeds without seed coat, (**c**) seeds at radicle protrusion and (**d**) seeds with a radicle length of 2 cm.



Supplementary Figure 2. Design for hybridization of samples on two-colour Agilent microarrays. The colours of the arrows indicate Cy3 (green) and Cy5 (red) dyes in this microarray experiment. Four different stages were analysed: dry seeds (Dry), seeds at early imbibition (EI), seeds at radicle protrusion (RP) and with a radicle length of 2 cm (R2) at three different temperatures: 20°C, 25°C and 35°C.



Supplementary Figure 3. Principal component analysis (PCA) based on transcript levels during germination of *R. communis* seeds in response to different temperatures. (**a)** subset of transcripts differentially expressed in EI seeds, (**b**) subset of transcripts differentially expressed in RP seeds, and (**c**) subset of transcripts differentially expressed in R2 seeds. Four different stages were analysed: dry seeds (Dry), seeds at early imbibition (EI), seeds at radicle protrusion (RP) and with a radicle length of 2 cm (R2). Seed germination was performed at three different temperatures: 20, 25 and 35ºC.

9 Supplementary table caption

Supplementary Table 1. Microarray results with average expression values for all genes with indications of differential expression.

Supplementary tables can be found at http://www.wageningenseedlab.nl/thesis/prribeiro/SI/

Chapter 5

Physiological and biochemical responses of *Ricinus communis* seedlings to different temperatures: a metabolomics approach

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Abstract

Compared with major crops, growth and development of *Ricinus communis* is still poorly understood. A better understanding of the biochemical and physiological aspects of germination and seedling growth is crucial for the breeding of high yielding varieties adapted to various growing environments. In this context, we analysed the effect of temperature on growth of young *R. communis* seedlings and we measured primary and secondary metabolites in roots and cotyledons. Three genotypes, recommended to small family farms as cash crop, were used in this study. Seedling biomass was strongly affected by the temperature, with the lowest total biomass observed at 20ºC. The response in terms of biomass production for the genotype MPA11 was clearly different from the other two genotypes: genotype MPA11 produced heavier seedlings at all temperatures but the root biomass of this genotype decreased with increasing temperature, reaching the lowest value at 35ºC. In contrast, root biomass of genotypes MPB01 and IAC80 was not affected by temperature, suggesting that the roots of these genotypes are less sensitive to changes in temperature. In addition, an increasing temperature decreased the root to shoot ratio, which suggests that biomass allocation between below- and above ground parts of the plants was strongly affected by the temperature. Carbohydrate contents were reduced in response to increasing temperature in both roots and cotyledons, whereas amino acids accumulated to higher contents. Our results show that a specific balance between amino acids, carbohydrates and organic acids in the cotyledons and roots seems to be an important trait for faster and more efficient growth of genotype MPA11. An increase in temperature triggers the mobilization of carbohydrates to support the preferred growth of the aerial parts, at the expense of the roots. A shift in the carbon-nitrogen metabolism towards the accumulation of nitrogen-containing compounds seems to be the main biochemical response to support growth at higher temperatures. The biochemical changes observed in response to the increasing temperature provide leads into understanding plant adaptation to harsh environmental conditions, which would be very helpful in developing strategies for *R. communis* crop improvement research.

Keywords: carbon-nitrogen balance, Castor bean, *Ricinus communis*, seedling establishment, temperature

1 Introduction

Ricinus communis is a member of the Euphorbiaceae and can be found throughout tropical, sub-tropical and warm temperate regions (Santiago et al., 2010; Scarpa and Guerci, 1982a). The oil extracted from this species has been primarily used as purgative or laxative in traditional medicine to counter constipation (Scarpa and Guerci, 1982b), but the commercial interest in *R. communis* is mainly increasing because its seeds contain high amounts of a unique oil consisting of up to 94% of the fatty acid ricinoleic acid (12-hydroxy-cis-9-octadecenoic acid) (Gong et al., 2005). This fatty acid confers unique and highly demanded properties to the oil and the biodiesel produced from it: its high calorific value and the high cetane number are of advantage along with the low content of phosphorous and carbon residues (Anjani, 2012; Conceicao et al., 2007; Salimon et al., 2010; Scholz and da Silva, 2008). It is at present one of the few commercially available sources of natural hydroxylated triglycerides (Derksen et al., 1995).

The Brazilian National Program for Production and Use of Biodiesel has identified *R. communis* as the ideal oil crop to promote social development in the semi-arid region of Brazil because of its versatility as a productive (oil) crop in various environments (Cesar and Batalha, 2010). For this reason *R. communis* is currently grown in the arid zones of Northeastern Brazil (Sausen and Goncalves Rosa, 2010). Genotypes MPA11, MPB01 and IAC80 were developed by the breeding program of the Campinas Agronomic Institute and by the *Empresa Baiana de Desenvolvimento Agrícola S.A* (EBDA), aiming at finding alternative high yielding crops for family farmers in the semi-arid region of Brazil.

Temperature is a critical environmental cue that influences seedling establishment and a difference of only a few degrees may already lead to a notable change in growth and survival rate (Salisbury and Ross, 1985). The effects of the temperature on the survival, growth, photosynthesis, and metabolism of young seedlings has been assessed for several plants (Booth and Bai, 1999; Karim et al., 2000; Niu et al., 2012; Wheeler et al., 2000), but growth and development of *R. communis* is still poorly understood, as compared with major crops. Therefore, a better understanding of the biochemical and physiological aspects of germination and seedling growth is crucial for the breeding of high yielding varieties adapted to various growing environments that could be used for family farmers worldwide (Severino and Auld, 2013).

Metabolite profiling of plants under different environmental conditions has provided important information about the biochemical and molecular changes related to temperature adaptation. Carbohydrate and amino acid metabolism appear to be part of the mechanisms by which plants adapt to different environmental conditions (Diamant et al., 2001; Kaplan et al., 2004; Panikulangara et al., 2004; Rizhsky et al., 2004). Temperature may also affect heat tolerance in terms of seedling growth, antioxidant response and cell death (Hameed et al., 2012), whilst in general, plants display considerable plasticity to respond to

short-term fluctuations of environmental factors (Callaway et al., 2003; Nicotra and Davidson, 2010; Valladares et al., 2007). Although metabolomics has been used to dissect plant responses to abiotic stresses, most of the studies regarding the temperature effect on seedling performance have focused on the ability of plants to maintain homeostasis at chilling temperatures (0 to 15ºC) or have investigated plant responses to high-temperature stress, mostly using Arabidopsis as model species (Arbona et al., 2013; Guy et al., 2008; Obata and Fernie, 2012). Plant metabolic plasticity in response to mild temperatures (20 to 35ºC) has received much less attention although it is an important trait for crop species (Lewicka and Pietruszka, 2006). *R. communis* possesses the ability to grow in various diverse environments which makes this species an ideal candidate to provide a better understanding of seedling performance and adaptation under different temperatures.

Initial vegetative growth is very important to the establishment of *R. communis* and since little is known about biochemical and molecular changes related to temperature adaptation in *R. communis*, we raise the question whether such a highly adaptable species may have a specific metabolic signature that may apply to other crops, as well. In this context, we set out to study the *R. communis* metabolome in young seedlings and its relationship with seedling growth performance at different temperatures.

2 Methods

2.1 Plant material

Seeds of three different *Ricinus communis* genotypes (IAC80, MPA11 and MPB01) used in this work were kindly supplied by "*Empresa Baiana de Desenvolvimento Agrícola S.A.*" (EBDA), Salvador-Bahia, Brazil. The genotype IAC80 was developed by the breeding program of the Campinas Agronomic Institute aiming to recommend it to small family farms that use manpower to carry out the harvest in several steps. This genotype enables high yields of *R. communis* seeds concomitantly with the production of food crops. Genotypes MPA11 and MPB01 were bred by *Empresa Baiana de Desenvolvimento Agrícola S.A* (EBDA) aiming at finding alternative crops for family farmers at the semi-arid region of Brazil.

For phenotypical assays, seeds were allowed to germinate using paper rolls as substrate at 25ºC in the dark. After 72 hours, germinated seeds were transferred to moist soil and were allowed to grow at four different temperatures (20ºC, 25ºC, 30ºC and 35ºC) in continuous light for 13 days. Fresh and dry weight of the cotyledons, first true leaves, cotyledons, stem and roots of the 14-day-old seedlings were measured together with stem height. Fifteen to twenty seedlings were used.

For metabolite profiling assays, seed coats were removed and the seeds were allowed to germinate using paper rolls as substrate at 25ºC in the dark. After 44-50 hours, germinated seeds were transferred to

moist vermiculite and were allowed to grow at 20ºC in continuous light for 10 days. This was done to reduce differences in developmental stages that were observed during the phenotypical characterization when plants grew over a period of 13 days in different temperatures. Then, half of the 10-day-old seedlings were then transferred to an incubator at 35ºC with continuous light. After 4 days, roots and cotyledons (three biological replicates of 15-18 seedlings each) were collected, immediately frozen in liquid nitrogen, freeze-dried, ground and stored at -80ºC prior to analysis.

2.2 Extraction and derivatization of primary metabolites for GC-TOF-MS analysis

Metabolite profiling analysis was performed as described previously (Louis Joosen et al., 2013). Approximately 20 mg of freeze-dried and ground roots or cotyledons were transferred to a 2-mL Eppendorf tube after which 400 µL methanol and 300 µL chloroform were added and vortexed briefly. Then, 130 µL of H_2O and 20 µL of the internal standard ribitol (1 mg/mL) were added, vortexed thoroughly and sonicated for 10 minutes. Thereafter, 200 µL of H_2O was added, vortexed thoroughly and centrifuged for 5 min at 17000 g in an Eppendorf centrifuge. Of the upper phase 600 µL were transferred to a new 2-mL Eppendorf tube. To the remaining material 500 µL of a 1:1 v/v mix of methanol and chloroform was added, vortexed thoroughly and kept on ice for 10 minutes. Then, 200 µL of H_2O was added and centrifuged for 5 min at 17000 g in an Eppendorf centrifuge. Of the upper phase 400 µL were transferred to the previously collected upper phase. An aliquot of 30 µL of the joint upper phase was dried overnight by vacuum centrifugation.

All samples were analyzed by gas chromatography coupled to a quadrupole time of flight mass spectrometry system (GC-TOF-MS) as TMS derivatives. TMS derivatives were obtained by online derivatization (Combi PAL autosampler - CTC Analytics) as described previously (Sumner et al., 2007). Initially, 12.5 µL of O-methylhydroxylamine hydrochloride (20 mg mL^{-1} in pyridine) was added to the vials and incubated for 30 min at 40ºC in a shaking incubator. Then, 17.5 µL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was added to the vials for 60 min at 40ºC in a shaking incubator. 5 µL of an alkane mixture (C10–C17 and C19–C33) was added to determine the retention index of the metabolites. Sample aliquots of 2 µL were injected at a split ratio of 20:1 into an Optic 3 high-performance injector (ATAS) at 70ºC, and then the injector was rapidly heated to 240ºC at 6ºC s^{-1}. Chromatography was performed in an Agilent 6890 gas chromatograph (Agilent Technologies) coupled to a Pegasus III time-of-flight mass spectrometer (Leco Instruments) using a VF-5ms capillary column (Varian; 30 m x 0.25 mm x 0.25 µm) including a 10-m guardian column with helium as carrier gas at a column flow rate of 1 mL min^{-1}. The oven temperature program was 2 min at 70ºC, followed by a 10ºC min^{-1} ramp to 310ºC, 5 min at 310ºC, and 6 min at 70ºC before the next injection. The transfer line temperature was set at 270ºC. The column effluent was ionized by electron impact at 70 eV. Mass spectra

were recorded at 20 scans s^{-1} within a mass-to-charge ratio range of 50 to 600 at a source temperature of 200ºC. A solvent delay of 295 s was set. The detector voltage was set to 1,650 V.

2.3 Extraction of secondary metabolites for GC-MS analysis

Approximately 10 mg of freeze-dried and ground roots or cotyledons were transferred to a 2- mL Eppendorf tube and 300 µL or 150 µL of a methanol:chloroform (1:1) solution was added, respectively. Subsequently, the tubes were sonicated for 10 minutes and centrifuged for 5 min at 17000 g in an Eppendorf centrifuge. The upper phase (100µL) was filtered and transferred to a 2-mL glass GC vial with a 200-µL glass insert. Residual water was removed with sodium sulfate during filtration. Hexadecane and heptadecane (1:1) in hexane were used as internal standards.

Chromatography was performed with an Agilent 7809A gas chromatograph (Agilent Technologies) coupled to a Triple-Axis detector (Agilent 5975C), using a ZB-5 (Phenomenex; 30 m x 0.25 mm) capillary column (0.25 mm film thickness) using helium as carrier gas at a flow rate of 1 mL min^{-1}. Three different split ratios were used: 29:1 with detector off between 18.8 and 19.2 min for *R. communis* green cotyledon samples, 9:1 for *R. communis* root samples and 149:1 during ricinine quantification. The inlet temperature of the injector was set to 250ºC. The initial oven temperature was 45ºC for 1 min, and was increased to 300ºC after 1 min at a rate of 10ºC min^{-1} and held for 5 min at 300ºC. A solvent delay of 240s was set.

2.4 Extraction of soluble carbohydrates and starch

Soluble carbohydrates were determined as described by Bentsink (2000), with minor modifications. Fifteen milligrams of freeze-dried and ground roots or cotyledons were transferred to a 2-mL Eppendorf tube and homogenized in 1 mL of methanol (80% v/v) with the addition of 10 µg of lactose as internal standard. Samples were incubated in a water bath for 15 minutes at 76°C. Samples were completely dried by vacuum centrifugation. Then, 500 µL of milliQ water was added, thoroughly vortexed and centrifuged for 5 min at 17000 g in an Eppendorf centrifuge. The supernatant was injected into a Dionex HPLC system (Dionex, Sunnyvale, CA) to analyze soluble carbohydrates, using a CarboPac PA100 4- x 250-mm column (Dionex) preceded by a guard column (CarboPac PA100, 4 x 50 mm), a gradient pump module (model GP40, Dionex) and an ED40-pulsed electrochemical detector (Dionex). Mono-, di-, and trisaccharides were separated by elution in an increasing concentration of NaOH (50–200 mM) with a flow rate of 1 mL per minute. Peaks were identified by co-elution of standards. Sugar quantity was corrected by means of the internal standard and transformed to micrograms of sugar per milligram of dry material.

Starch was determined as glucose, using an EnzyPlus glucose test kit (BioControl, art.nr. EZS 781+), based on NADPH + H^+ formation that is measured with a spectrophotometer at 340 nm. The remaining pellets from the previous carbohydrate analysis were used to quantify starch. Pellets were washed 3x with milliQ water in order to remove all remaining soluble carbohydrates. Samples were completely dried by vacuum centrifugation after the final washing step. Ten mg of dried material were weighed and homogenized with 50 µL 8N HCl and 200 µL DMSO. Samples were incubated in a water bath for 60 minutes at 60°C. After this 300 µL milliQ water and 80 µL 5 N NaOH were added, vortexed immediately, followed by addition of 370 µL citric acid (pH 4.60), thoroughly vortexed and centrifuged for 1 min at 17000 g. The clear supernatant was used for starch determination according to the instructions provided with the kit.

2.5 GC-MS data processing and compound identification

Initially, raw data were first processed by ChromaTOF software 2.0 (Leco Instruments), and further baseline correction, accurate mass calculation, data smoothing and noise reduction, followed by alignment between chromatograms were performed using the MetAlign software (Lommen, 2009). MSClust was used to remove metabolite signal redundancy in aligned mass peaks tables and to retrieve mass spectral information of metabolites using mass peak clustering (Tikunov et al., 2012). This resulted in a set of reconstructed metabolites (representative masses). The mass spectra of the representative masses were used for tentative identification by matching to the spectral libraries (National Institute of Standards and Technology [NIST08]; Golm metabolome database [http://gmd.mpimp-golm.mpg.de/]) and by comparison of the retention index calculated using a series of alkanes. Authentic reference standards were used to confirm the identity of the metabolites. Levels of identification are presented at Supplementary Tables S1 and S2 according to Summer (2007).

2.6 Multivariate statistical analysis

Mass intensity values of the representative masses were normalized by the internal standard and by the weight. Normalized and log transformed data were subjected to statistical analysis using Canoco 5 (http://www.canoco5.com/). In order to compare the overall variation in metabolite composition explained by differences in temperature, tissue and genotype, as well as to evaluate the importance of individual axes of constrained (Redundancy Analysis - RDA) and unconstrained ordination (Principal Component Analysis - PCA), we performed a “compared-constrained-unconstrained” analysis in Canoco 5. Temperature, tissue and genotype were used as explanatory variables.

Normalized data were also uploaded at MetaboAnalyst 2.0, a web-based analytical pipeline for high-throughput metabolomics studies (http://www.metaboanalyst.ca/MetaboAnalyst/) (Xia and Wishart,

2011). Row-wise normalization was performed to allow general-purpose adjustment for differences among samples. Log transformation and auto-scaling were performed to make features more comparable. Uni- and multivariate analysis were performed using log transformed and auto-scaled data. Metabolite-metabolite correlation analysis was also performed on the entire data set of metabolites using Pearson`s correlation.

3 Results

3.1 Growth and biomass production are strongly affected by environmental conditions in *Ricinus communis* seedlings

In order to explore the effect of temperature on *R. communis* seedling growth attributes we measured the dry weight of cotyledons, true leaves and roots of 14-day-old seedlings, grown at 4 different temperatures (20, 25, 30 and 35ºC) (Figure 1).

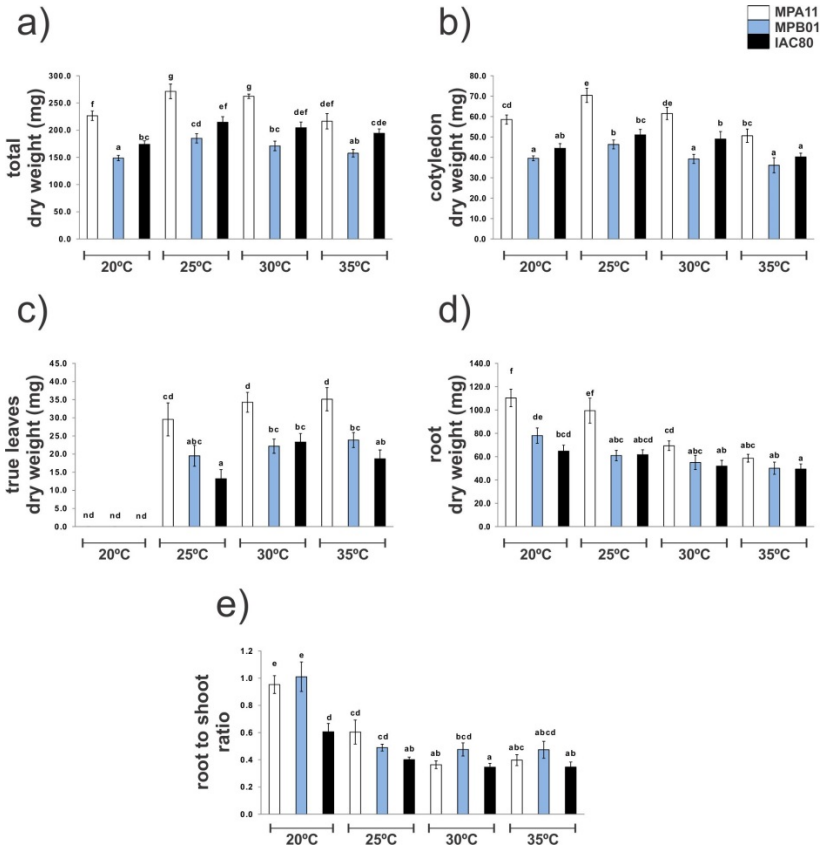

Figure 1. ***R. communis* seedling growth performance**. Total (a), cotyledon (b), first true leaves (c) and roots (d) dry weight and root to shoot ratio (e) of 14-day-old seedlings of three different genotypes, growing at 4 different temperatures (20, 25, 30 and 35ºC). Results for genotypes MPA11 (white bars), MPB01 (blue bars) and IAC80 (black bars) are shown.

For this purposes we used three different genotypes: MPA11, MPB01 and IAC80. The genotype MPA11 displayed a greater dry biomass which makes it clearly different from the other two genotypes. The lowest total biomass of the genotype MPA11 was observed at 20 and 35ºC and the highest at 25 and 30ºC. For genotypes MPB01 and IAC80 the lowest total biomass was observed at 20ºC and no differences in total biomass were observed between seedlings grown at 25, 30 and 35ºC (Figure 1a).

The cotyledon biomass for the genotype MPB01 and IAC80 showed minor changes in response to the temperature, while for genotype MPA11 the highest cotyledon biomass was observed at 25 and 30ºC (Figure 1b). The first pair of true leaves of seedlings growing at 20ºC could not develop and consequently the 14-day-old seedlings showed no pair of true leaves. The dry weight of the first pair of true leaves of the genotypes MPA11 and MPB01 was not significantly affected by the temperature, and for genotype IAC80 growing at 30ºC the first pair of true leaves was slightly heavier that those growing at 25 and 35ºC (Figure 1c).

For genotype MPA11, root biomass decreased with increasing temperature reaching the lowest value at 35ºC (Figure 1d). The root weight was negatively influenced by the temperature, apparently to the benefit of the aboveground part. On the other hand, root biomass of genotypes MPB01 and IAC80 was not appreciably affected by the temperature suggesting that the root of these genotypes are less sensitive to the temperature (Figure 1d). However, the decrease of the root to shoot ratio with temperature was consistent and appeared to be independent of the genotype (Figure 1e).

3.2 Changes in primary metabolite content in root and cotyledons in response to two different growth temperatures

In order to unravel metabolic changes associated with the temperature during seedling establishment we measured metabolite levels in roots and cotyledons of young seedlings growing at 20 and 35ºC. We detected over 100 peaks in root samples, of which 54 could be annotated, while in cotyledons samples we detected over 200 peaks of which 69 were annotated (Supplementary Table S1). Initially, redundancy analysis (RDA) was used to compare the overall variation in metabolite composition associated with temperature, tissue and genotype differences (Supplementary Figure S1). Principal component 1 discriminated root and cotyledon samples explaining nearly 94% of the total variance. Principal component 2 separated samples that were grown at 20 and 35ºC and explained only 2% of the total variance . The RDA plot shows that most of the variation in the metabolite composition must be attributed to differences between the tissues rather than between temperatures. Therefore, in order to assess whether the temperature has an effect on the metabolite composition of a given tissue, we decided to analyze cotyledon and root samples separately. When analyzed separately, for both cotyledon and root, principal component 1 discriminated samples that were grown at 20 and 35ºCwhile principal component 2

discriminated the natural variance contribution of the different genotypes. Based on the RDA analysis, genotypes MPB01 and IAC80 are closely related, whereas genotype MPA11 differed more in terms of metabolome. However, just a few metabolites appear to be responsible for this difference (Figure 2, Supplementary Table S3).

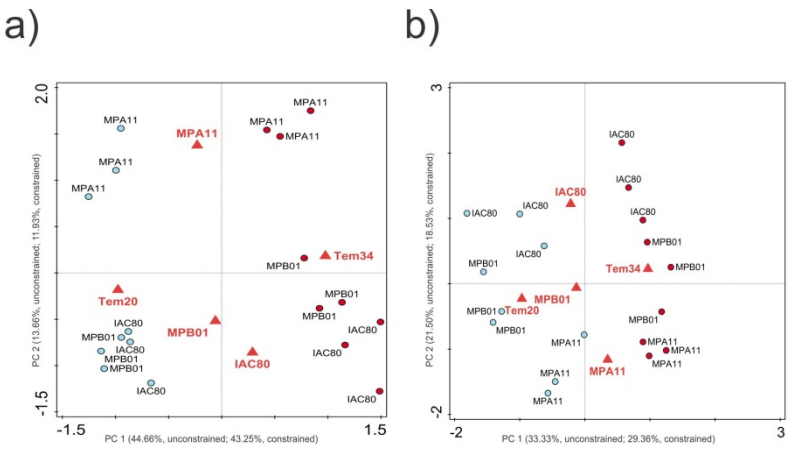

Figure 2. **Redundancy analysis based on polar metabolite profiles in response to an increase in temperature**. RDA plot in the cotyledons (a) and roots(b) based on polar metabolite profiles. The distance between genotypes approximates the average dissimilarity of metabolite composition between the two sample classes being compared as measured by their Euclidean distance, whereas the distance between replicates approximates the dissimilarity of their metabolite content as measured by their Euclidean distance. The distance of selected sample symbol (circles) from temperature and genotypes symbols (triangles) predicts the sample membership in one of the classes. It can also be seen as the dissimilarity between metabolite composition of that sample and average metabolite composition of samples belonging to individual classes. Score scaling is focused on standardized metabolites scores.

3.2.1 Overall changes in amino acid content in response to an increase in temperature

A total of 21 and 14 amino acids were identified in cotyledons and roots, respectively (Supplementary Table S1). In the RDA plots, they are strongly associated with the temperature of 35ºC, which is in agreement with the fact that the levels of almost all identified amino acids increased at 35ºC (Figure 3; Supplementary Figure S2). In the cotyledons, most of the amino acid increased 2 to 10-fold at 35ºC, however methionine, tyrosine and tryptophan showed a steeper increase, varying from 20 to 220 fold change. Statistically significant differences in methionine levels were only observed in the genotype IAC80 (53-fold) (Supplementary Table S4). Although glutamate content did not vary significantly, one of its biosynthetic derivatives, 4-aminobutyric acid (GABA) showed significant changes related to the temperature (FDR < 0.01). GABA increased 1.9-fold at 35ºC for genotype IAC80 (Supplementary Tables S1 and S4).

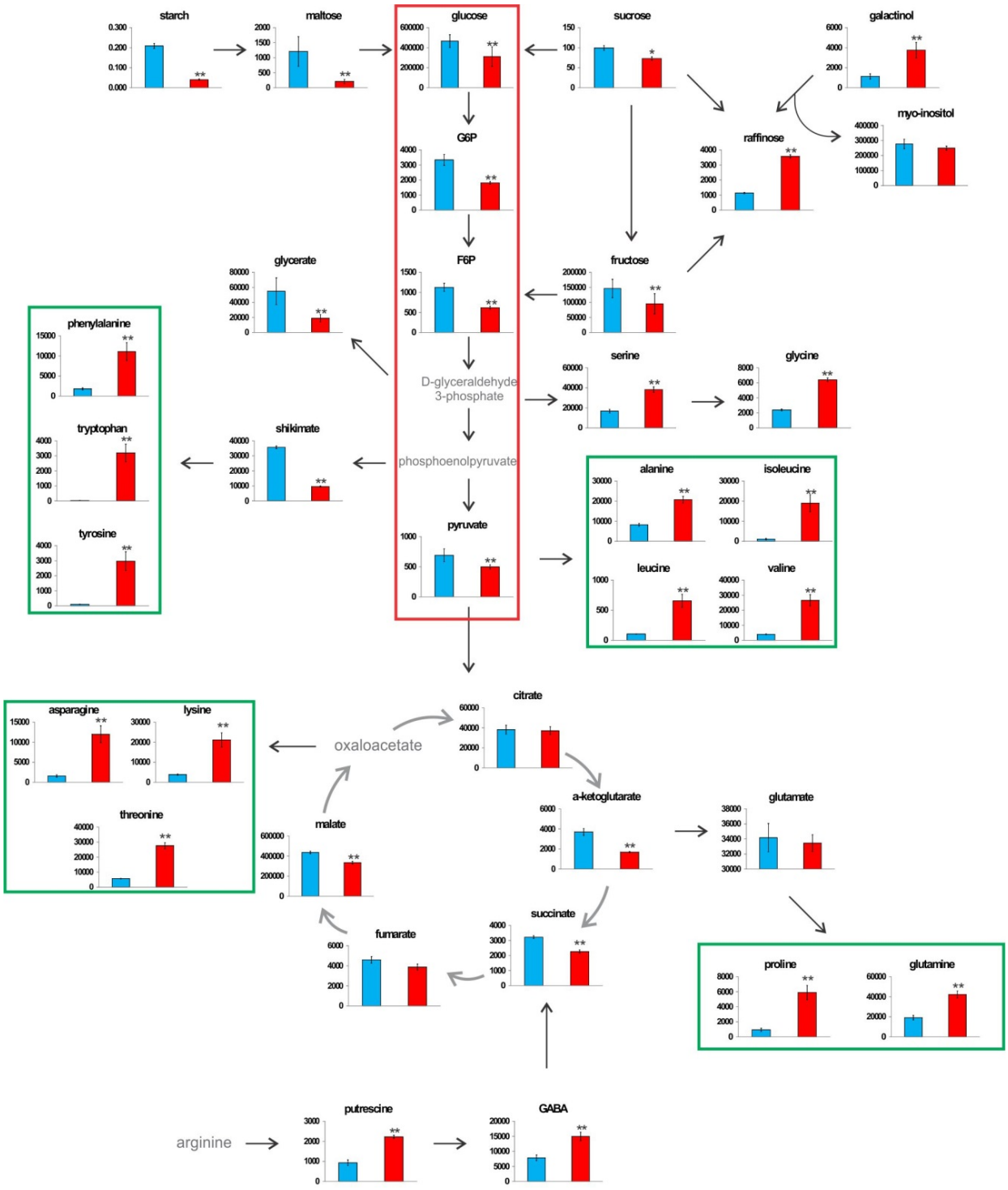

Figure 3. **Mapping of relative metabolite concentrations on known pathways for cotyledons of *Ricinus communis* genotype MPA11**. Seedlings were grown at 20ºC (blue columns) or at 35ºC (red columns). Data means and standard errors of three biological replicates containing 15-18 seedlings each are shown. Metabolites in grey (without column graphs) were not detected. Sucrose content was determined by HPLC. * $p < 0.05$ ** FDR < 0.01.

Amino acids were strongly and positively correlated with each other as well as with some sugars (raffinose and galactinol) and weakly correlated with some organic acids (fumarate and citrate). In addition, strong positive correlations were found for *β*-alanine, putrescine, vitamin E and other tocopherols, urea, 3-hydroxy-3-methylglutarate and ricinine (Figure 4).

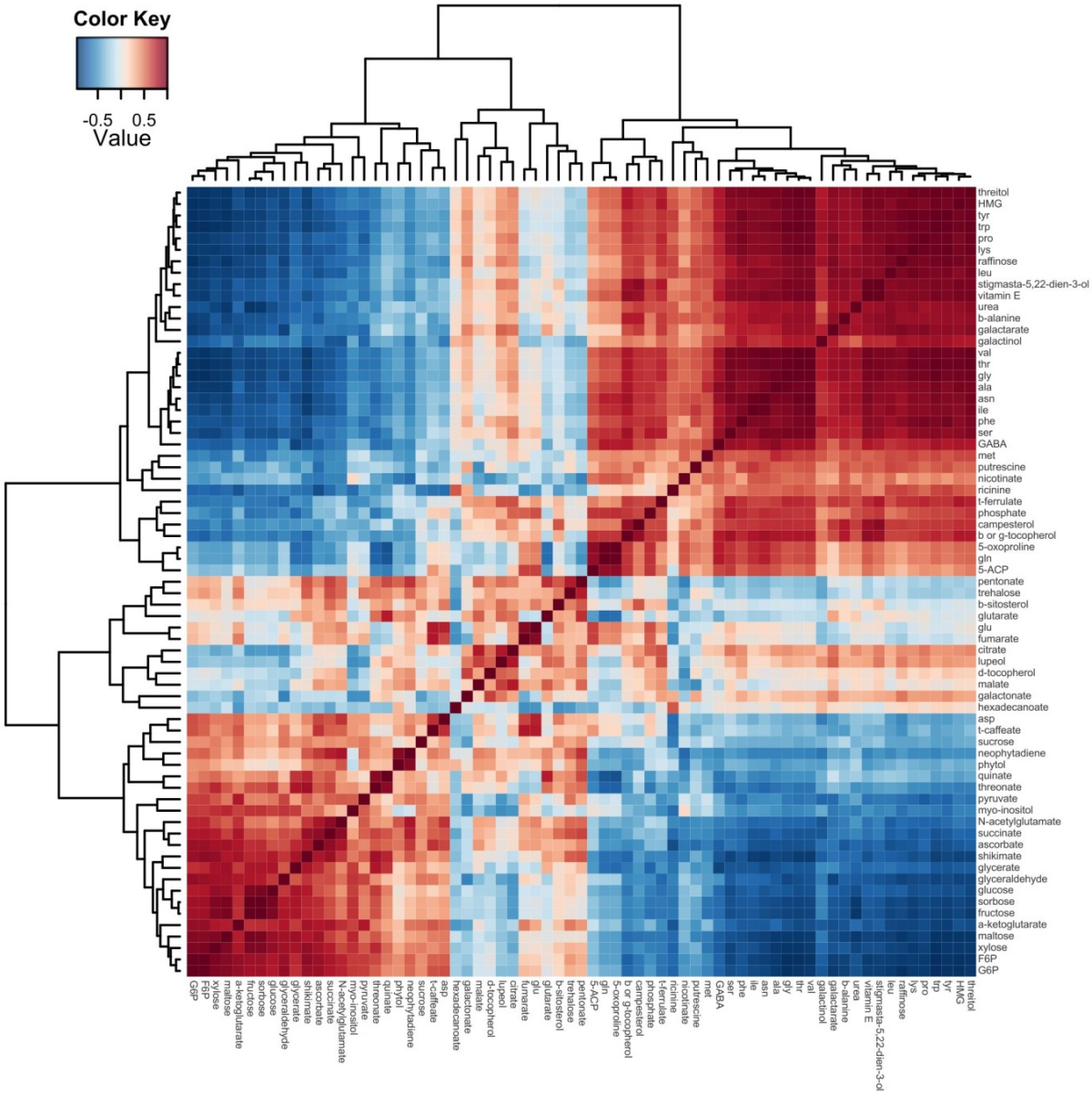

Figure 4. **Hierarchical cluster analysis**. Heatmap representation of the metabolite-metabolite correlations in response to the temperature treatment in cotyledon samples of three *R. communis* genotypes. Correlations coefficients were calculated based on Pearson`s correlation.

In the roots, much less variation in amino acid content was found (FDR < 0.01) in response to the temperature. Only 6 out of 14 of the detected amino acids were affected by the temperature (FDR < 0.01), but to a lesser extent than in cotyledons (Supplementary Table S1). Amino acids were strongly and positively correlated with each other and weakly correlated with some organic acids (fumarate and citrate). In addition, strong positive correlations were found with vitamin E and other tocopherols, urea, 3-hydroxy-3-methylglutarate and ricinine (Supplementary Figure S3).

3.2.2 Overall carbohydrate changes in response to an increase in temperature

The initial reduction in the levels of starch in cotyledons (up to 5.3 fold) in response to the increasing temperature (Figure 3) suggests that as soon as the seedlings are transferred to higher temperatures, carbohydrate mobilization and metabolism is up-regulated. In the roots, no starch could be detected. In both cotyledons and roots, RDA shows that carbohydrates are strongly associated with the temperature of 20ºC, which is in agreement with the fact that the levels of the majority of identified carbohydrates were higher at 20ºC (Figures 3; Supplementary Figure S2). Glucose, fructose, galactose, sorbose, sucrose and xylose levels decreased in cotyledons (1.3 to 3-fold), but to a much greater extent in the roots (5.8 to 20 times) at 35ºC (Supplementary Table S1). These carbohydrates were negatively correlated with amino acids, suggesting that a shift in the carbon-nitrogen metabolism occurs in response to the temperature change (Figure 4; Supplementary Figure S3). Interestingly, maltose was the only carbohydrate that decreased more in the cotyledons than in the roots at 35ºC. Raffinose and galactinol were the only carbohydrates that increased (up to 4-fold) at 35ºC in cotyledons and roots as compared to 20ºC (Supplementary Table S1) and they were negatively correlated with the others carbohydrates (Figure 4; Supplementary Figure S3).

3.2.3 Changes in tricarboxylic acid cycle (TCA) and glycolytic intermediates in response to an increase in temperature

Glycolysis and the TCA cycle are key metabolic routes by which organisms generate energy from carbohydrates, amino acids and fatty acids. TCA cycle and glycolytic intermediates were found to be affected by temperature (FDR < 0.01) and RDA suggests a close relationship among these compounds (Figure 3 and 4; Supplementary Figure S3). The glycolytic intermediates glucose-6-phosphate (G6P), fructose-6-phosphate and pyruvate were up to 3-fold reduced in the cotyledons of seedlings grown at 35ºC, but only G6P was detected in the roots and showed the same behavior as in the cotyledons. In both tissues, four TCA cycle intermediates were detected: fumarate, citrate, succinate and malate and α-ketoglutarate was detected in the cotyledons only. All TCA cycle intermediates detected in the roots showed a decrease (from 1.5 to 3.5-fold) at 35ºC; however, in the cotyledons only α-ketoglutarate and

succinate showed the same behavior. Fumarate, citrate and malate were slightly increased in the cotyledons (2-fold) at 35ºC (Figure 3; Supplementary Table S1). Metabolite-metabolite correlations of amino acids, glycolytic and TCA cycle intermediates confirm the strong relationship between these metabolic pathways (Figure 4).

3.3 Changes in contents of secondary metabolites in response to temperature increase

As an increase in ricinine content of growing Ricinus seedlings had been reported (Robinson, 1965), we decided to assess the effect of temperature on the relative amounts of ricinine and other secondary metabolites in roots and cotyledons of young seedlings. We detected over 100 peaks in root samples and over 50 peaks in cotyledon samples. Unfortunately, we could identify only 15 and 12 metabolites in roots and cotyledons, respectively (Supplementary Table S2). Except for IAC80 ricinine levels showed an increase in cotyledons was observed at 35ºC, while in roots it decreased (Figure 5). Some tocopherols were detected in both root and cotyledon samples and higher levels were detected in the seedlings grown at 35ºC (Supplementary Table S2). Phytosterols are steroid compounds similar to cholesterol which naturally occur in plants and play important roles in plant adaptation to temperature. Plant sterols are also involved in the regulation of temperature-driven membrane dynamics (Dufourc, 2008). In this study, several phytosterols were affected by the temperature. Levels of these compounds were more affected in roots compared to cotyledons. Campesterol, squalene and stigmasta-5,22-dien-3-ol levels increased at 35ºC, while *β*-sitosterol decreased (Supplementary Table S2).

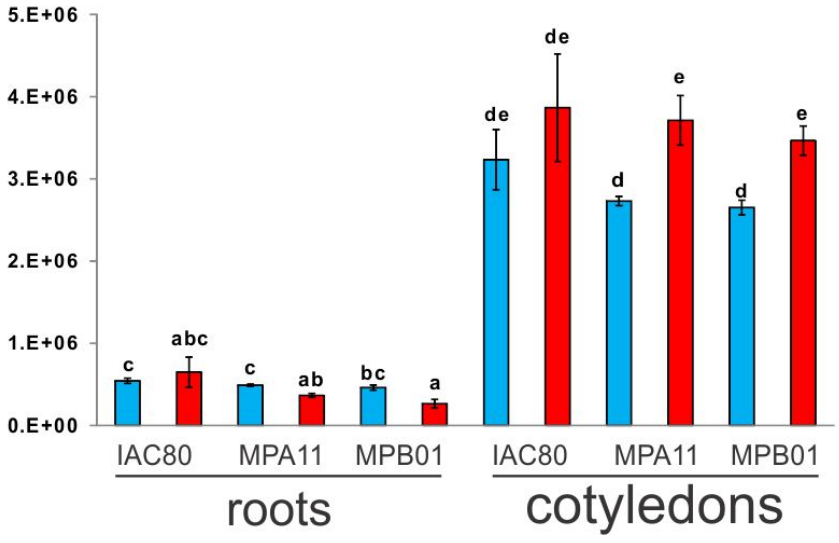

Figure 5. **Relative ricinine levels in root and cotyledon samples of *Ricinus communis***. Seedlings were grown at 20ºC (blue columns) or at 35ºC (red columns). Data means and standard errors of three biological replicates containing 15-18 seedlings each are shown.

3.4 Phenotypic and biochemical strategies of different genotypes to cope with the increase temperature

Genotypes MPA11 and MPB01 were developed by the breeding program of the *Empresa Baiana de Desenvolvimento Agrícola S.A* (EBDA-Brazil), while genotype IAC80 was developed by the breeding program of the Campinas Agronomic Institute. All three genotypes were produced aiming to recommend it to small family farms. MPB01 is a relatively small tree (reaching up to 1m), has a short flowering time (49 days) and produces high yields of seed (2500 kg/ha). MPA11 is 1.30 m high on average, displays a longer flowering time (96 days) and produces lower yields of seed (1900 kg/ha). IAC80 can reach up to 3 m high, has a long life cycle (240 days) and its yield can vary from 1.500 to 4.000 kg/ha. Genotype MPA11 could be clearly differentiated, both phenotypically and biochemically, from genotypes MPB01 and IAC80. Biomass production of genotype MPA11 was far higher than for the other two genotypes at all temperatures. This phenotypic observation was confirmed at the biochemical level. The metabolomes of the genotypes MPB01 and IAC80 were more similar to each other than to the metabolome of MPA11 (Figure 2). Little variation on the metabolite profile due to genotypic differences was observed between genotypes MPB01 and IAC80. Only four metabolites varied significantly between these two genotypes: malate, N-acetylglutamate, sucrose and leucine. In the cotyledons, leucine levels at 20ºC were higher for genotype MPB01 than for genotype IAC80, while no differences were observed at 35ºC. In the roots, sucrose levels at 20ºC were higher for genotype MPB01 than for genotype IAC80, and at 35ºC N-acetylglutamate increased and malate decreased in the genotype MPB01 (Supplementary Table S1 and S3).

Comparisons between genotype MPA11 and genotypes MPB01 and IAC80 revealed considerably higher significant variation. The levels of some amino acids were reduced in root samples of genotype MPA11 while the levels of most of the identified organic acids increased in the cotyledon samples when compared to the other two genotypes. Thus, increased levels of organic acids in the cotyledons, especially those belonging to the TCA cycle, and reduced levels of amino acids in the roots seem to be an important trait for faster and more efficient growth of genotype MPA11 (Supplementary Table S1 and S3).

4 Discussion

4.1 Growth and biomass production are strongly affected by an increase in temperature in *R. communis* seedlings

So far, metabolite profiling studies in *R. communis* are limited to two studies describing the use of LC-MS, HPLC-UV and ^{1}H NMR-based methodologies to reveal differences in the seed metabolome that could be used to characterize both provenance and cultivar (Ovenden et al., 2010; Pigott et al., 2012). In the first study, the strongest contributions to the differences in cultivar and provenance were associated

with sucrose, phenylalanine, ricinine, *N*-demethyl and *O*-demethyl ricinine (Pigott et al., 2012). In the second study, the ratio between ricinine and *N*-demethyl or *O*-demethyl ricinine was found to be important for specimen determination (Ovenden et al., 2010). The effect of temperature on plant growth, especially under stress conditions, has been extensively studied (Booth and Bai, 1999; Dufourc, 2008; Guy et al., 2008; Kaplan et al., 2004; Tacarindua et al., 2013). However, there is still a lack of studies that correlate physiological events, such as germination or seedling establishment, with metabolic changes under different environmental conditions. We hereby present a first large scale metabolite profiling study in *R. communis* genotypes that were produced by breeding programs as temperature/drought resistant varieties for family farmers of the semi-arid region of Brazil. This report adds new insights to the understanding of biomass allocation and adaptation to different temperatures during seedling establishment.

Ricinus communis showed high plasticity in response to changes of the environmental conditions during initial vegetative growth. It appears that an increasing temperature leads to preferred growth of the stem and true leaves, at the expense of cotyledons and roots. Consequently, a reduction of the root to shoot ratio was observed, which indicates that seedlings are experiencing improved growing conditions. This reduction is also observed as a result of favorable weather, fertilization, irrigation, aeration, or pest control (Harris, 1992). This growth preference is correlated with a shift from carbon to nitrogen metabolism.

Under drought stress conditions, plants usually produces more root biomass, increasing their root to shoot ratio, as an important trait to acquire drought tolerance (Wittenmayer and Merbach, 2005). In the light of a usual concomitance of drought with increased temperature, it is surprising that *R. communis* does not invest in its root system in our experiments, unless root growth is not stimulated by high temperature but predominantly by drought. This would be in agreement with the fact that under the prevailing (laboratory) growth conditions there was no drought.

4.2 *R. communis* seedlings adjust their metabolism in order to support growth at elevated temperature, making this species a good candidate for crop production in the lowland tropics

A common feature shared by several temperature-responsive metabolite profiling studies to date is the fact that carbohydrate and amino acid metabolisms seem to be key responsive elements of plasticity and tolerance mechanisms (Cook et al., 2004; Diamant et al., 2001; Guy et al., 2008; Kaplan et al., 2004; Obata and Fernie, 2012; Panikulangara et al., 2004; Rizhsky et al., 2004). Carbohydrates may act as nutrients, osmotic regulators, or signalling molecules through their interaction with phytohormonal networks (Matsoukas et al., 2013). Both heat shock and cold acclimation lead to a coordinated increase in the content of amino acids, TCA intermediates (fumarate and malate), amine-containing metabolites (*β*-alanine, GABA, and putrescine) and some carbohydrates, such as maltose, sucrose, raffinose, galactinol and myoinositol (Kaplan et al., 2004; Rizhsky et al., 2004). Therefore, an indistinct and unidirectional

increase in the content of most of the identified metabolites seems to be the main response to a variety of environmental stimuli. Additionally, temperature shock-responsive metabolites did not seem to be specific to one particular phase in the development of acquired tolerance (Kaplan et al., 2004).

Ricinus communis showed high plasticity during initial vegetative growth, which was also reflected in the metabolome of the seedlings. An increase in the temperature did not lead to an indiscriminate accumulation of the identified metabolites. Instead, a shift in their carbon-nitrogen balance was observed, in order to adjust development and growth at higher temperature. In *R. communis* seedlings carbohydrate levels were reduced in response to an increasing temperature in both roots and cotyledons. Starch is produced by most green plants as an energy storage compound, which is degraded to produce maltose and glucose by β-amylases (BAM) and disproportionating enzyme (DPE1) in the chloroplast (Critchley et al., 2001; Lao et al., 1999). The reduction of starch levels in response to an increase in the temperature suggests that carbohydrate catabolism is triggered when seedlings are experiencing higher temperatures. Starch catabolism-derived signaling has been associated with the juvenile-to-adult phase transition during normal growth and development (Matsoukas et al., 2013). Glucose is a key metabolite of many plants, animals, and microorganisms, as an extraordinarily versatile precursor, capable of supplying a vast array of metabolic intermediates for biosynthetic reactions. Glucose, glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) and pyruvate levels are reduced in response to the increase in the temperature indicating that downstream reactions in glycolytic pathway may be up-regulated at 35ºC. Transcript levels of several enzymes of the glycolytic and alcohol fermentation pathways were examined in rice seedlings and it was reported that enzymes in this pathway have a sufficient degree of flexibility to adjust to increased energy demands and supply of intermediates for acclimatizing to higher temperatures (Minhas and Grover, 1999). The observation of a variation in glycolytic and TCA cycle intermediates in this study confirms the strong relationship between glycolysis and the TCA cycle and indicates that, in response to an increase in temperature, *R. communis* seedlings activate catabolic pathways to generate energy through the oxidization of acetate. Taken together, we conclude that an increase in temperature triggers the mobilization of carbohydrates from the roots, which are then transported through the hypocotyl to the aerial parts, leading to preferred growth of the true leaves, apparently at the expense of the roots (Figure 6).

The carbon skeleton backbones used for amino acid biosynthesis in plants are derived from organic acids produced in glycolysis, the oxidative pentose phosphate pathway and the TCA and Calvin cycles. Amino acids were mostly increased in the cotyledons of young *R. communis* seedlings, whereas in the roots just a few amino acids were identified, but decreasing in content, at elevated temperature. Here, we observed that a decrease in shikimate levels is associated with an increase in its amino acid derivatives tryptophan, tyrosine and phenylalanine as a result of an increasing temperature. In plants, these amino

acids are precursors for the production of several important compounds such as phytohormones, electron carriers, enzyme cofactors and antioxidants (tocopherols). *R. communis* seedlings seem to shift their metabolic pool from carbohydrates to amino acids as a dominant biochemical response to adjust growth and developmental processes to the higher temperatures and, likely, to maintain cellular homeostasis. Apparently, fluxes of *R. communis* carbohydrate and amino acid metabolic pathways increase as the increase in temperature increase respiration and growth rate. However, not all crops are able to cope with such high temperatures as 35ºC or higher (Wheeler et al., 2000). In fact, most crops show a decrease in total dry matter and seed yield when experiencing increased temperatures (Tacarindua et al., 2013). This makes *R. communis* a good candidate for crop production in the lowland tropics.

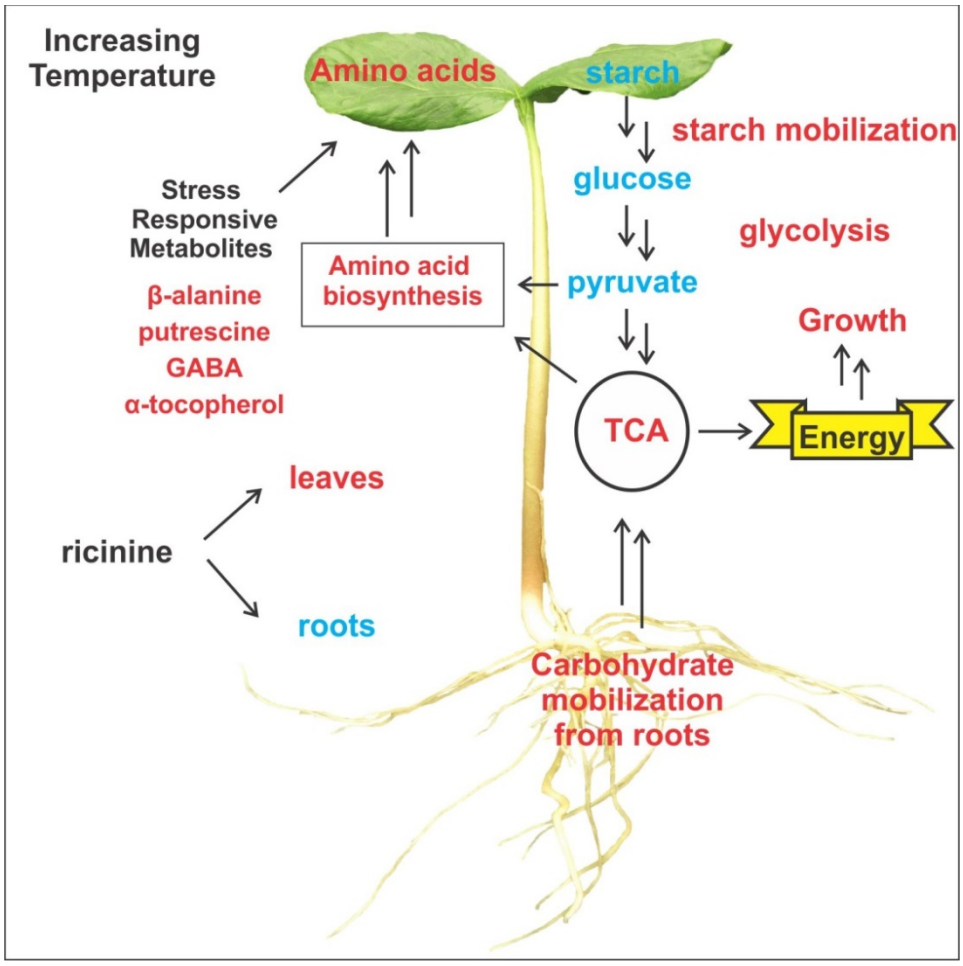

Figure 6. **Schematic changes in *Ricinus communis* seedlings in response to an increasing temperature**. Compounds highlighted in blue show a decrease in response to an increased temperature, while compounds highlighted in red had increased. Pathways highlighted in red are assumed to be up-regulated by the increasing temperature

4.3 *R. communis* seedlings prevent oxidative damage by enhancing the biosynthesis of osmoprotectants molecules and ricinine with increasing temperature

Raffinose family oligosaccharides (RFO) are known to act as osmoprotectants in plant cells, in which high levels of galactinol and raffinose are associated with increased tolerance to stress and are implicated in membrane protection and radical scavenging (Nishizawa-Yokoi et al., 2008; Nishizawa et al., 2008). RFO are also produced in response to elevated cellular metabolic rate, which is known to produce reactive oxygen species (ROS) as natural byproducts. ROS are highly reactive causing damage to proteins, lipids, carbohydrates and DNA (Gill and Tuteja, 2010).

Polyols can act as stabilizing macromolecules and as effective hydroxyl radical scavengers, thereby preventing oxidative damage of membranes and enzymes (Smirnoff and Cumbes, 1989). Galactinol is produced from UDP-galactose and myo-inositol by galactinol synthase. This explains why myo-inositol levels were reduced at 35ºC, whereas galactinol content increased.

Alpha-tocopherol levels increased in the cotyledons in response to the temperature treatment, but no variation was found in the roots. Delta- and *β*-tocopherol also showed an increase, but to a lesser extent. Tocopherols have many biological functions of which their antioxidant function is the most important and best known. It is generally assumed that increases of α-tocopherol contribute to protect the plant against oxidative damages (Munne-Bosch, 2005). Taken together these results suggest that raffinose, galactinol and tocopherols may function to protect *R. communis* cells from oxidative damage caused by the increase in temperature.

Ricinine is an alkaloid with an α-pyridone ring found in *R. communis* seeds, seedlings and yellow cotyledons (Holfelder et al., 1998; Waller and Negi, 1958), which is involved in the senescence of cotyledons (Lee and Waller, 1972). In the early stages of senescence, ricinine is degraded to N-demethylricinine and O-demethylricinine (Lee and Waller, 1972) and at the end of the senescence neither ricinine nor ricinine derivatives can be detected because they are translocated to young healthy growing tissue via the phloem (Holfelder et al., 1998; Waller and Negi, 1958). Ricinine also plays an important role in defense mechanisms against phloem feeders such as aphids (Olaifa et al., 1991). Apart from a possible contribution to defense mechanisms, ricinine may play a role in engaging or modifying primary metabolism, which may be an important prerequisite for successful seedling establishment and adaptation. Ricinine content in genotype IAC80 showed no variance related to temperature, both in the roots and in the cotyledons. However, for genotypes MPB01 and MPA11 an increase in ricinine content in the cotyledons was observed at 35ºC, while in the roots ricinine content decreased at 35ºC. Therefore, ricinine levels also reflect differences in the seed/seedling metabolome that may be associated with growth rate under different environmental conditions.

5 Conclusion

By employing a GC-TOF-MS metabolite profiling approach this study has clearly shown that carbohydrate and amino acid metabolism are tightly coordinated and that they are strongly affected by the temperature during *R. communis* seedling establishment. Due to an increase in temperature, seedlings trigger carbohydrate catabolic pathways as we can infer from the reduced carbohydrate content observed in both roots and cotyledons. Starch is mobilized providing glucose for further utilization in the glycolytic pathway and TCA cycle. Glucose oxidation through glycolysis provides energy and also precursors for amino acid biosynthesis. Carbohydrates are assumed to be mobilized from the roots, through the hypocotyl, to the aerial parts leading to preferred growth of the true leaves, at the expense of the roots. In this paper we raised the question whether this species may have a metabolic signature that may apply to other crops, as well. Here we report that as a result of an increasing temperature, young *R. communis* seedlings show a shift in their carbon-nitrogen metabolism as the main biochemical response to adjust growth and developmental processes to higher temperatures and likely to maintain cellular homeostasis. These observations are extremely important for crops that experience varying environmental conditions during initial vegetative growth, because most crops are not able to cope with temperatures of 35ºC or higher, which normally leads to a reduction of biomass production and yield.

Although all three genotypes showed similar responses to the temperatures, we showed that increased levels of organic acids in the cotyledons and reduced levels of amino acids in the roots might contribute to faster and more efficient growth of genotype MPA11. These findings provide insight into the mechanisms underlying temperature tolerance and adaptation during seedling establishment. Furthermore, the metabolic changes observed in response to the increasing temperature provide leads into plant adaptation to varying environmental conditions, which would be very helpful in developing strategies for *R. communis* crop improvement research.

6 Authors' contributions

PRR carried out the physiological and metabolite profiling experiments, data processing, statistical analysis and draft of the manuscript. LGF, RDC, WL and HWMH participated in the design of the study, coordination and critical reading of the manuscript. All authors read and approved the final manuscript.

7 Conflict of interest

The authors declare no conflict of interest.

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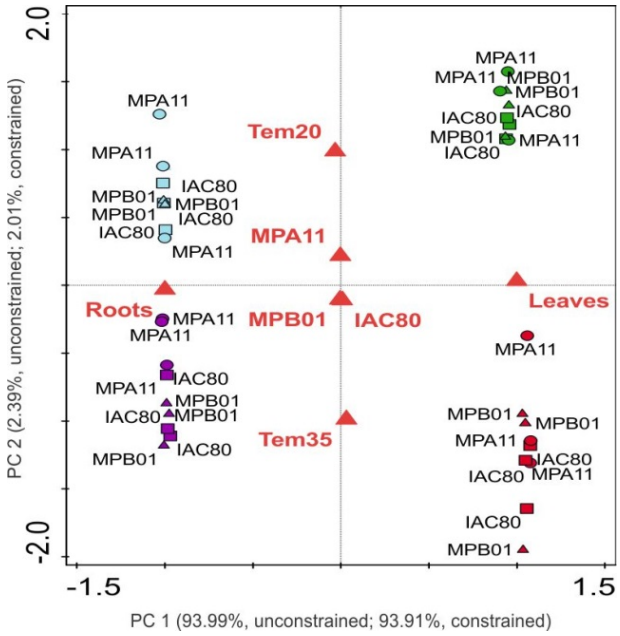
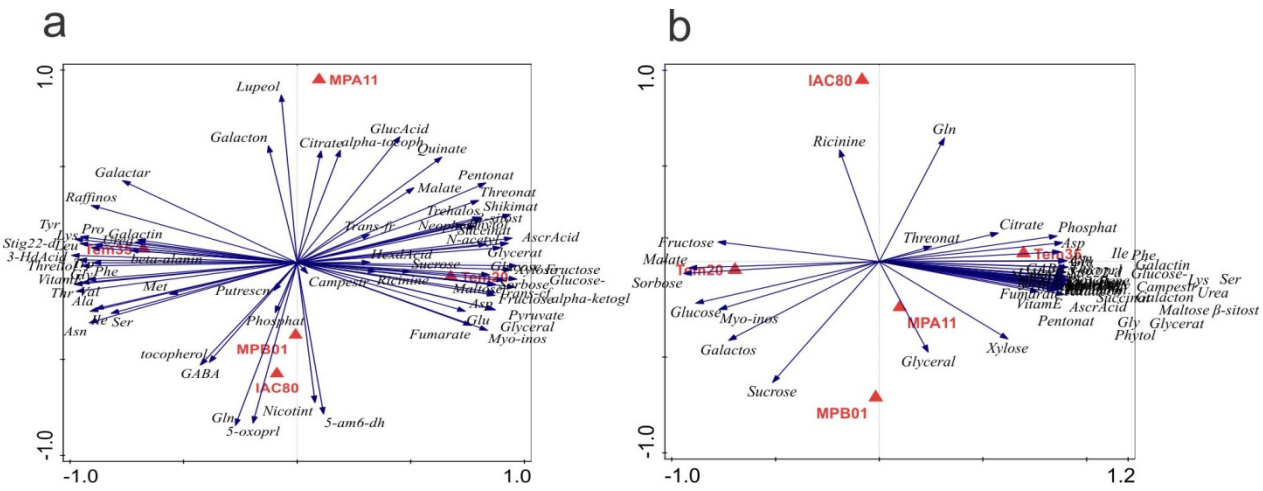
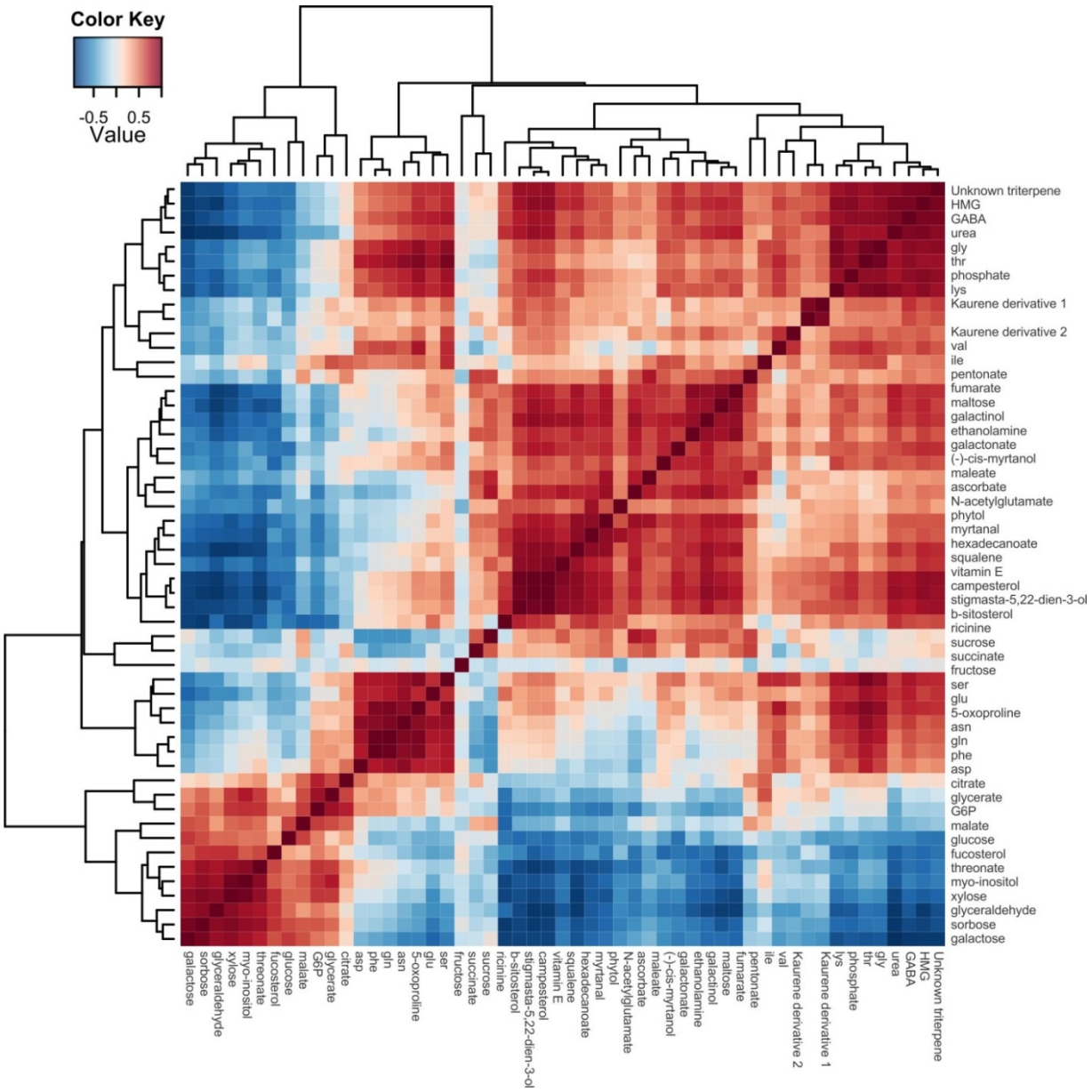
9 Supplementary figures



Supplementary Figure S1 **Redundancy analysis based on polar metabolite profiles in response to an increase in temperature**. The distance between genotypes approximates the average dissimilarity of metabolite composition between the two sample classes being compared as measured by their Euclidean distance, whereas the distance between replicates approximates the dissimilarity of their metabolite content as measured by their Euclidean distance. The distance of selected sample symbol (circles) from temperature and genotypes symbols (triangles) predicts the sample membership in one of the classes. It can also be seen as the dissimilarity between metabolite composition of that sample and average metabolite composition of samples belonging to individual classes. Score scaling is focused on standardized metabolites scores.



Supplementary Figure S2. **Redundancy analysis based on polar metabolite profiles in response to an increase in temperature**. summarizes the variation in metabolite content that is explained by the increase in temperature and differences between genotypes in the (a) leaves and (b) roots.



Supplementary Figure S3. **Hierarchical cluster analysis in root samples**. Heatmap representation of the metabolite-metabolite correlations in response to the temperature treatment in root samples of three *R. communis* genotypes. Correlations coefficients were calculated based on Pearson`s correlation.

10 Supplementary Table Captions

Supplementary Table S1. Primary metabolites in *R. communis* cotyledon and root samples.

Supplementary Table S2. Secondary metabolites in *R. communis* cotyledon and root samples.

Supplementary Table S3. Comparison of metabolite content in different *R. communis* genotypes.

Supplementary Table S4. Significance of metabolite variation in cotyledons and roots of *R. communis.*

Supplementary tables can be found at http://www.wageningenseedlab.nl/thesis/prribeiro/SI/

Chapter 6

Effect of temperature on biomass allocation in seedlings of two contrasting genotypes of the oilseed crop *Ricinus communis*

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Abstract

Ricinus communis is becoming an important crop for oil production, and studying the physiological and biochemical aspects of seedling development may aid in the improvement of crop quality and yield. The objective of this study was to assess the effect of temperature on biomass allocation in two *R. communis* genotypes. Biomass allocation was assessed by measuring dry weight of roots, stems, and cotyledons of seedlings grown at three different temperatures. Root length of each seedling was measured. Biomass allocation was strongly affected by temperature. Seedlings grown at 25ºC and 35ºC showed greater biomass than seedlings grown at 20ºC. Cotyledon and stem dry weight increased for both genotypes with increasing temperature, whereas root biomass allocation showed a genotype-dependent behaviour. Genotype MPA11 showed a continuous increase in root dry weight with increasing temperature, while genotype IAC80 was not able to sustain further root growth at higher temperatures. Based on metabolite and gene expression profiles, genotype MPA11 increases its level of osmoprotectant molecules and transcripts of genes encoding for antioxidant enzymes and heat shock proteins to a higher extent than genotype IAC80. This might be causal for the ability to maintain homeostasis and support root growth at elevated temperatures in genotype MPA11.

Keywords: antioxidant enzymes, heat stress, metabolite profiling, phenotypic plasticity, seedling performance

1 Introduction

Ricinus communis L. is widespread throughout tropical, sub-tropical and warm temperate regions (Santiago et al., 2010; Scarpa and Guerci, 1982). Commercial interest in this species is increasing because its seeds contain high amounts of oil consisting of up to 94% of the fatty acid ricinoleic acid (Gong et al., 2005). This oil is mainly used for pharmaceutical and industrial applications (Severino et al., 2012). *R. communis* grows in dry and hot environments, where most other crops would not grow, and still displays good yield (Vijaya Kumar et al., 1997). Further investigation of its growth and development in stressful environments, however, is necessary to develop high-yielding varieties adapted to various environmental conditions.

Temperature is a critical environmental cue that affects seedling establishment. Differences of only a few degrees lead to a notable change in growth and survival rate (Salisbury and Ross, 1985). Yield losses up to 16% are expected, in tropical and subtropical regions, for every 1°C increase in seasonal temperatures (Lobell et al., 2008). Understanding the complexities of heat tolerance mechanisms is, therefore, becoming increasingly important to manipulate thermotolerance in plants. The effect of low or high temperature on seedling performance and development has been assessed for several species. *Phaseolus vulgaris* seedlings growing at low temperatures (10ºC) showed a strong delay in root growth (Badowiec and Weidner, 2014). Maize seedlings grown at 37°C showed reduced root growth compared with plants grown at 28°C (Trachsel et al., 2010). High temperature reduced the shoot and root dry weight, chlorophyll content and membrane stability index in wheat plants (Gupta et al., 2013). *R. communis*, however, possesses the ability to grow in various diverse environments. This species, therefore, could provide a better understanding of seedling performance and adaptation under different temperatures.

Metabolite profiling of plants grown under different environmental conditions has unravelled biochemical and molecular mechanisms related to temperature adaptation. Adaptation mechanisms of plants to diverse environmental conditions involve several stress-responsive molecules, such as γ-aminobutyric acid (GABA), β-alanine, and putrescine (Kaplan et al., 2004; Ribeiro et al., 2014b; Ribeiro et al., 2015a; Rizhsky et al., 2004). GABA is a non-protein amino acid that is accumulated in response to a number of environmental stresses in plants (Kinnersley and Turano, 2000). Additionally, two nitrogen-containing metabolites, β-alanine and putrescine, appeared to accumulate upon heat stress (Guy et al., 2008). Metabolomics has been used to dissect plant responses to abiotic stresses, however, most studies have focused on the ability of plants to maintain homeostasis at chilling temperatures (0ºC to 15ºC) or have investigated plant responses to high-temperature stress, mostly using Arabidopsis as model species (Arbona et al., 2013; Guy et al., 2008; Obata and Fernie, 2012). Plant metabolic plasticity in response to

mild temperatures (20ºC to 35ºC) has received much less attention although it is an important trait for crop species (Lewicka and Pietruszka, 2006).

Uncontrolled generation of reactive oxygen species (ROS) is one consequence of high-temperature stress. ROS are usually generated via aerobic respiration in mitochondria, photosynthesis in chloroplasts, and photorespiration in peroxisomes (Foyer and Noctor, 2005; Gill and Tuteja, 2010). Plants have evolved efficient antioxidant systems that scavenge ROS and thereby avoid cellular damages. Antioxidant systems can be divided into two groups: small antioxidants molecules (De Tullio and Arrigoni, 2003; Munné-Bosch, 2005) and antioxidant enzymes (Jaleel et al., 2009). Antioxidant enzymes are the most active and efficient protective mechanism against oxidative stress. Enhanced or altered expression of antioxidant enzymes is reported in response to abiotic stresses (Rizhsky et al., 2004). Knowledge of the antioxidant system at the molecular level could, therefore, provides valuable information to understand heat-tolerance mechanisms. A better understand of these mechanisms can potentially lead to improved breeding strategies.

We addressed the question how temperature affects *R. communis* biomass allocation, especially to the root system. Our results provide leads for the understanding of the underlying mechanisms that are not only required to support vigorous seedling growth, but also for adaptation to harsh environmental conditions in semi-arid areas worldwide.

2. Material and methods

2.1 Plant material and germination conditions

Seeds were surface sterilized with 1% NaClO for 20 min and rinsed five times with distilled water. Seeds were germinated using paper roll as substrate at 25°C in the dark. Germinated seeds (72 h) were transferred to three different temperatures (20°C, 25°C and 35°C) and allowed to grow for seven days. Biomass allocation was assessed by measuring dry weight of roots, stems, and cotyledons of seedlings. Root length of each seedling was measured. Roots were collected, immediately frozen in liquid nitrogen, freeze-dried, ground, and stored at -80ºC.

2.2 GC-TOF-MS analysis

Primary metabolites were analyzed by gas chromatography coupled to a quadrupole time of flight mass spectrometry system (GC-TOF-MS) as TMS derivatives following Ribeiro et al. (2014b). Approximately 20 mg of freeze-dried roots were used. Ribitol (1 mg/mL) was used as internal standard. All analyses were performed on three biological replicates of 10 seedlings each.

2.3 GC-MS data processing and compound identification

Data processing and compound identification were performed following Ribeiro et al. (2014b). Metabolite identification was performed by matching the mass spectra of the representative masses to the spectral libraries and by comparing retention index calculated using a series of alkanes. Authentic reference standards confirmed the identity of the metabolites. Levels of identification (Sumner et al., 2007) are presented in Supplementary Table S1.

2.4 Carbohydrate analysis

Carbohydrates were determined following Ribeiro et al. (2014b). Samples were injected into a Dionex HPLC system (Dionex, Sunnyvale, CA) to analyze soluble carbohydrates using a CarboPac PA100 4- x 250-mm column (Dionex) preceded by a guard column (CarboPac PA100, 4 x 50 mm), a gradient pump module (model GP40, Dionex) and followed by an ED40-pulsed electrochemical detector (Dionex). Approximately 20 mg of freeze-dried seeds were used. Melezitose (40 µg/mL) was used as internal standard. Authentic reference standards confirmed the identity of the metabolites. All analyses were performed on three biological replicates of 10 seedlings each.

2.5 Uni- and multivariate statistical analysis

Normalized data were uploaded at MetaboAnalyst 2.0; a web-based analytical pipeline for high-throughput metabolomics studies (Ribeiro et al., 2014b; Ribeiro et al., 2015a; Xia and Wishart, 2011). Row-wise normalization allowed general-purpose adjustment for differences among samples. Log transformation and auto-scaling allowed comparison of features. Multivariate analyses were performed using log transformed and auto-scaled data. ANOVA was performed to assess overall variation in metabolite levels, followed by post-hoc analyses (Bonferroni correction, FDR < 0.05).

2.6 Protein extraction and quantification

Approximately 100 mg of freeze-dried and ground roots were transferred to a 2-mL Eppendorf tube after which 500 µL of the extraction buffer were added and vortexed till the powder was completely homogenized. Extraction buffer consisted of 50 mM HEPES pH 8.0, 1 mM EDTA, 0.5 M NaCl, 1 tablet of protease cocktail (Roche), 40 µL DNase(10 U/µL) and 10 µL RNase(10 U/µL) per 10 mL extraction buffer. Samples were kept on ice for two hours. Then, samples were centrifuged for 20 min at 13000 g. The clear supernatant was collected and transferred to new sterile Eppendorf tube and kept on ice. Protein quantification was performed using the BIORAD DC kit according to the manufacturer's instructions. Absorbance was measured in a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices)

at 750 nm. A standard curve ranging from 0 to 2 mg/mL of bovine serum albumin was used to calculate the absolute concentration in the samples. All analyses were performed on three biological replicates of 10 seedlings each.

2.7 Determination of antioxidant enzyme activities

In order to determine antioxidant enzyme activities, 40 mg of root sample were homogenized in 1 ml of chilled 100 mM K- phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1 % (v/v) Triton X-100 and 2 % (w/v) polyvinyl pyrrolidone (PVP) (Bhoomika et al., 2013; Madhava Rao and Sresty, 2000). The homogenate was filtered and centrifuged at 12 000 *g* for 10 min. The supernatant obtained was diluted 100x in H_2O and used as enzyme extract for follow-up experiments. Protein quantification was performed on these samples using the BIORAD DC kit according to the manufacturer's instructions.

Catalase (CAT) activity was determined according to the method of Beers Jr and Sizer (1952). The reaction was done in 200 µL containing 200 mM phosphate buffer (pH7.0), 12.5 mM H_2O_2, and 1 µL of enzyme extract. Decomposition of H_2O_2 was recorded as decrease in absorbance at 240 nm using a UV-VIS spectrophotometer. Enzyme specific activity was expressed as µmol H_2O_2 reduced/min mg of total protein.

Total superoxide dismutase (SOD) activity was determined using the method of Beauchamp and Fridovich (1971) based on the inhibition of photochemical reduction of p-nitro blue tetrazolium chloride (NBT) by $O_2^{\cdot-}$. The 200 µl reaction mixture contained 40 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium, 2 mM riboflavin, 0.1 mM EDTA and 1 µL of enzyme extract (Madhava Rao and Sresty, 2000). The reaction was started by placing samples below a light source consisting of two 15-W fluorescent lamps. Activity of Mn SOD was determined by incubating the enzyme extracts separately with 5 mM H2O2 for 30 min at 4ºC prior to the assay. The reaction was allowed to take place for 15 min and was stopped by switching off the light.The absorbance of the reaction mixture was measured at 560 nm. The non-irradiated reaction mixture was used as control. Enzyme specific activity was expressed as one unit of SOD activity (U) per mg total protein. One unit of SOD activity is expressed as the amount of enzyme required to cause 50 % inhibition of NBT reduction under the experimental conditions.

2.8 RNA extraction, DNase digestion, RNA quality control and cDNA synthesis

Total RNA was extracted from 20 mg of dry material using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNeasy Plant Mini Kit includes the RNase-Free DNase Set

(Qiagen), which was used for digestion of genomic DNA. RNA quantification and quality control were performed following Ribeiro et al. (2014a). First strand cDNA was synthesized from 1 µg of total RNA using the iScriptTM cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Reverse transcription was performed at 37°C for 40 minutes followed by 5 min at 85°C. cDNA was diluted 20 times and stored at -20°C prior to further analysis by RT-qPCR.

2.9 Target gene selection, primer design and analysis of amplification efficiency

Genes encoding for four antioxidants enzymes were selected: catalase (*RcCAT1* and *RcCAT2*), superoxide dismutase (*Rc Cu-Zn SOD1* and *Rc Cu-Zn SOD2*), glutathione reductase (*RcGR1* and *RcGR2*), peroxidase 21 precursor (*RcPER21*), and peroxidase 63 precursor (*RcPER63*). Additionally, genes encoding for two heat shock proteins (*RcHSP1* and *RcHSP2*) were selected. Primers with melting temperatures of 58°C–62°C, lengths of 18–22 bp and amplicon lengths of 80-200 bp were designed using CLCbio software (CLC bio, Aarhus, Denmark). Amplification efficiency was evaluated based on a two-fold serial dilution series of a pooled cDNA. Primer specificity was verified by separating the products on a 2.5% agarose gel and melting curve analysis. Actin (*RcACT*) and Pob (*RcPOB*) were used as reference genes (Ribeiro et al., 2014a). Gene identifier number (GI), gene name, gene description, amplicon length, primer efficiency, and primer sequences are listed in Supplementary Table S2.

2.10 RT-qPCR conditions

RT-qPCR was performed in a total volume of 10 µL containing 2.5 µL of cDNA, 0.5 µL of primer mix (10 µM), 5 µL of iQ SYBR Green Supermix (Bio-Rad), and 2 µL of milliQ. Experiments were run on a CFX (Bio-Rad). The following program was used: 95°C for 3 min, followed by 40 cycles of 95°C for 15s, and 60°C for 30s. Melting curves were obtained by increasing the temperature stepwise by 0.5°C every 5 s from 55°C to 95°C (Ribeiro et al., 2014a).

3 Results and discussion

3.1 The ability of *R. communis* seedlings to sustain biomass production at high temperatures is genotype-dependent

In order to explore the effect of temperature on *R. communis* seedling performance, we measured the dry weight of roots, stem and cotyledons, as well as the root length of 10-day-old seedlings grown at 3 different temperatures (20ºC, 25ºC and 35°C) (Figure 1). For this purposes, we used two different genotypes: MPA11 and IAC80. For genotype MPA11, no differences in root length of seedlings grown at 25ºC and 35ºC were observed. They were, however, longer than roots of seedlings grown at 20ºC. For

genotype IAC80, root length increased with increasing temperature (Figure 1a). For genotype MPA11, root dry weight increased with increasing temperature, reaching the highest value at 35ºC (Figure 1b). Roots of genotype IAC80 were heavier at 25ºC (3.3-fold) than at 20ºC (Figure 1b), which is comparable with the result observed for roots of genotype MPA11. Genotype IAC80, however, was not able to sustain this increase in root growth at 35ºC and root dry weight of seedlings grown at 35ºC was not different from 25ºC. These results highlight the fact that genotype MPA11 possesses higher plasticity in response to changes of the environmental conditions during initial vegetative growth than genotype IAC80. Stem and cotyledon dry weight increased with increasing temperature for both genotypes, except for a small decrease in cotyledon dry weight of genotype IAC80 at 35ºC compared with 25ºC (Figure 1c,d). We, therefore, decided to focus on the differential ability of these two genotypes to sustain root biomass production at higher temperatures. For that, we calculated the ratio between root dry weight and root length at each temperature. This ratio provides insight into biomass allocation to the roots in response to temperature (Figure 1e), since it indirectly measures the thickness of the root, i.e. dry weight per unit length of root. For genotype MPA11, this ratio increased with increasing temperature, whereas it decreased for genotype IAC80.

3.2 Biochemical and molecular changes in *R. communis* roots in response to temperature

3.2.1 Changes in primary metabolite composition of R. communis roots in response to temperature

Primary metabolites were measured in roots of seedlings grown at 20ºC, 25ºC, and 35ºC to assess metabolic changes associated with temperature that could provide further insights into the mechanisms underlying root biomass allocation. Twenty-one amino acids, six carbohydrates and four tricarboxylic acid cycle intermediates were identified, along with eleven other metabolites (Supplementary Table S1).

Principal component analysis was applied to the whole data set, using both identified and unidentified metabolites, aiming at finding directions that explained the variance in the data set. Principal component 1 (PC1) explained 34.5% of the variance, whereas principal component 2 (PC2) explained 26.1% (Supplementary Figure S1). For genotype MPA11, a clear separation due to temperature was observed among samples grown at 20ºC, 25ºC, and 35ºC. For genotype IAC80, no clear separation due to temperature was observed between samples grown at 20ºC and 25ºC. Samples grown at 35ºC were differentiated from samples grown at 20ºC and 25ºC. Using the concept that proximity means similarity, root samples grown at 20ºC and 25ºC seem to be closer related in terms of metabolome, than those grown at 35ºC.

Levels of twenty-nine metabolites varied (Bonferroni correction, FDR < 0.05) in response to temperature or genotype differences (Supplementary table 3). For genotype MPA11, glutamine and threonine levels increased with increasing temperature (up to 3.92-fold), whereas asparagine levels were greater in roots grown at 25ºC and 35ºC than at 20ºC (up to 12.29-fold).

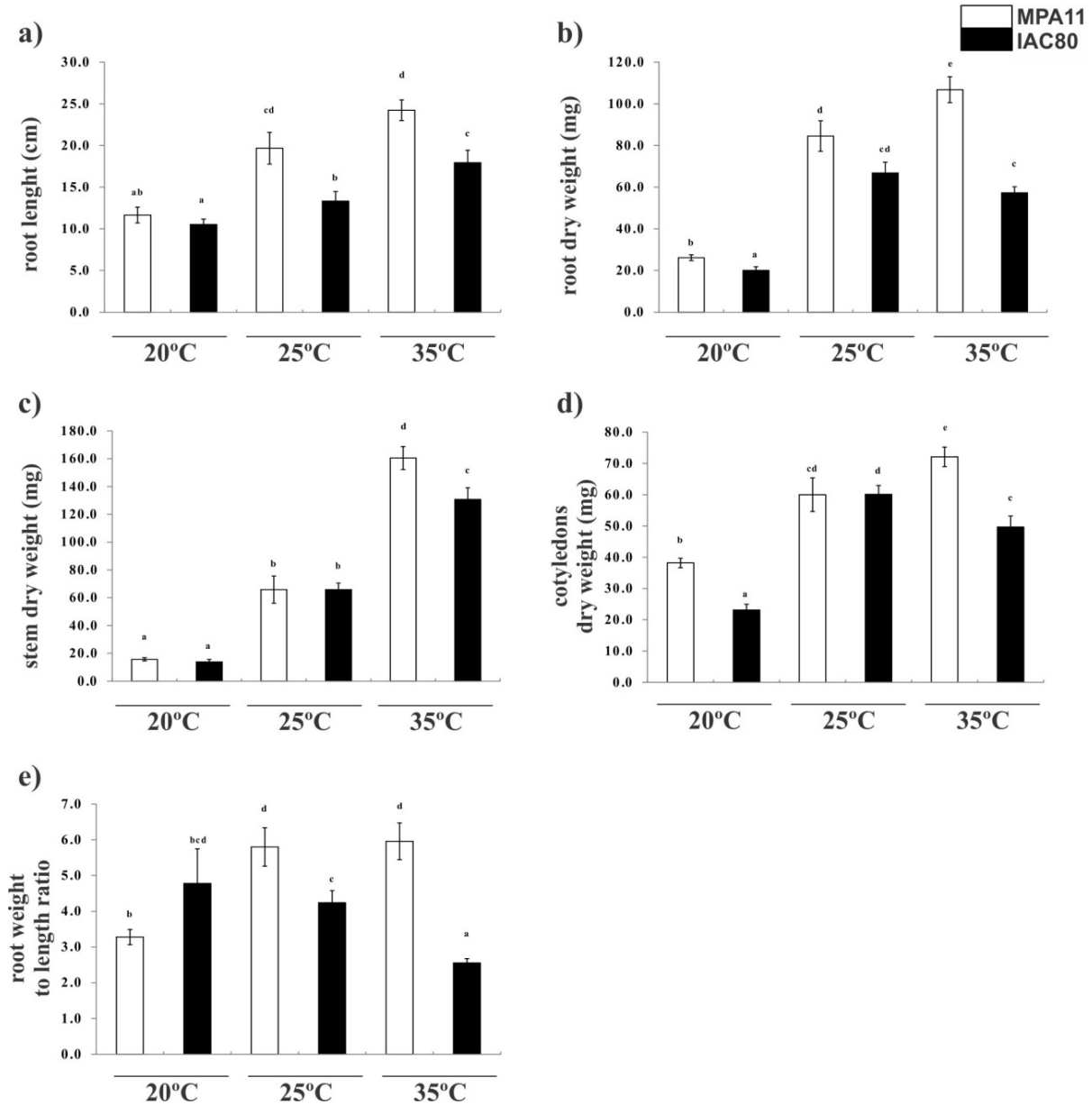

Figure 1. *R. communis* performance in response to temperature. (a) root length, (b) root dry weight, (c) stem dry weight, (d) cotyledons dry weight and (e) ratio between root dry weight and root length of 10-day-old seedlings grown at three different temperatures (20°C, 25°C and 35°C). Results for genotypes MPA11 (white bars) and IAC80 (black bars) are shown. Different letters above the bars indicate significant differences between samples by Turkey's HSD ($p<0.05$).

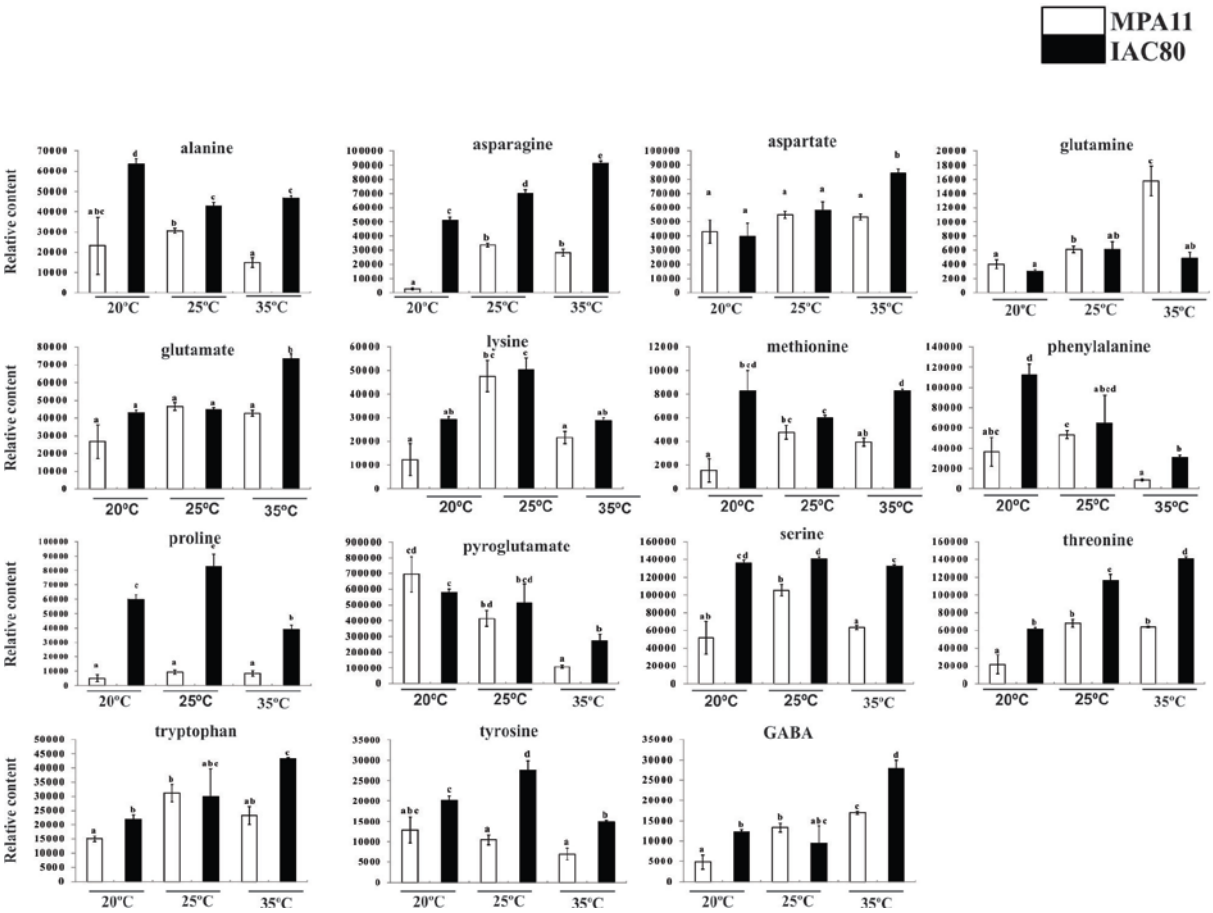

Figure 2. Relative concentration of identified amino acids in roots of 10-day-old seedlings grown at three different temperatures (20°C, 25°C and 35°C). Results for genotypes MPA11 (white bars) and IAC80 (black bars) are shown. Average and standard error of three biological replicates containing 10 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Turkey's HSD ($p<0.05$).

A decreasing pattern was observed in pyroglutamate levels with the increasing temperature (up to 6.45-fold). Little variation was observed for other identified amino acids (Figure 2). For genotype IAC80, an increasing pattern was observed for asparagine, aspartate, glutamate, threonine and tryptophan levels with increasing temperature (up to 3.11-fold). A decreasing pattern, however, was observed for alanine, proline, phenylalanine and pyroglutamate (up to 3.63-fold). A peak in lysine and tyrosine levels was observed in roots grown at 25ºC. Little variation was observed for glutamine, methionine and serine (Figure 2). For both genotypes, fructose and xylose levels showed little difference between root samples grown at 20ºC and 25ºC, but lower levels were observed in roots grown at 35ºC. For genotype MPA11, levels of trehalose were lower in roots grown at 35ºC than at 20ºC, whereas for genotype IAC80 levels of trehalose were lower in roots grown at 35ºC than at 25ºC. For genotype MPA11, glucose levels were lower in samples grown at 35ºC than at 25ºC, whereas for genotype IAC80 glucose levels were lower in

roots grown at 35ºC than at 20ºC, For genotype MPA11, a peak in sucrose levels was observed in roots grown at 25ºC, whereas for genotype IAC80 the lowest level was observed in roots grown at 35ºC (Figure 3).

R. communis seedlings have a specific metabolic signature to adjust growth and developmental processes to higher temperatures, likely to maintain cellular homeostasis (Ribeiro et al., 2014b; Ribeiro et al., 2015a; Ribeiro et al., 2015b). A shift in their carbon-nitrogen metabolism towards the accumulation of nitrogen-containing compounds is the main biochemical response to high temperatures. However, no further insights that could explain biomass production of *R. communis* genotypes could be inferred based on these results.

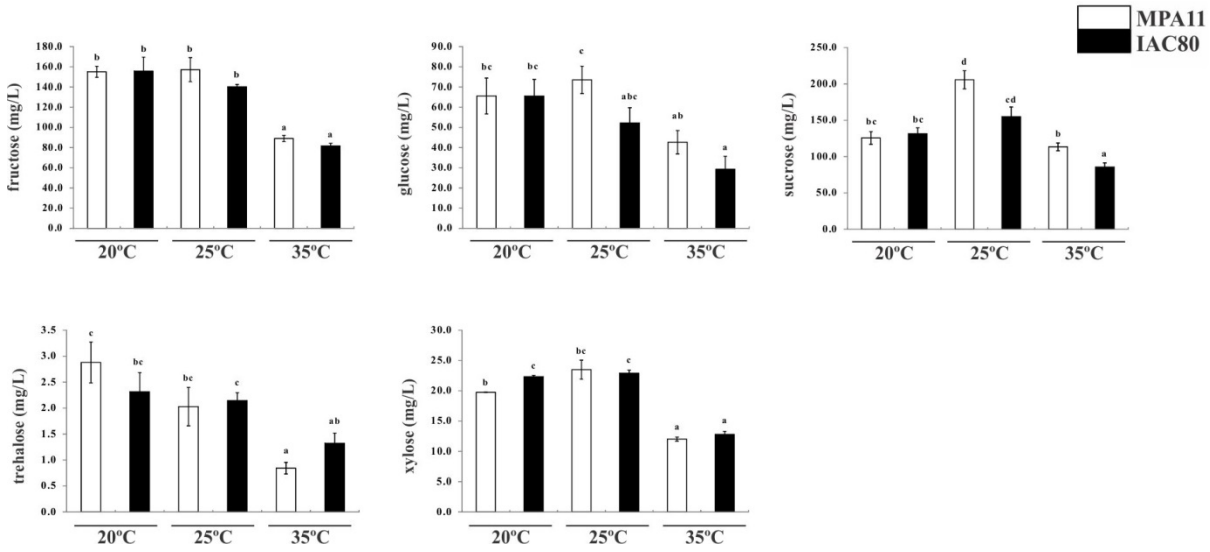

Figure 3. Relative concentration of identified carbohydrates in roots of 10-day-old seedlings grown at three different temperatures (20°C, 25°C and 35°C). Results for genotypes MPA11 (white bars) and IAC80 (black bars) are shown. Average and standard error of three biological replicates containing 10 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Turkey's HSD ($p<0.05$).

Glycolysis and the tricarboxylic acid cycle (TCA) are metabolic routes by which organisms generate energy from carbohydrates, amino acids and fatty acids. Four TCA cycle intermediates were detected: citrate, fumarate, malate, and succinate (Figure 4a). For genotype MPA11, fumarate levels did not vary between roots grown at 20ºC and 25ºC. Fumarate levels, however, were greater in roots grown at 35ºC (up to 2.5-fold) than at 20ºC and 25ºC. Citrate, malate and succinate levels hardly showed any variation among root samples. For genotype IAC80, an increasing pattern was observed for fumarate and

malate with increasing temperature (up to 6.80-fold), whereas little variation was observed for citrate and malate (Figure 4a). These results indicate that *R. communis* seedlings activate catabolic pathways to generate energy through the oxidation of acetate in the TCA cycle in response to an increase in temperature.

γ-Aminobutyric acid (GABA) is a non-protein amino acid associated with abiotic stresses, signalling, and nitrogen storage in plants (Kinnersley and Lin, 2000; Kinnersley and Turano, 2000). GABA may act as a signalling molecule in nitrogen metabolism, as a regulator of the cytosolic pH and as osmoprotectant to prevent oxidative damage in response to various abiotic stresses (Renault et al., 2010). GABA accumulates in response to the water content of the seeds during the initial phase of imbibition and in response to temperature during early seedling establishment (Ribeiro et al., 2014b; Ribeiro et al., 2015b). For both genotypes, levels of GABA and putrescine were greater in roots grown at 35ºC than at 20ºC and 25ºC. GABA levels in roots of genotype IAC80 grown at 35ºC were, however, greater than in roots of genotype MPA11 grown at the same temperature (Figure 2 and 4b). This indicates that genotype IAC80 perceives a stronger stress than genotype MPA11.

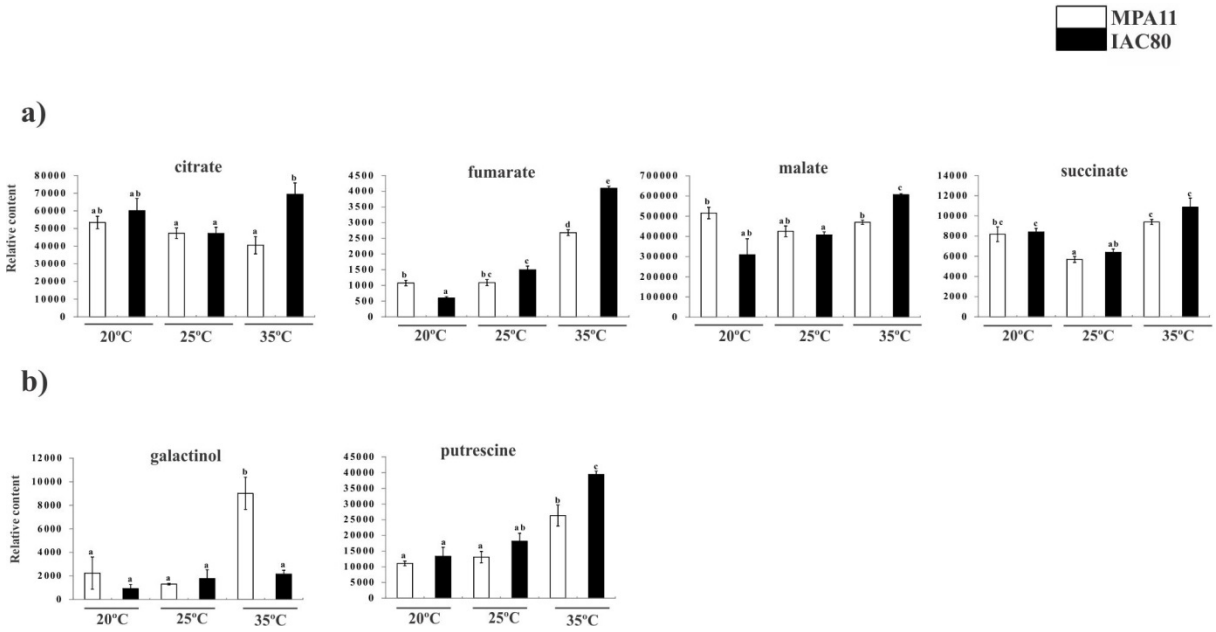

Figure 4. Relative concentration of selected metabolites in roots of 10-day-old seedlings grown at three different temperatures (20°C, 25°C and 35°C). (a) organic acids and (b) galactinol and putrescine. Results for genotypes MPA11 (white bars) and IAC80 (black bars) are shown. Average and standard error of three biological replicates containing 10 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Turkey's HSD ($p<0.05$).

Galactinol is a member of the raffinose family oligosaccharides (RFOs) and in plant cells it acts as an osmoprotectant molecule. For genotype MPA11, galactinol levels were greater in roots grown at 35ºC than at 20ºC and 25ºC. For genotype IAC80, however, galactinol levels did not vary in response to temperature (Figure 4b). Greater levels of galactinol at 35ºC, therefore, indicate that genotype MPA11 avoids oxidative damages caused by heat-stress and thereby maintain homeostasis to support higher root growth at elevated temperatures. High intracellular levels of galactinol in transgenic Arabidopsis plants overexpressing galactinol synthase genes (*GolS1*, *GolS2* and *GolS4*) were correlated with increased tolerance to several abiotic stresses (Nishizawa et al., 2008). Galactinol and raffinose protected plant cells from oxidative damages by scavenging hydroxyl radicals (Nishizawa et al., 2008).

Total soluble protein content was assessed, but hardly any differences were found in response to temperature (Supplementary Figure S2). In fact, no differences were found between roots of genotypes MPA11 and IAC80 grown at 35ºC.

3.2.2 Transcript levels of genes encoding antioxidant enzymes and heat-shock proteins

Plants have developed two main antioxidant defence mechanisms to overcome oxidative stress: non-enzymatic and enzymatic. Non-enzymatic mechanisms consist of small molecules, such as vitamins A, C and E, glutathione, carotenoids, and phenolic compounds. Enzymatic mechanisms involve several enzymes, such as catalase, peroxidase, glutathione reductase, and superoxide dismutase. These enzymes share the ability to scavenge superoxide and hydrogen peroxide radicals (Jaleel et al., 2009). Transcript levels of genes encoding for antioxidant enzymes were assessed in an additional attempt to identify possible underlying mechanisms that could explain *R. communis* differential ability to sustain biomass production at high temperatures.

Catalases are enzymes responsible for protecting cells from oxidative damages caused by reactive oxygen species (ROS). Three catalase genes have been identified in Angiosperms (Mhamdi et al., 2012). The *R. communis* genome contains two sequences putatively annotated as catalase, *RcCAT1* and *RcCAT2* (http://www.ncbi.nlm.nih.gov/). *RcCAT1* is 1479 bp long and encodes a predicted enzyme of 492 amino acids, whereas *RcCAT2* is 2925 bp long and encodes a predicted enzyme of 974 amino acids (Supplementary Table 2). Transcript levels of *RcCAT1* were 10.9 and 18.2-fold greater in roots grown at 35ºC than at 20ºC for MPA11 and IAC80, respectively. Transcript levels of *RcCAT2* were 5.09 and 2.93-fold greater in roots grown at 35ºC than at 20ºC for MPA11 and IAC80, respectively (Figure 5a). *RcCAT1* was 3-fold upregulated in IAC80 root samples grown at 25ºC as compared with 20ºC, but there were no further differences found in the transcript levels of *RcCAT1* and *RcCAT2* between root samples grown at 20ºC and 25ºC for both genotypes (Figure 5a). Both genotypes are, therefore, able to efficiently increase

the transcript levels of catalase genes at higher temperatures. This is in agreement with the fact that no difference in catalase activity was found in roots of MPA11 and IAC80 grown at 35ºC (Figure 5b).

Glutathione reductase (GR) plays a vital role in maintenance of ascorbate and glutathione pools in a reduced state (Yousuf et al., 2012). The *R. communis* genome contains two sequences putatively annotated as glutathione reductase, *RcGR1* and *RcGR2* (http://www.ncbi.nlm.nih.gov/). *RcGR1* has a coding region of 1491 bp and encodes a predicted enzyme of 496 amino acids, whereas *RcGR2* has a coding region of 1683 bp and encodes a predicted enzyme of 560 amino acids (Supplementary Table 2). Transcript levels of *GR1* were 2.68 and 1.47-fold greater in roots grown at 35ºC than at 20ºC for MPA11 and IAC80, respectively. Transcript levels of *GR2* were 2.26 and 1.61-fold greater in roots grown at 35ºC than at 20ºC for MPA11 and IAC80, respectively. No differences were found in transcript levels of *GR1* and *GR2* between root samples grown at 20ºC and 25ºC for both genotypes (Figure 5c). These results show that the degree of increase in transcript levels of both glutathione reductase genes at higher temperatures is slightly higher for genotype MPA11 than for genotype IAC80.

Peroxidase precursor 21 (*PER21*) and 63 (*PER63*) are responsible for the removal of generated H_2O_2 and they are upregulated in response to environmental stresses such as wounding, pathogen attack and oxidative stresses (Yong et al., 2002). The homolog of *PER21* in *R. communis*, *RcPER21*, has a coding region of 666 bp and encodes a predicted enzyme of 221 amino acids. *RcPER63* has a coding region of 810 bp and encodes a predicted enzyme of 269 amino acids (Supplementary Table 2). For genotype MPA11, transcript levels of *RcPER21* were greater in root samples grown at 35ºC than at 20ºC (3.9-fold). For genotype IAC80, no differences were observed in the transcript levels of *RcPER21* between root samples grown at 35ºC and 20ºC (Figure 5d). For genotype MPA11, transcript levels of *RcPER63* did not vary between root samples grown at 20ºC and 35ºC. For genotype IAC80, they were lower in root samples grown at 35ºC than at 20ºC (Figure 5d). Genotype MPA11 was, therefore, able to increase the transcript levels of *RcPER21* and to maintain the transcript levels of *RcPER63* at 35ºC as compared with 20ºC, whereas genotype IAC80 was unable to efficiently increase the transcript levels of both genes at higher temperature.

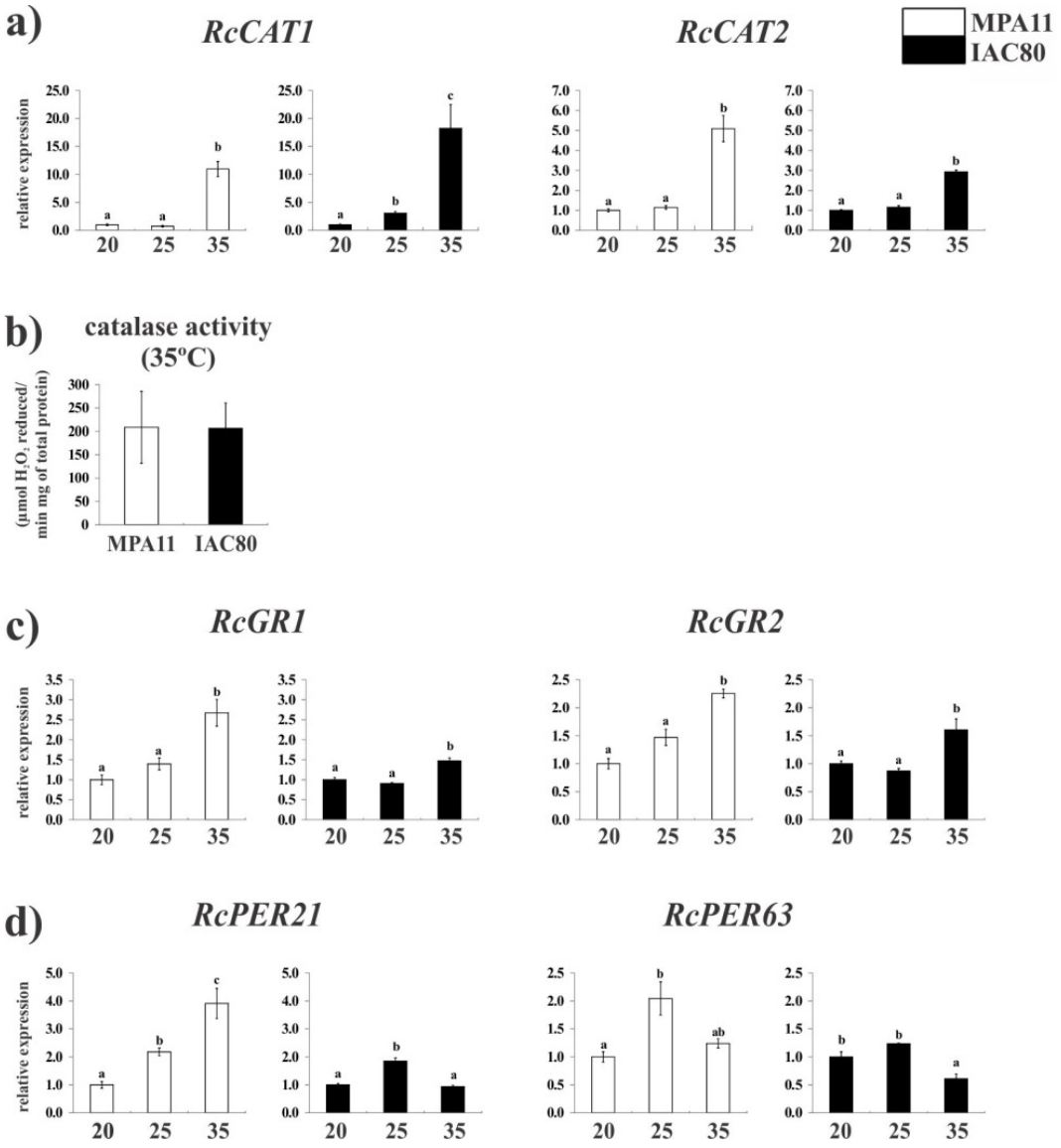

Figure 5. Transcript levels of genes encoding for antioxidant enzymes and catalase activity in roots of 10-day-old seedlings grown at three different temperatures (20°C, 25°C and 35°C). (a) Transcript levels of catalase (*RcCAT1* and *RcCAT2*), (b) catalase activity, (c) transcript levels of glutathione reductase (*RcGR1* and *RcGR2*), (d) transcript levels of peroxidase 21 precursor (*RcPER21*) and peroxidase 63 precursor (*RcPER63*). Results for genotypes MPA11 (white bars) and IAC80 (black bars) are shown. Average and standard error of three biological replicates containing 10 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Turkey's HSD ($p<0.05$).

Superoxide dismutase [cu-zn] binds copper and zinc ions and is responsible for the dismutation of superoxide radicals (O_2^-) into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). The *R. communis* genome contains three sequences putatively annotated as superoxide dismutase [cu-zn] (http://www.ncbi.nlm.nih.gov/). Primers suitable to measure transcript levels could only be obtained for

two of the genes and, therefore, expression of these two genes was assessed. *Rc Cu-Zn SOD1* has a coding region of 459 bp and encodes a predicted enzyme of 152 amino acids. *Rc Cu-Zn SOD2* has a coding region of 438 bp and encodes a predicted enzyme of 145 amino acids (Supplementary Table 2). For genotype MPA11, transcript levels of *Rc Cu-Zn SOD1* were 3.66 and 2.39-fold greater in root samples grown at 35ºC than at 20ºC and 25ºC, respectively. For genotype IAC80, hardly any differences were observed in transcript levels of *Rc Cu-Zn SOD1* (Figure 6a). For genotype MPA11, transcript levels of *Rc Cu-Zn SOD2* had a similar pattern of expression as *RcSODCu-Zn1*. For genotype IAC80, transcript levels of *Rc Cu-Zn SOD2* were 2.10 and 1.90-fold greater in roots grown at 35ºC than at 20ºC and 25ºC, respectively. No differences were found in the transcript levels of *Rc Cu-Zn SOD1* and *Rc Cu-Zn SOD2* between root samples grown at 20ºC and 25ºC for both genotypes (Figure 5d). Genotype MPA11 efficiently increased transcript levels of *Rc Cu-Zn SOD1* and *Rc Cu-Zn SOD2* at 35ºC as compared with 20ºC; however, genotype IAC80 was unable to increase the transcript levels of both genes at higher temperatures. This is in agreement with the fact total SOD activity, and the activity of Mn SOD and Cu-Zn SOD isoforms were higher in roots of MPA11 than in roots of IAC80, both grown at 35ºC (Figure 6b-c). No differences in the activity of Fe SOD was observed between the two genotypes.

Transcript levels of two genes encoding small heat-shock proteins (HSP1 and HSP2) were assessed. *R. communis* genome contains several sequences putatively annotated as heat shock proteins (HSPs), but these two *HSP* genes were used based on a previous gene expression study of *R. communis* germination at different temperatures (Ribeiro et al., 2015b). Levels of both transcripts were higher in seeds imbibed at 35ºC than at 20ºC and 25ºC for all developmental stages (Ribeiro et al., 2015b). These genes are homologs of Arabidopsis genes that are known to be expressed in seeds and to be induced upon heat stress in the seedling stage. *RcHSP1* has a coding region of 612 bp and encodes a predicted enzyme of 203 amino acids. *RcHSP2* has a coding region of 573 bp and encodes a predicted enzyme of 190 amino acids (Supplementary Table 2). For genotype MPA11, transcript levels of *RcHSP1* were 9.2 and 13.4-fold greater in root samples grown at 35ºC than at 20ºC and 25ºC, respectively. For genotype IAC80, transcript levels of *RcHSP1* were only 5.8 and 8.0-fold greater in root samples grown at 35ºC than at 20ºC and 25ºC, respectively (Figure 6d). For genotype MPA11, transcript levels of *RcHSP2* were 20.0 and 11.5-fold greater in root samples grown at 35ºC than at 20ºC and 25ºC, respectively. For genotype IAC80, transcript levels of *RcHSP1* were only 4.6 and 4.3-fold greater in root samples grown at 35ºC than at 20ºC and 25ºC, respectively (Figure 6d).

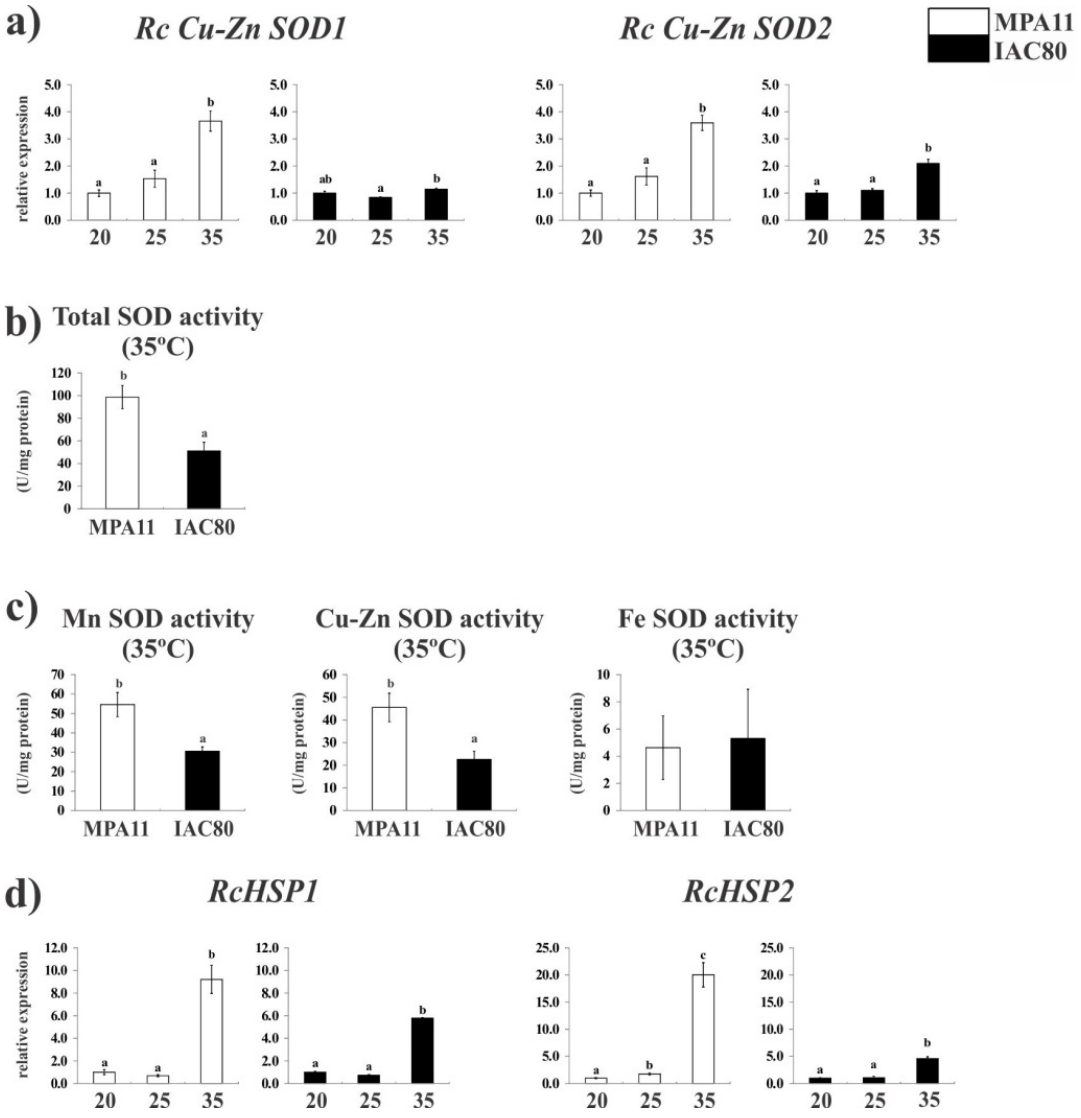

Figure 6. Transcript levels of genes encoding for antioxidant enzymes and heat-shock proteins and SOD activity in roots of 10-day-old seedlings grown at three different temperatures (20°C, 25°C and 35°C (a) Transcript levels of superoxide dismutase (*RcCu-ZnSOD1* and *RcCu-ZnSOD2*), (b) total SOD activity, (c) Mn SOD, Cu-Zn SOD and Fe SOD activity, (d) transcript levels of heat-shock proteins (*RcHSP1* and *RcHSP2*). Results for genotypes MPA11 (white bars) and IAC80 (black bars) are shown. Average and standard error of three biological replicates containing 10 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Turkey's HSD ($p<0.05$).

Taken together, these results show that under high-temperature conditions genotype MPA11 is able to more efficiently up-regulate protective mechanisms against oxidative damages than genotype IAC80. This includes genes encoding heat-shock proteins and antioxidant enzymes. Additionally, genotype MPA11 contains greater levels of osmoprotectant molecules than genotype IAC80, which might

also contribute to better seedling performance of MPA11 in terms of root biomass production at higher temperatures. This is in agreement with the findings of Zhu et al. (2010) who reported that increased activities of superoxide dismutase, catalase, and peroxidase alleviated the damage caused by temperature stress on maize plants colonized by the arbuscular mycorrhizal fungus, *Glomus etunicatum*, by reducing membrane lipid peroxidation and membrane permeability and increasing the accumulation of osmotic adjustment compounds and antioxidant enzyme activity. This led to increased host biomass and promoted plant growth (Zhu et al., 2010). Heat stress caused higher production of ROS, greater membrane damage, and declines in photosynthetic rate and activities of antioxidant enzymes, which resulted in lower grain yields for sorghum (Djanaguiraman et al., 2010). The importance of the antioxidant defense mechanism was demonstrated by foliar application of selenium, which decreased membrane damage by enhanced antioxidant defense and increased photosynthetic rates and grain yield in heat-stressed plants (Djanaguiraman et al., 2010).

4 Conclusions

Our results provide important leads for the mechanisms underlying root biomass production in response to varying temperatures that are required to support vigorous seedling growth under high temperature. In summary, biomass allocation in *R. communis* seedlings was strongly affected by temperature. The differential ability to sustain biomass production of genotype MPA11 seems to be associated with a more efficient up-regulation of protective mechanisms against oxidative damage caused by ROS. These protective mechanisms include antioxidant enzymes and heat shock proteins. Therefore, higher levels of osmoprotectant molecules and up-regulation of other protective mechanisms might be cause or consequence of higher root growth. Evidently, post-transcriptional, translational and post-translational control cannot be ruled out. Our results provide further insights into the mechanisms underlying plant adaptation to harsh environmental conditions during seedling establishment, which might be helpful to develop germination and seedling production protocols for the sustainable exploitation of *R. communis*.

5 Authors' contributions

P.R. Ribeiro, R.F. Zanotti and C. Deflers carried out the physiological experiments. P.R. Ribeiro performed the metabolite profiling analysis, data processing and compound identification. P.R. Ribeiro and R.F. Zanotti carried out the gene expression experiments, carbohydrate measurements and wrote the manuscript. L.G. Fernandez and R.D. de Castro participated in the critical reading and discussion of the manuscript. W. Ligterink and H.W.M. Hilhorst participated in the design of the study, coordination and critical reading of the manuscript. All authors read and approved the final manuscript.

6 Conflict of interest

The authors declare no conflict of interest.

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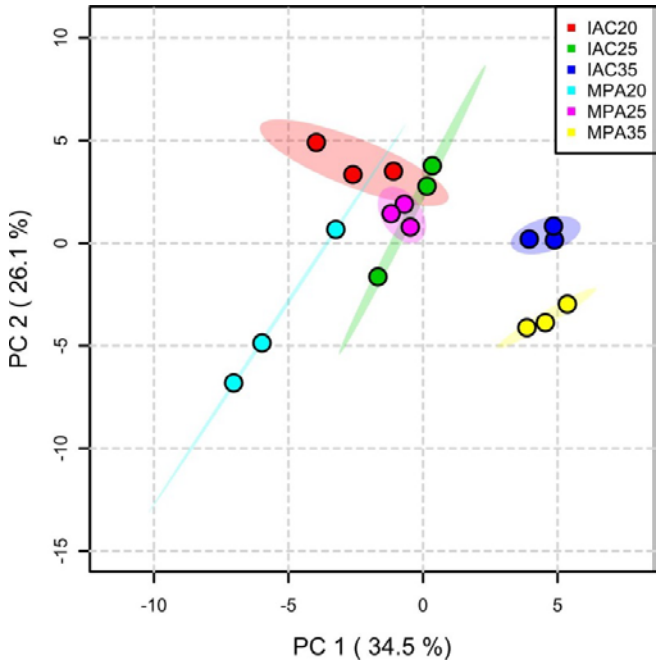
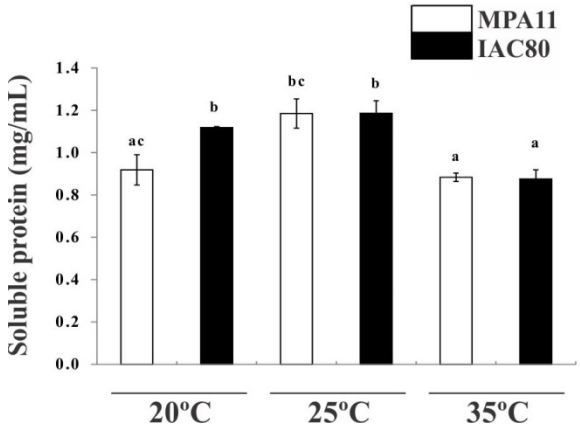


Supplementary Figure S1. Principal component analysis (PCA) based on polar metabolite content (identified and non-identified metabolites) in root samples of *R. communis* seedlings in response to different temperatures. Root samples of genotypes MPA11 and IAC80 were used. Temperatures are depicted in the plot.



Supplementary Figure S2. Total soluble protein in response to temperature and/or genotype. Results for genotypes MPA11 (white bars) and IAC80 (black bars) are shown. Average and standard error of three biological replicates containing 10 seedlings each are shown. Different letters above the bars indicate significant differences between samples by Turkey's HSD ($p<0.05$).

8 Supplementary table captions

Supplementary Table S1. Metabolite content and levels of identification.

Supplementary Table S2. Description of target genes used for RT-qPCR.

Supplementary tables can be found at http://www.wageningenseedlab.nl/thesis/prribeiro/SI/

Chapter 7

Expression profiles of genes related to carbohydrate metabolism provide new insights into carbohydrate accumulation in seeds and seedlings of *Ricinus communis* in response to temperature

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Abstract

Ricinus communis is an important oilseed crop, which possesses a specific metabolic signature to adjust growth and developmental processes in response to temperature. Carbohydrates are accumulated at low temperatures, whereas amino acids are accumulated at elevated temperatures. This is a robust signature that has been observed during seed imbibition, seed germination and seedling development. Our objective was to assess tissue-specific changes in transcript levels of genes related to carbohydrate biosynthesis and catabolism in response to temperature. For that, we measured transcript levels of genes encoding enzymes involved in starch biosynthesis, starch catabolism, and gluconeogenesis in *R. communis* leaves, roots, and seeds grown at 20ºC and 35ºC. Transcript levels of genes involved in starch catabolism were higher in leaves grown at 20ºC than at 35ºC, but up-regulation of genes involved in starch biosynthesis seems to compensate for this and, therefore, are the likely explanation for higher levels of starch in leaves grown at 20ºC. Higher levels of soluble carbohydrates in leaves grown at 20ºC may be caused by a coordinated up-regulation of starch catabolism and gluconeogenesis pathways. In roots, starch catabolism and gluconeogenesis seem to be enhanced at elevated temperatures. Higher levels of starch in seeds germinated at low temperatures is associated with higher transcript levels of genes involved in starch biosynthesis. Similarly, higher transcript levels of *RcPEPCK* and *RcFBPase* are most likely causal for fructose and glucose accumulation in seeds germinated at 20ºC. This study provides important insights in the understanding of the plasticity of *R. communis* in response to temperature that may apply to other species as well.

Keywords: carbohydrate metabolism, gluconeogenesis, starch biosynthesis, starch catabolism

1 Introduction

Temperature is a critical environmental cue that influences seed germination, seedling establishment, and plant growth. In general, a progressive increase in temperature shortens the time required for germination to occur until it reaches an optimum temperature and maximum final germination percentage (Bewley et al., 2013; Kendall and Penfield, 2012). A difference of only a few degrees may already lead to a notable change in growth, survival rate, and yield (Salisbury and Ross, 1985). Plants may experience high-temperature stress during one or more stages of their life cycle, especially in tropical and sub-tropical regions worldwide. Losses due to global warming are expected in the production of several crops, including wheat, rice and maize in both temperate and tropical regions (Challinor et al., 2014; Peng et al., 2004; Pratibha et al., 2015). For example, the optimum mean temperature for grain formation and grain yield of rice is 25ºC. Grain yield is reduced 10% per 1ºC temperature increase above 25ºC until 35 to 36ºC mean temperature when no yield is obtained (Hatfield et al., 2011). For cauliflower, cabbage and rutabaga a 10% yield loss was observed for every 10 days that the temperature reached 30°C or higher during the growing season (Warland et al., 2006). High temperatures during seed maturation reduced canola yield by up to 52% for a sensitive cultivar due to a reduction in seed weight (Aksouh-Harradj et al., 2006). Understanding heat tolerance mechanisms is, therefore, increasingly important in order to improve thermotolerance of plants.

The effect of high temperatures on seedling development and establishment has been assessed for several species. For example, maize seedlings grown at 37°C showed reduced root growth compared with plants grown at 28°C (Trachsel et al., 2010). Gupta et al. (2013) showed that heat stress reduced shoot and root dry weight, chlorophyll content and membrane stability in wheat. In this study, heat stress conditions were created by exposing seedlings to 45°C for 2h after 7 days of germination (Gupta et al., 2013). *Ricinus communis* possesses the ability to grow in various and adverse environments which makes this species an ideal candidate to provide a better understanding of seedling performance and adaptation under different temperatures. Temperature affected water uptake of *R. communis* seeds from as early as 2 hours of imbibition. Faster imbibition occurred in seeds imbibing at 35ºC as compared with those imbibing at 20 and 25ºC (Ribeiro et al., 2015a). *R. communis* seeds germinate to a high percentage and faster at 35ºC than at lower temperatures, but with compromised seedling establishment. During later stages of seedling establishment, however, seedlings are able to cope with high temperatures at later stages of seedling establishment if germination occurred at lower temperatures (Ribeiro et al., 2015b). Biomass allocation between below- and aboveground parts of 14-day-old seedlings was strongly affected by the temperature. An increasing temperature resulted in preferred growth of the stem and true leaves, at the expense of

cotyledons and roots. Consequently, a reduction of the root to shoot ratio was observed (Ribeiro et al., 2014b).

Carbohydrate metabolism is a key responsive element of thermoplasticity and thermotolerance mechanisms in a diverse range of plant species. For example, a low soil temperature increased starch content of Norway spruce seedlings and delayed the loss of starch at the end of the growing season (Repo et al., 2004). Starch was stored while plants experienced low soil temperature, but it was released to support growth of shoots and roots under favorable conditions (Repo et al., 2004). Total and reducing soluble carbohydrates and polyol content in zucchini fruits were generally higher during storage at 4°C than at 20°C (Palma et al., 2014). Fructose, glucose, sucrose, and starch accumulated in tomato seedlings subjected to a lower night temperature (Qi et al., 2011). Similarly, young tomato seedlings accumulated fructose, glucose, sucrose, and starch when growth was limited by low temperatures. These carbohydrates, however, were mobilized when plants were transferred to higher temperatures (Klopotek and Kläring, 2014).

R. communis has a specific metabolic signature during growth and development at different temperatures. Accumulation of carbohydrates at low temperatures is the main biochemical response of *R. communis* seeds and seedlings to temperature. Levels of starch and soluble carbohydrates were higher in seeds and seedlings grown at 20ºC than at 35ºC (up to 20-fold). This biochemical response has been observed during *R. communis* seed imbibition (Ribeiro et al., 2015a), germination (Ribeiro et al., 2015b) and early seedling growth (Ribeiro et al., 2014b). Little is known, however, about tissue-specific changes in transcript levels of genes related to carbohydrate biosynthesis and catabolism in response to temperature. Therefore, we raise the question whether carbohydrate accumulation in *R. communis* leaves, roots, and seeds, grown at low temperatures results from up-regulation of biosynthetic pathways, from down-regulation of catabolic pathways, or both. We measured expression levels of genes encoding key enzymes involved in starch biosynthesis, starch catabolism, and gluconeogenesis. Hence, this study provides important insights in the understanding of *R. communis* plasticity in response to temperature during seed germination and seedling establishment that may apply to other species as well.

2 Materials and Methods

2.1 Plant material and germination conditions

Samples used in this work were grown and selected following Ribeiro et al. (2014b; 2015b). Three different tissues were used: leaves, roots, and entire seeds. To collect leaves and roots, seeds were allowed to germinate using wet paper rolls as substrate at 25ºC in the dark. After 44-50 hours, germinated

seeds were transferred to moist vermiculite and were allowed to grow at 20ºC in continuous light for 10 days. Half of the 10-day-old seedlings was then transferred to an incubator at 35ºC in continuous light. After 4 days, leaves and roots (three biological replicates of 15-18 seedlings each) were collected, immediately frozen in liquid nitrogen, freeze-dried, ground and stored at -80ºC prior to analysis. To collect germinated seeds with a radicle of 2 cm, seeds were allowed to germinate using wet paper rolls as substrate at two different temperatures (20ºC and 35ºC) in the dark. Seeds were collected as soon as the radicle reached 2 cm in length at each temperature (three biological replicates of 15-20 seeds each), immediately frozen in liquid nitrogen, freeze-dried, ground and stored at -80ºC prior to analysis.

2.2 RNA extraction, DNase digestion, RNA quality control and cDNA synthesis

Total RNA was extracted from 20 mg of dry material using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNeasy Plant Mini Kit includes the RNase-Free DNase Set (Qiagen), which was used for degradation of genomic DNA. RNA quantification and quality control were performed following Ribeiro et al. (2014a). First strand cDNA was synthesized from 1 µg of total RNA using the iScriptTM cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Reverse transcription was performed at 37°C for 40 minutes followed by 5 min at 85°C. cDNA was diluted 20 times with H_2O and stored at -20°C prior to further RT-qPCR analysis.

2.3 Target gene selection, primer design and analysis of amplification efficiency

Twenty-one genes encoding for enzymes involved in starch biosynthesis were selected: two phosphoglucoisomerases (*RcPGI1* and *RcPGI2*), seven phosphoglucomutases (*RcPGM1*, *RcPGM2*, *RcPGM3*, *RcPGM4*, *RcPGM5*, *RcPGM6*, and *RcPGM7*), six glucose-1-phosphate adenylyltransferases (*RcAPG1*, *RcAPG2*, *RcAPG3*, *RcAPG4*, *RcAPG5*, and *RcAGP6*), and six starch synthases (*RcSS1*, *RcSS2*, *RcSS3*, *RcSS4*, *RcSS5, and RcSS6*). Sixteen genes encoding for enzymes involved in starch catabolism were selected: three isoamylases (*RcISA1*, *RcISA2*, and *RcISA3*), eight β-amylases (*Rcβ-amylase1*, *Rcβ-amylase2*, *Rcβ-amylase3*, *Rcβ-amylase4*, *Rcβ-amylase5*, *Rcβ-amylase6*, *Rcβ-amylase7*, and *Rcβ-amylase8*), and five hexokinases (*RcHK1*, *RcHK2*, *RcHK3*, *RcHK4*, and *RcHK5*). Additionally, nine genes encoding for enzymes involved in gluconeogenesis were selected: three ATP-dependent phosphoenolpyruvate carboxykinases (*RcPEPCK1*, *RcPEPCK2*, and *RcPEPCK3*), and six fructose-1,6-bisphosphatases (*RcFBPase1*, *RcFBPase2*, *RcFBPase3*, *RcFBPase4*, *RcFBPase5*, and *RcFBPase6*). Primers with melting temperatures of 58°C–62°C, lengths of 18–22 bp and amplicon lengths of 80-200 bp were designed using CLCbio software (CLC bio, Aarhus, Denmark). Amplification efficiency was evaluated based on a two-fold serial dilution series of a pooled cDNA sample. Primer specificity was verified by separating the PCR products on a 2.5% agarose gel and melting curve analysis. Genes for actin

(*ACT*), serine/threonine protein phosphatase 2a regulatory subunit A (*PP2AA1*) and protein pob (*POB*) were used as reference genes (Ribeiro et al., 2014a). Gene identifier number (GI), gene name, gene description, amplicon length, primer efficiency, and primer sequences are listed in Supplementary Table S1 and S2.

2.4 RT-qPCR conditions

RT-qPCR was performed in a total volume of 10 µL containing 2.5 µL of cDNA, 0.5 µL of primer mix (10 µM), 5 µL of iQ SYBR Green Supermix (Bio-Rad), and 2 µL of milliQ. Experiments were run on a CFX (Bio-Rad). The following program was used: 95°C for 3 min, followed by 40 cycles of 95°C for 15s, and 60°C for 30s. Melting curves were obtained by increasing the temperature stepwise by 0.5°C every 5 s from 55°C to 95°C (Ribeiro et al., 2014a).

2.5 Phosphate measurements

Phosphate levels were measured as described by He et al. (2014). Approximately 20 mg of freeze-dried material was boiled at 100°C for 15 min in 0.5 mL 0.5 M HCl. t-Aconitase (40 µg/mL) was used as internal standard. After centrifuging for 2 min at 17000 g , 200 µL of the supernatant was transferred to an HPLC-vial. Samples were injected into a Dionex ICS2500 system with an AS11-HC column and an AG11-HC guard column and eluted with NaOH. The elution procedure was: 0-15 min linear gradient of 25-100mM NaOH, then 15-20 min 500 mM NaOH followed by 20–35 min 5 mM NaOH. Flow rates were 1 mL/min throughout the run. Contaminating anions in the eluents were removed using an ion trap column (ATC), installed between the pump and the sample injection valve. Anions were determined by conductivity detection. Background conductivity was decreased using an ASRS suppressor, with water as counterflow. Peaks were identified by co-elution of standards. Three biological replicates of 20 seeds each were used for this analysis.

2.6 Prediction of subcellular location

TargetP 1.1 is a web-based tool that predicts subcellular location of eukaryotic proteins. Subcellular location assignment is based on the presence of any the three predicted N-terminal sequences: chloroplast transit peptide, mitochondrial targeting peptide or secretory pathway signal peptide. Strength of the prediction was expressed as reliability class (RC). RC ranged from 1 to 5, where 1 indicates the strongest prediction (http://www.cbs.dtu.dk/services/TargetP/).

3 Results

3.1 Transcript levels of genes involved in starch biosynthesis

The *R. communis* genome contains two sequences annotated as phosphoglucoisomerase (*RcPGI1* and *RcPGI2*), seven as phosphoglucomutase (*RcPGM1*, *RcPGM2*, *RcPGM3*, *RcPGM4*, *RcPGM5*, *RcPGM6*, and *RcPGM7*), six as glucose-1-phosphate adenylyltransferase (*RcAPG1*, *RcAPG2*, *RcAPG3*, *RcAPG4*, *RcAPG5*, and *RcAGP6*), and six as starch synthase (*RcSS1*, *RcSS2*, *RcSS3*, *RcSS4*, *RcSS5*, and *RcSS6*) (Supplementary Table S1; http://www.ncbi.nlm.nih.gov/).

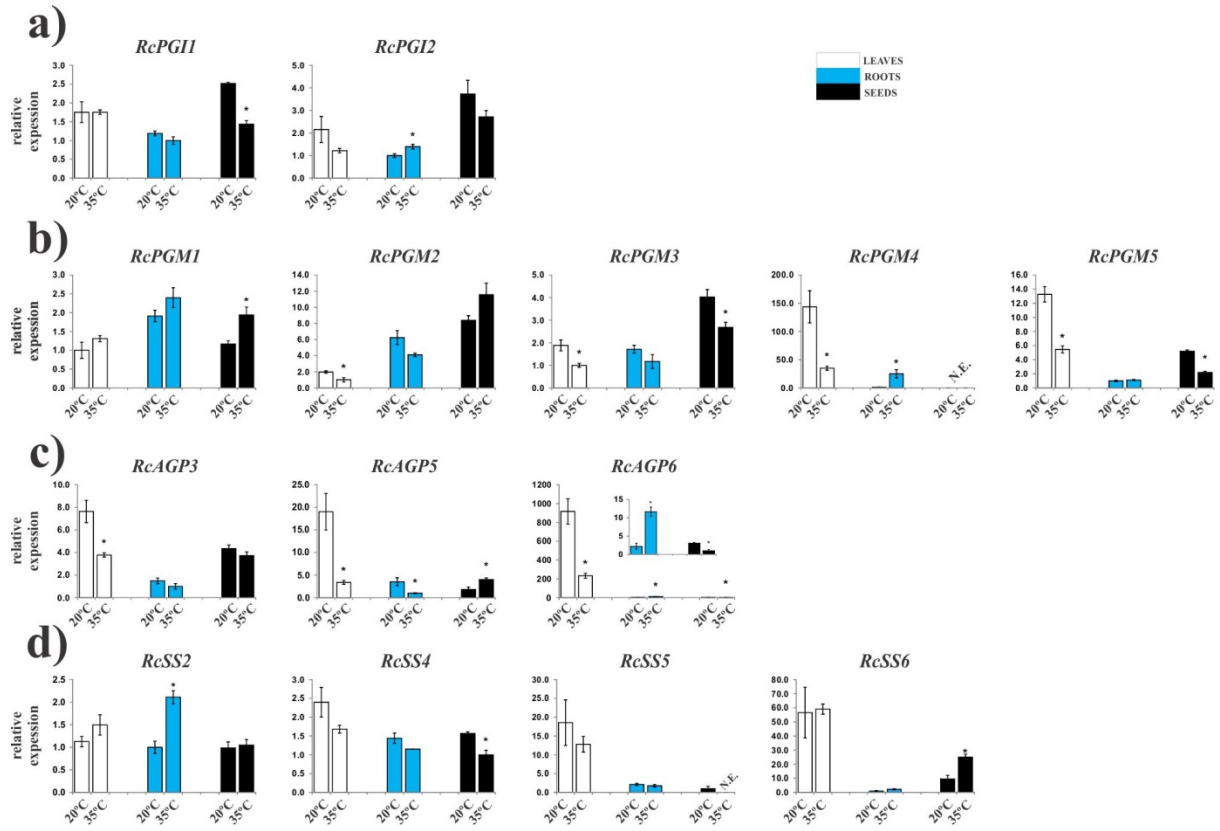

Figure 1. Transcript levels of genes related to starch biosynthesis. (a) Phosphoglucoisomerase (*RcPGI1* and *RcPGI2*), (b) phosphoglucomutase (*RcPGM1*, *RcPGM2*, *RcPGM3*, *RcPGM4*, *RcPGM5*), (c) glucose-1-phosphate adenylyltransferase (*RcAPG3*, *RcAPG5*, and *RcAGP6*), and (d) starch synthase (*RcSS2*, *RcSS4*, *RcSS5*, *and RcSS6*). Average and standard errors of three biological replicates containing 15-20 seeds or seedlings each are shown. Asterisks above the bars indicate significant differences between different temperatures within a given tissue by Student t-test ($p<0.05$).

RcPGI1 contains a predicted chloroplast transit peptide. *RcPGI2* does not contain any predicted subcellular localisation signal peptide (Supplementary Table 1) and, therefore, is most likely located in the

cytosol. Only few differences in the transcript levels of *RcPGI1* and *RcPGI2* were found among the tested samples in dependence of the temperature. Expression of *RcPGI1* was higher in seeds germinated at 20ºC than at 35ºC (1.8-fold), whereas expression of *RcPGI2* was higher in roots of seedlings grown at 35ºC than at 20ºC (1.4-fold) (Figure 1a).

RcPGM1 contains a predicted mitochondrial targeting peptide, whereas *RcPGM4* and *RcPGM5* contain a predicted chloroplast transit peptide. *RcPGM2*, *RcPGM3, RcPGM6,* and *RcPGM7* are most likely located in the cytosol. Expression of *RcPGM1* was higher in seeds germinated at 35ºC than at 20ºC(1.7-fold) (Figure 1b). Expression of *RcPGM1* did not differ between leaves and roots in response to temperature. Expression of *RcPGM2* and *RcPGM3* was higher in leaves of seedlings grown at 20ºC than at 35ºC (up to 2.0-fold). Expression levels of *RcPGM2* and *RcPGM3* in roots and seeds did not differ between the different temperatures. Expression of *RcPGM4* was higher in leaves of seedlings grown at 20ºC than at 35ºC (4.1-fold). In roots, however, expression of *RcPGM4* was higher in seedlings grown at 35ºC than at 20ºC (24.9-fold). Expression of *RcPGM5* was higher in leaf and seed samples collected at 20ºC than at 35ºC (2.4-fold). No amplification was detected for *RcPGM6* and *RcPGM7* (Figure 1b).

Each *RcAGP* isoform (except *RcAGP5*) contains a predicted chloroplast transit peptide. *RcAGP5,* however, is most likely located in the cytosol. Expression of *RcAGP3* was higher in leaves of seedlings grown at 20ºC than at 35ºC (2.0-fold) (Figure 1c). There were no differences in expression levels of *RcAGP3* between the two temperatures in roots and seeds. Expression of *RcAGP5* was higher in leaves and roots of seedlings grown at 20ºC than at 35ºC (up to 5.6-fold). In seeds, however, expression of *RcAGP5* was higher in samples grown at 35ºC than at 20ºC (2.2-fold). Expression of *RcAGP6* was higher in leaf and seed samples collected at 20ºC than at 35ºC (up to 4.0-fold). In roots, however, expression of *RcAGP6* was higher in seedlings grown at 35ºC than at 20ºC (5.4-fold) (Figure 1c). No expression was detected for *RcAGP1*, *RcAGP2* and *RcAGP4*. Since AGP is known to be allosterically inhibited by inorganic phosphate (Pi) (Kleczkowski, 1999), phosphate levels were measured in *R. communis* leaves, roots, and seeds grown at 20ºC and 35ºC. Phosphate levels were higher in leaves, roots, and seeds grown at 35ºC than at 20ºC (Figure 2).

Each *RcSS* isoform (except *RcSS1*) contains a predicted chloroplast transit peptide. *RcSS1* contains a predicted mitochondrial targeting peptide (Supplementary Table 1). Expression of *RcSS2* was higher in roots of seedlings grown at 35ºC than at 20ºC (2.1-fold) (Figure 1d). Expression of *RcSS4* was higher in seeds germinated at 20ºC than at 35ºC (1.6-fold). Transcripts of *RcSS5* were detected in seeds germinated at 20ºC, but not in seeds germinated at 35ºC. Expression of *RcSS6* was higher in seeds germinated at 35ºC than at 20ºC (2.6-fold) (Figure 1d). No expression was detected for *RcSS1* and *RcSS3*.

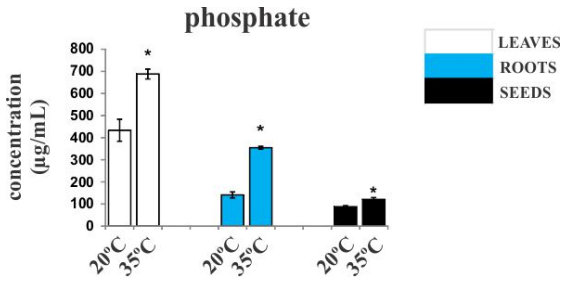

Figure 2. Phosphate levels in *R. communis* leaves, roots, and seeds grown at 20ºC and 35ºC. Average and standard errors of three biological replicates containing 15-20 seeds or seedlings each are shown. Asterisks above the bars indicate significant differences between different temperatures within a given tissue by Student t-test ($p<0.05$).

3.2 Transcript levels of genes involved in starch catabolism

The *R. communis* genome contains three sequences putatively annotated as isoamylase (*RcISA1*, *RcISA2*, and *RcISA3*), eight as *β*-amylase (*Rcβ-amylase1*, *Rcβ-amylase2*, *Rcβ-amylase3*, *Rcβ-amylase4*, *Rcβ-amylase5*, *Rcβ-amylase6*, *Rcβ-amylase7*, and *Rcβ-amylase8*), and five as hexokinase (*RcHK1*, *RcHK2*, *RcHK3*, *RcHK4*, and *RcHK5*) (Supplementary Table S1; http http://www.ncbi.nlm.nih.gov/).

Each *RcISA* isoforms contains a predicted chloroplast transit peptide (Supplementary Table 1). Expression of *RcISA1* and *RcISA3* was higher in leaves of seedlings grown at 20ºC than at 35ºC (up to 4.7-fold) (Figure 3a). In roots, however, higher expression of *RcISA1* and *RcISA2* was found in seedlings grown at 35ºC than at 20ºC (up to 5.3-fold). In seeds, the expression of none of the *RcISA* isoforms was affected by the temperature at which the seeds germinated. The expression of *RcISA* isoforms did not differ in seeds in response to temperature.

In leaves, expression of *Rcβ-amylase3* and *Rcβ-amylase7* was higher in seedlings grown at 20ºC than at 35ºC (up to 57.7-fold); however, expression of *Rcβ-amylase4* and *Rcβ-amylase5* was higher in seedlings grown at 35ºC than at 20ºC (up to 3.8-fold) (Figure 3b). The expression of *Rcβ-amylase1, Rcβ-amylase2, and Rcβ-amylase6* did not differ in leaves in response to temperature. In roots, expression of each *Rcβ-amylase* isoform (except *Rcβ-amylase6*) was higher in seedlings grown at 35ºC than at 20ºC (up to 12.3-fold). In seeds, expression of *Rcβ-amylase7* was higher at 20ºC than at 35ºC (1.4-fold); however, expression of *Rcβ-amylase2* was higher at 35ºC than at 20ºC (up to 3.9-fold). The expression of *Rcβ-amylase1*, *Rcβ-amylase3*, *Rcβ-amylase4*, *Rcβ-amylase5*, and *Rcβ-amylase6* did not differ in seeds in response to temperature (Figure 3b). No expression was detected for *Rcβ-amylase8*.

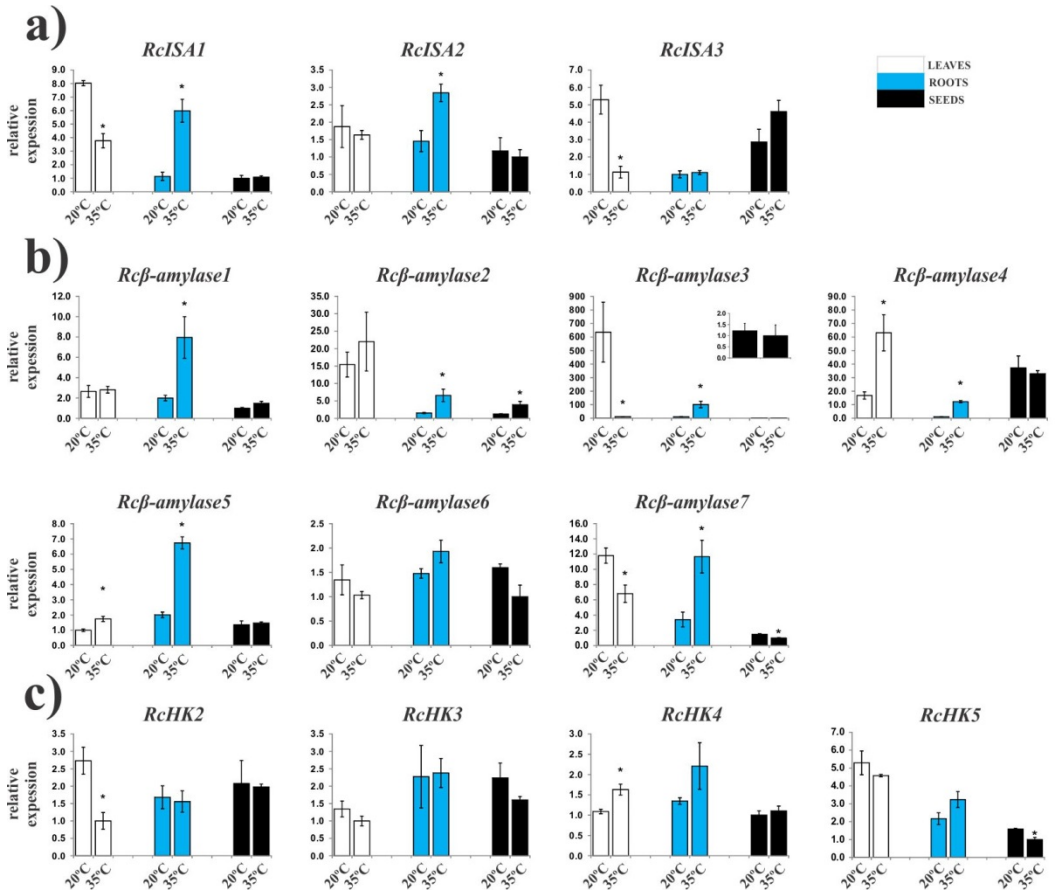

Figure 3. Transcript levels of genes related to starch catabolism. (a) isoamylase (*RcISA1*, *RcISA2*, and *RcISA3*), (b) β-amylase (*Rcβ-amylase1*, *Rcβ-amylase2*, *Rcβ-amylase3*, *Rcβ-amylase4*, *Rcβ-amylase5*, *Rcβ-amylase6*, and *Rcβ-amylase7*), and (c) hexokinase (*RcHK2*, *RcHK3*, *RcHK4*, and *RcHK5*). Average and standard errors of three biological replicates containing 15-20 seeds or seedlings each are shown. Asterisks above the bars indicate significant differences between different emperatures within a given tissue by Student t-test ($p<0.05$).

Expression of *RcHK2* was higher in leaves of seedlings grown at 20ºC than at 35ºC (2.7-fold); expression of *RcHK4*, however, was higher in leaves of seedlings grown at 35ºC than at 20ºC (1.5-fold) (Figure 3c). The expression of *RcHK2* and *RcHK4* did not differ in roots and seeds in response to temperature. Expression of *RcHK5* was higher in seeds germinated at 20ºC than at 35ºC (1.6-fold). The expression of *RcHK3* did not differ in leaves, roots and seeds in response to temperature. No expression was detected for *RcHK1*.

3.3 Transcript levels of genes involved in gluconeogenesis

R. communis genome contains three sequences putatively annotated as ATP-dependent phosphoenolpyruvate carboxykinases (*RcPEPCK1*, *RcPEPCK2*, and *RcPEPCK3*) and six as fructose-1,6-

bisphosphatases (*RcFBPase1*, *RcFBPase2*, *RcFBPase3*, *RcFBPase4*, *RcFBPase5*, and *RcFBPase6*) (Supplementary Table S1; http://www.ncbi.nlm.nih.gov/).

The expression of *RcPEPCK1*, *RcPEPCK2*, *and RcPEPCK3* did not differ in leaves in response to temperature (Figure 4a). In roots, expression of *RcPEPCK2*, and *RcPEPCK3* was higher in seedlings grown at 35ºC than at 20ºC (up to 4.3-fold). In seeds, expression of all three *RcPEPCK* isoforms was higher in samples collected at 20ºC than at 35ºC (up to 6.5-fold) (Figure 4a).

In leaves, expression of *RcFBPase1*, *RcFBPase2*, and *RcFBPase3* was higher in seedlings grown at 20ºC than at 35ºC (up to 3.2-fold) (Figure 4b). In roots, expression of *RcFBPase1* and *RcFBPase2* was higher in seedlings grown at 35ºC than at 20ºC (up to 2.9-fold). In seeds, expression of *RcFBPase2* and *RcFBPase3* was higher in samples collected at 20ºC than at 35ºC (up to 3.1-fold). The expression of *RcFBPase1* did not differ in roots and seeds in response to temperature (Figure 4b). *RcFBPase5* and *RcFBPase6* were not tested because of lack of full length sequences. No expression was detected for *RcFBPase4*.

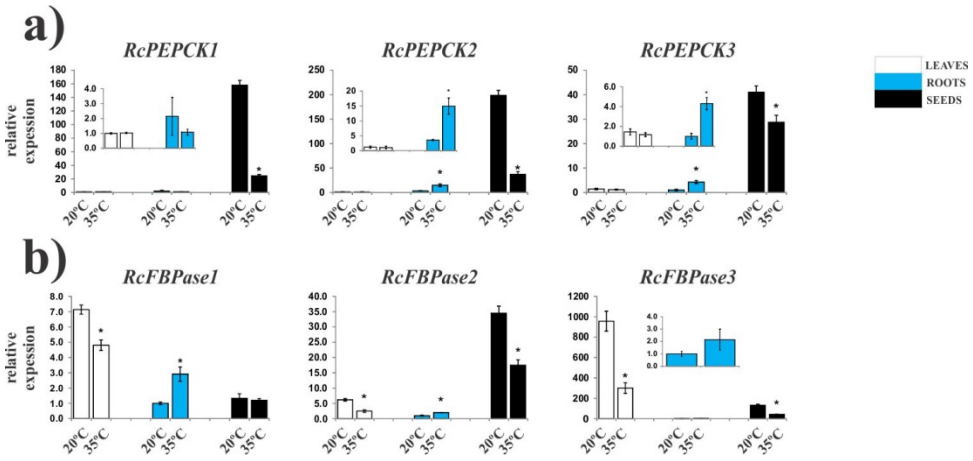

Figure 4. Transcript levels of genes related to gluconeogenesis. (a) ATP-dependent phosphoenolpyruvate carboxykinase (*RcPEPCK1*, *RcPEPCK2*, and *RcPEPCK3*), and (b) fructose-1,6-bisphosphatase (*RcFBPase1*, *RcFBPase2*, and *RcFBPase3*). Average and standard errors of three biological replicates containing 15-20 seeds or seedlings each are shown. Asterisks above the bars indicate significant differences between different temperatures within a given tissue by Student t-test ($p<0.05$).

4 Discussion

4.1 Transcript levels of genes involved in starch biosynthesis

Starch is the main storage carbohydrate in vascular plants and it is present in plastids of photosynthetic and non-photosynthetic tissues (Bahaji et al., 2014; Macdonald and ap Rees, 1983). In photosynthetic tissues, chloroplasts are able to fix carbon (as CO_2) for starch biosynthesis, whereas in non-photosynthetic tissues starch biosynthesis occurs in amyloplasts and is depending on the incoming supply of carbon precursors and energy from the cytosol (Bahaji et al., 2014).

4.1.1 Photosynthetic tissues

In the classic model, starch biosynthesis is linked to the Calvin-Benson cycle by means of plastidial phosphoglucoisomerase (Figure 5a). Phosphoglucoisomerases (PGI) exist as cytosolic (cPGI) and chloroplastic (pPGI) isoforms. PGI catalyzes the reversible isomerization of fructose 6-phosphate (F6P) to glucose-6-phosphate (G6P) (Backhausen et al., 1997; Gottlieb and Greve, 1981). Phosphoglucomutases (PGMs) catalyze the interconversion of G6P and glucose-1-phosphate (G1P). Similarly to PGI, PGM exists as cytosolic (cPGM) and as plastidial (pPGM) isoforms (Malinova et al., 2014; Periappuram et al., 2000). Subsequently, ADPG pyrophosphorylase (AGP) converts G1P and ATP to ADP-glucose (ADPG) and pyrophosphate (PPi) (Batra and Mehta, 1981). AGP catalyzes the first rate-limiting step in starch biosynthesis (Schwarte et al., 2015). In Arabidopsis leaves, the AGP holoenzyme mainly consist of *AtAPS1* and *AtAPL*1 (Crevillén et al., 2003; Hädrich et al., 2012). Reactions catalyzed by pPGI, pPGM, and AGP are reversible, but the reaction catalyzed by AGP is rendered irreversible upon hydrolytic breakdown of PPi by plastidial alkaline pyrophosphatase (Bahaji et al., 2014). Starch is then produced by starch synthase (SS) using ADPG as the sugar donor molecule. In Arabidopsis, each class of SS is represented by a single gene (*AtSSI*, *AtSSII*, *AtSSIII*, and *AtSSIV*) and the granule-bound starch synthase (*AtGBSS*). GBSS synthesizes amylose, whereas SSI to III catalyze distinct, but partly overlapping steps of amylopectin biosynthesis (Schwarte et al., 2015). SSIV is important for the initiation of starch granule biosynthesis, but can be functionally replaced by SSIII to some extent (Schwarte et al., 2015).

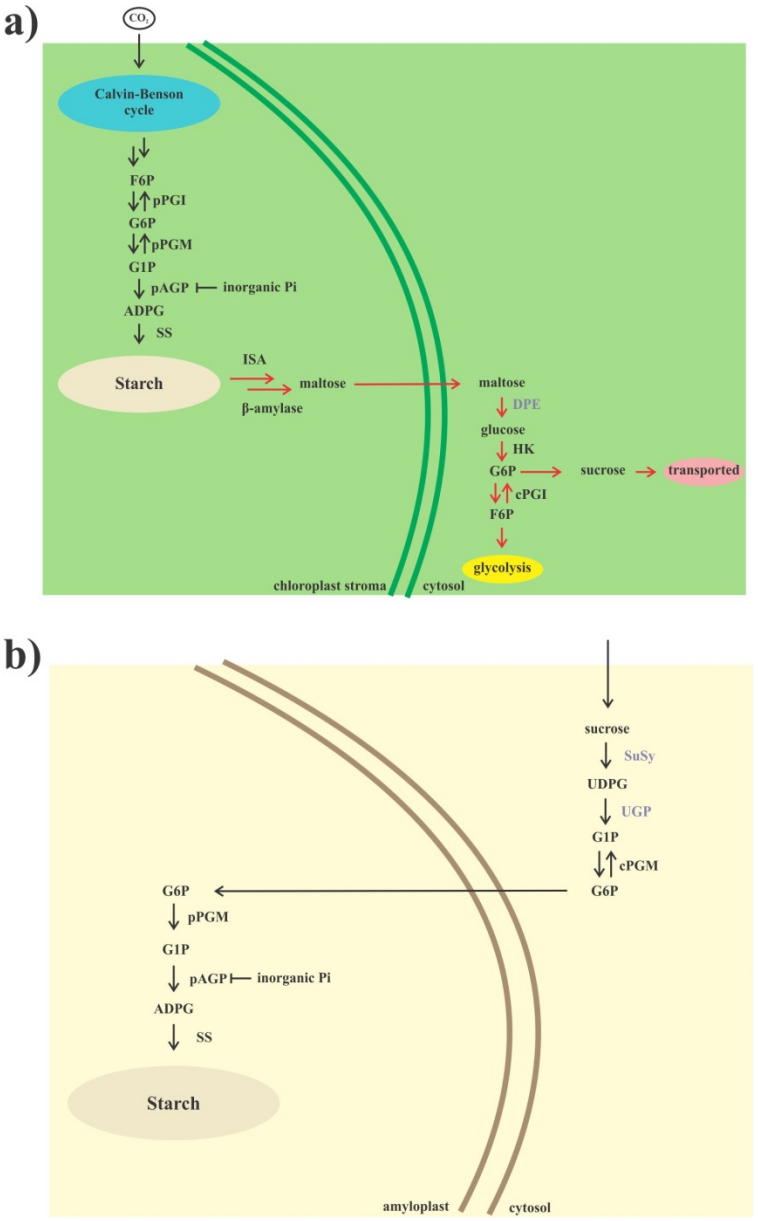

Figure 5. Schematic representation of genes related to (a) starch biosynthesis and (b) starch catabolism. Abbreviations in alphabetical order: **ADPG**: ADP-glucose. **PPi**: pyrophosphate. **AGP**: ADPG pyrophosphorylase. **F6P**: fructose 6-phosphate. **G1P**: glucose-1-phosphate. **G6P**: glucose-6-phosphate. **HK**: hexokinase. **ISA**: isoamylase. **cPGI**: cytosolic phosphoglucoisomerase. **pPGI**: chloroplastic phosphoglucoisomerase. **cPGM**: cytosolic phosphoglucomutases. **pPGM**: chloroplastic phosphoglucomutases. **SS**: starch synthase. **SuSy**: sucrose synthase. **UGP**: UDPG pyrophosphorylase.

Starch levels were higher in *R. communis* leaves of seedlings grown at 20ºC than at 35ºC (5.3-fold) (Ribeiro et al., 2014b). In this study, we show that expression of phosphoglucoisomerase (*RcPGI1* and *RcPGI2*) and starch synthase (*RcSS2*, *RcSS4*, *RcSS5*, and *RcSS6*) in *R. communis* leaves was not

affected by temperature. The expression of *RcPGM* and *RcAGP*, however, seems to be tightly regulated by temperature. Expression of *RcPGM* and *RcAGP* was higher in leaves of seedlings grown at 20ºC than at 35ºC. There is, therefore, a correlation between higher levels of starch and expression of *RcPGM* and *RcAGP* genes in *R. communis* leaves grown at 20ºC. Evidently, post-transcriptional, translational and post-translational control of these genes cannot be ruled out. For example, AGP is known to be allosterically inhibited by inorganic phosphate (Pi) (Kleczkowski, 1999). In *R. communis* leaves, levels of Pi were higher in seedlings grown at 35ºC than at 20ºC (1.6-fold) (Figure 4), which might suggest that not only *AGP* expression, but above that, also AGP activity is reduced at 35ºC as compared with 20ºC.

4.1.1 Non-photosynthetic tissues

In non-photosynthetic tissues, starch biosynthesis depends on sucrose that is imported from the aerial parts of the plant (Figure 5b). Sucrose is broken down in the cytosol of heterotrophic cells by sucrose synthase (SuSy) to produce fructose and UDPglucose (UDPG). UDPG is then converted to G1P and PPi by UDPG pyrophosphorylase (UGP). G1P is subsequently metabolized to G6P by means of cPGM (Bahaji et al., 2014). G6P is then transported to the amyloplasts for subsequent conversion into starch (Yu et al., 2000). In the amyloplasts, G6P is re-converted to G1P by means of pPGM. Similar to the reactions that take place in the chloroplasts, ADPG pyrophosphorylase (AGP) converts G1P and ATP into ADP-glucose (ADPG) and pyrophosphate (PPi) (Batra and Mehta, 1981). Unlike chloroplasts, amyloplasts are unable to photosynthetically generate ATP and therefore, cytosolic ATP must enter the amyloplast to produce ADPG by means of AGP (Bahaji et al., 2014). Starch is then produced by starch synthase (SS) using ADPG as the sugar donor molecule.

In this study, expression levels of only few genes involved in starch biosynthesis changed in response to temperature in roots of *R. communis*. In fact, no starch could be detected in its roots (Ribeiro et al., 2014b), indicating that starch does not accumulate in roots of this species. This is in agreement with the findings of Poiré et al. (2010) who reported that *R. communis* roots contained negligible amounts of starch.

Levels of starch were higher in seeds germinated at 20ºC than at 35ºC (2.2-fold) (Ribeiro et al., 2015b), which could possibly be explained by the higher expression of *RcPGM3*, *RcPGM5*, *RcAGP6*, *RcSS4*, and *RcSS5* in seeds germinated at 20ºC. Besides these changes in transcript levels, levels of Pi were higher in seeds germinated at 35ºC than at 20ºC (1.4-fold) (Figure 2), which might suggest that AGP activity is also reduced at 35ºC as compared with 20ºC. Transcripts of *RcSS5* were not detected in seeds that germinated at 35ºC, instead they were only found in seeds that germinated at 20ºC. This suggests that *RcSS5* plays a critical role in starch biosynthesis in *R. communis* seeds in response to temperature. Several

reports describe starch biosynthesis from oil reserves in germinating seeds (Chapman and Galleschi, 1985; Jordy and Favre, 2003). For example, during germination and post-germinative growth of *Pinus pinaster* seeds, triacylglycerols are hydrolyzed to fatty acids, which in turn are used for starch biosynthesis in the embryo (Jordy and Favre, 2003). Our data suggest that something similar may occur in *R. communis* seeds. Although a good correlation between starch levels and transcript levels of *RcPGM*, *RcAGP*, and *RcSS* genes was observed, their role in starch biosynthesis in response to temperature needs to be further investigated.

4.2 Transcript levels of genes involved in starch catabolism

Starch catabolism provides the supply of carbohydrates that is essential for normal plant growth (Smith et al., 2005). In leaves, starch catabolism occurs in the chloroplasts and the major products are G6P and sucrose. In the endosperm, starch catabolism takes place in the amyloplasts and the major product is glucose, which is taken up by the embryo to fuel its growth (Smith, 2012). The first step of starch catabolism in Arabidopsis leaves is the breakdown of starch granules, which is catalyzed by isoamylases (ISA) and β-amylases (Smith, 2012). *ISA3* and *β-amylase 3* (*BAM3*) are major players in starch catabolism in Arabidopsis leaves, but other isoforms may contribute as well (Fulton et al., 2008; Wattebled et al., 2005). These enzymes produce mainly maltose and small amounts of maltotriose (Fulton et al., 2008; Smith, 2012; Smith et al., 2005). Maltose is then exported from the chloroplast to the cytosol (Weise et al., 2004), where a glucanotransferase (DPE2) catalyzes the cleavage of the α1,4 linkage of β-maltose to produce glucose (Chia et al., 2004). In germinating barley grains, however, maltose is hydrolyzed via an α-glucosidase to glucose that is taken up by the embryo (Stanley et al., 2011). Glucose released from maltose via DPE2 is then converted to G6P via hexokinase (Smith et al., 2005).

In *R. communis* leaves, expression of *ISA1*, *ISA3*, *β-amylase 3*, and *β-amylase 7* was higher in seedlings grown at 20ºC than at 35ºC. Therefore, the higher levels of starch in leaves of seedlings grown at 20ºC as compared to 35°C seem to be the result from a coordinated up-regulation of genes involved in starch biosynthesis, which more than compensates for the up-regulation of genes involved in starch catabolism. In contrast, starch catabolism in *R. communis* roots seems to be enhanced at elevated temperatures, because most *ISA* and *β-amylase* genes are up-regulated at 35ºC as compared to 20ºC. These results suggest that in roots, high temperatures may have a greater effect on genes related to starch catabolism than on genes related to starch biosynthesis. In seeds, however, the expression of *ISA* and *β-amylase* was hardly affected by temperature. These results suggest that in seeds elevated temperatures may have a greater effect on genes related to starch biosynthesis than on genes related to starch catabolism.

4.3 Transcript levels of genes involved in gluconeogenesis

Mono- and disaccharides can be produced via photosynthesis, via the breakdown of stored starch, and via gluconeogenesis (Kobr and Beevers, 1971; Sánchez-Linares et al., 2012; Weitbrecht et al., 2011). Gluconeogenesis is the most likely source of mono- and disaccharides during germination of *R. communis* seeds (Beevers, 1969; Canvin and Beevers, 1961; Runquist and Kruger, 1999; Stewart and Beevers, 1967). Therefore, due to the fact that not only starch, but also fructose, glucose, and sucrose accumulated in *R. communis* seeds and seedlings grown at lower temperatures (Ribeiro et al., 2014b; Ribeiro et al., 2015a; Ribeiro et al., 2015b), expression of genes involved in gluconeogenesis were measured (Figure 6).

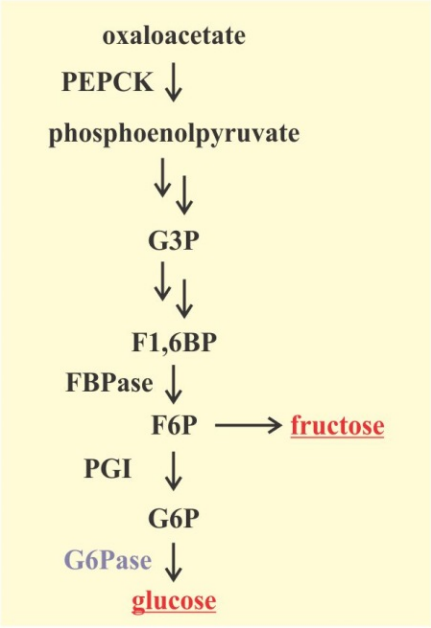

Figure 6. Schematic representation of genes related to gluconeogenesis. Abbreviations in alphabetical order: **F1,6BP**: fructose-1,6-bisphosphate. **F6P**: fructose 6-phosphate. **G3P**: glyceraldehyde-3-phosphate. **G6P**: glucose-6-phosphate. **G6Pase**: glucose-6-phosphatase. **PEPCK**: phosphoenolpyruvate carboxykinase. **PGI**: phosphoglucoisomerase.

Four-carbon compounds, such as oxaloacetate and succinate, can be converted to hexoses via gluconeogenesis and subsequently used for cell wall biosynthesis, or converted to sucrose and transported to other growing tissues of the seedling (Canvin and Beevers, 1961). Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the ATP-dependent decarboxylation of oxaloacetate to phosphoenolpyruvate, which is

a key step in photosynthetic CO_2 assimilation in leaves of some C4 plants and in gluconeogenesis following germination of triglyceride-storing seeds (Figure 6) (Leegood and Ap Rees, 1978; Walker and Leegood, 1996). PEPCK is regulated by phosphorylation, which alters its substrate affinity and its sensitivity to regulation by adenylates (Leegood and Walker, 2003). Phosphoenolpyruvate is further converted to fructose-1,6-bisphosphate (F1,6BP) via a few consecutive enzymatic reactions (Leegood and Ap Rees, 1978; Runquist and Kruger, 1999; Stewart and Beevers, 1967). Fructose-1,6-bisphosphatase is an important enzyme of gluconeogenesis that catalyzes the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate and Pi (Figure 6) (Nel and Terblanche, 1992).

In this study, we showed that expression of phosphoenolpyruvate carboxykinase (*RcPEPCK1*, *RcPEPCK2* and *RcPEPCK3*) were not affected by temperature in *R. communis* leaves. Genes encoding fructose-1,6-bisphosphatase, however, seem to be tightly regulated by temperature. Expression of *RcFBPase1*, *RcFBPase2*, and *RcFBPase3* were higher in leaves of seedlings grown at 20ºC than at 35ºC (up to 3.2-fold). Up-regulation of genes encoding for *RcFBPase*, therefore, is most likely causal for glucose, fructose, and sucrose accumulation in leaves grown at 20ºC. In contrast, gluconeogenesis in *R. communis* roots seems to be enhanced at elevated temperatures, because most *RcPEPCK* and *RcFBPase* genes were up-regulated at 35ºC (up to 4.3-fold) as compared to 20ºC. This is in agreement with the fact that carbohydrates are assumed to be mobilized from the roots, through the hypocotyl, to the aerial parts leading to preferred growth of the true leaves, at the expense of the roots (Ribeiro et al., 2014b). This mobilization of carbohydrates from roots to the aerial parts has been observed for several species. For example, mobilization of starch from *Caragana korshinskii* roots to support above-ground tissue growth after partial shoot removal is an important compensatory mechanism for growth and reproduction (Fang et al., 2006).

In seeds, expression of most of the *RcPEPCK* and *RcFBPase* genes is higher at 20ºC than at 35ºC (up to 6.5-fold). The glyoxylate cycle plays a central role in the conversion of storage triacylglycerol (TAG) to carbohydrates during early seedling growth in oil-rich seeds. Fatty acids released from TAG by lipolysis are usually transported across the glyoxysome by the comatose ABC transporter (*CTS*) and activated by *LACS* in the glyoxysome lumen where *β*-oxidation and part of the glyoxylate cycle occurs (Graham, 2008). Up-regulation of genes encoding for *RcPEPCK* and *RcFBPase* is, therefore, most likely causal for glucose, fructose, and sucrose accumulation in *R. communis* seeds germinated at 20ºC. Taken together, these results provide new insights into tissue-specific changes in transcript levels of genes related to gluconeogenesis and their correlation with carbohydrate content in response to temperature.

5 Conclusions

In this paper we raised the question whether carbohydrate accumulation in *R. communis* leaves, roots and seeds grown at low temperatures results from up-regulation of starch biosynthetic pathways, from down-regulation of its catabolic pathways, or both. Based on the steady-state levels of the expression of genes related to carbohydrate metabolism, we were able to dissect the regulation of the metabolic signature of *R. communis* that may apply to other crops, as well. Although some genes involved in starch catabolism were up-regulated in leaves grown at 20ºC compared with 35ºC, up-regulation of genes involved in starch biosynthesis seems to compensate for this and, therefore, is the likely explanation for higher levels of starch in leaves of seedlings grown at 20ºC. Higher levels of fructose, glucose, and sucrose in leaves of seedlings grown at 20ºC may be caused by a coordinated up-regulation of starch catabolism and gluconeogenesis pathways. In roots, temperature had a greater effect on genes related to starch catabolism rather than starch biosynthesis. In fact, starch catabolism and gluconeogenesis seem to be enhanced at elevated temperatures. At elevated temperature, carbohydrates are assumed to be mobilized from the roots to the shoots, leading to preferred growth of the aerial parts. In seeds, temperature had a greater effect on genes related to starch biosynthesis as compared to starch catabolism. Higher levels of starch in *R. communis* seeds germinated at low temperatures could be explained by higher expression levels of genes involved in starch biosynthesis, such as *RcPGM3, RcPGM5, RcAGP6, RcSS4*, and *RcSS5*. Up-regulation of genes encoding for *RcPEPCK* and *RcFBPase* are most likely causal for the observed fructose and glucose accumulation in seeds germinated at 20ºC. Taken together, these results provide new insights into tissue-specific changes in expression of genes related to gluconeogenesis, starch biosynthesis and catabolism and their relation with glucose, fructose, sucrose, and starch content in response to temperature.

6 Authors' contribution

P.R. Ribeiro designed the experiment, carried out gene expression assays, performed statistical analysis, and wrote the manuscript. W. Ligterink and H.W.M. Hilhorst supervised the experiments. All authors read and approved the final manuscript.

7 Conflict of interest

The authors declare no conflict of interest.

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9 Supplementary Table Captions

Supplementary Table 1. Description of target genes used for expression analysis.

Supplementary Table 2. Description of primers of target genes that were not expressed in R. communis leaves, roots and seeds.

Supplementary tables can be found at http://www.wageningenseedlab.nl/thesis/prribeiro/SI/

Chapter 8

General discussion

The overall objective of this thesis was to provide a detailed analysis of the temperature-responsive biochemical and molecular mechanisms that play a role during seed germination and seedling establishment of *Ricinus communis*. I focused my work on the effect of temperature on important metabolic pathways such as those involved in amino acid and carbohydrate metabolism. This chapter discusses the main findings of this thesis and presents future perspectives.

Seed germination and seedling establishment

Seed germination is one of the most important steps in the life cycle of higher plants. It begins with the uptake of water by the seed (imbibition), is followed by reactivation of metabolism, and ends with visible protrusion of the radicle through the surrounding layers (Bewley et al., 2013). In higher plants, seed germination is controlled by a combination of genetic and environmental factors. The endogenous balance in the levels of two plant hormones, gibberellin (GA) and abscisic acid (ABA), regulates the process of seed germination. GAs promote seed germination (Lee et al., 2002; Leubner-Metzger, 2001; Linkies and Leubner-Metzger, 2012), whereas ABA inhibits it (Garciarrubio et al., 1997; Hu et al., 2010; Subbiah and Reddy, 2010). Not only the endogenous balance, but also the sensitivity to these two hormones regulates seed germination. For example, *abi4* and *abi5*, two ABA-insensitive mutants of *Arabidopsis thaliana* showed germination percentages between 91 and 100% in 3 µM ABA, whereas only 1% of the seeds of the wild-type germinated under these conditions (Finkelstein, 1994). Environmental factors affecting seed germination include osmotic stress, light, and temperature. Osmotic stress can delay or even block seed germination and also prevent seedling growth (Farooq et al., 2009; Kaya et al., 2006). For example, sunflower seeds germinated in a polyethylene glycol solution of -0.9MPa showed impaired seedling development since radicle growth stopped after radicle emergence (Kaya et al., 2006). Light is another important regulator of seed germination in several plant species. Light perception is mediated by phytochromes which represent a class of photoreceptors that induce seed germination by modulating endogenous levels of GA and ABA, as well as GA responsiveness (Seo et al., 2009). Finally, temperature is a critical environmental cue that influences seed germination and seedling establishment. In general, a difference of only a few degrees may already lead to a notable change in germination rate and final percentage, but also in growth and survival rate of the seedling emerging from the seed (Salisbury and Ross, 1985).

Environmental factors may also indirectly affect seed germination during seed maturation (Cendán et al., 2013; He et al., 2014; Mondoni et al., 2014; Sales et al., 2013; Vu et al., 2015). For example, low temperature (15°C) during maturation of Arabidopsis seeds resulted in seeds with decreased seed longevity, and decreased germination in salt and mannitol compared with seeds obtained under control conditions (25ºC) (He et al., 2014).

Following imbibition, cells of the embryo change to a metabolically active state in which several physiological, molecular, and biochemical events occur such as cell elongation, cell cycle activation, transcription, translation, cellular respiration, repair mechanisms and organelle reassembly (Graham, 2008; Miransari and Smith, 2014; Rajjou et al., 2012; Rosental et al., 2014; Weitbrecht et al., 2011). These processes are generally supported by the initiation of central metabolism for energy generation and the production of building blocks for cellular structures and hormonal balance (Rosental et al., 2014). Nevertheless, when germination is completed the young seedling must rapidly establish its root system, as well as the photoautotrophic capability appropriate to its surrounding environment (Chen et al., 2008). Therefore, seed germination also determines whether seedling establishment will be successful or not. Cernac et al. (2006) suggested that seed germination and seedling establishment are metabolically distinct. However, such conclusion is only true to a certain extent. For example, the energy for germination in Arabidopsis is mostly derived from stored reserves other than lipids, and the seed oil becomes vital for continued growth and seedling establishment after the radicle has emerged (Cornah et al., 2004; Eastmond et al., 2000). However, in this thesis I showed that for *R. communis* a robust metabolic signature was observed during seed germination and seedling development. Most importantly, I showed that disturbance of pivotal metabolic pathways by high temperatures during seed germination has a profound impact on seedling development. This metabolic signature and the effect of temperature on important metabolic pathways will be discussed in the following sections.

R. communis is a drought tolerant species and this tolerance seems to be associated with an efficient stomatal control and the capacity to keep high net CO_2 fixation rates under drought stress conditions (Sausen and Rosa, 2010). However, there is a lack of studies that assess the effect of temperature on *R. communis* seed germination and seedling growth. Therefore, it is of pivotal importance to study physiological, molecular, and biochemical aspects of *R. communis* seed germination and seedling growth in response to temperature. Since *R. communis* possesses the ability to grow in various diverse environments it makes this species an ideal candidate to provide a better understanding of seedling performance and adaptation under different temperatures.

Identification of reference genes for gene expression studies during seed germination and seedling establishment in *Ricinus communis*

The agronomic importance of *R. communis* has spurred molecular research related to seed maturation (Cagliari et al., 2010; Loss-Morais et al., 2013; Sánchez-García et al., 2010), plant development (O'Leary et al., 2011), and responses to biotic and abiotic stresses (Maciel et al., 2011; Wei et al., 2010). Characterization and expression profiles of several genes from *R. communis* have been reported (Cagliari et al., 2010; Chileh et al., 2010; Maciel et al., 2011; Mhaske et al., 2013; Wei et al., 2010). There is, however, a lack of studies that provide a reliable set of reference genes for Real Time Quantitative PCR (RT-qPCR) studies of *R. communis* seed germination and seedling growth. Several reports show a reckless selection of reference genes for RT-qPCR studies, most of them without confirming if the candidate gene can indeed be used as reference gene. For example, Li et al. (2012) identified 47 members of the WRKY family in *R. communis* and assessed their expression profile during plant development. For that, they used 18S RNA as the reference gene. Cagliari et al. (2010) reported the identification of 26 genes that are involved in different steps of triacylglycerol (TAG) biosynthesis in *R. communis*. They characterized the expression profiles of these genes in relation to accumulation of ricinoleic acid and TAG during seed development. They claimed to use two genes with the most stable expression pattern throughout seed development as reference genes: EF1B/elongation factor 1-beta and Ubi9/ubiquitin. They did not show, however, how they assessed the stability of these genes or even how "stable" the expression of these two genes was.

In Chapter 2 a detailed analysis of the expression profile of 17 candidate reference genes was performed across a set of 14 samples that included different tissues, developmental stages, and environmental conditions. Furthermore, to illustrate the usefulness and to validate the candidate reference genes for analysis of gene expression purposes, I analyzed the expression levels of three *R. communis* genes which are involved in primary energy metabolism. More importantly, my data showed that some of the widely used reference genes were not suitable reference genes for my samples, highlighting the importance of a proper validation of candidate reference genes for each study. This is the first systematic report on the selection of reference genes for *R. communis* and provides useful guidelines for future accurate gene expression profiling experiments by RT-qPCR.

GABA as a drought stress-responsive molecule

The first step in the process of seed germination occurs when dry seeds come into contact with water under favorable conditions. This step is called 'seed imbibition' and it is driven by the matrix potential of the seed. In dry seeds the matrix potential is very low (down to more than -100 MPa) causing

a rapid influx of water (Woodstock, 1988). As seed imbibition progresses, the matrix potential increases and the water influx is reduced (Bewley et al., 2013). Based on the measurement of the water content of *R. communis* seeds during imbibition I showed that a progressive increase in temperature leads to faster imbibition. In fact, the water content was greater in seeds imbibed for 6 hours at 35^{o}C than at 25^{o}C or 20^{o}C. Faster imbibition at elevated temperatures had two important consequences: it accelerated germination and reduced levels of GABA in 6h-imbibed seeds. First, I will discuss the possible role of GABA accumulation during early imbibition and later suggest possible implications of faster imbibition on seed germination and seedling establishment.

Several reports have shown that GABA accumulates rapidly in plant tissues exposed to a variety of stresses (Bartyzel et al., 2004; Kinnersley and Turano, 2000; Locy et al., 1996). Possible functions of GABA include regulation of cytosolic pH, nitrogen storage, plant development, plant defence, and carbon-nitrogen metabolism (Akçay et al., 2012; Bouché and Fromm, 2004; Kinnersley and Turano, 2000). GABA also acts as an osmolyte to mitigate drought stress (Kramer et al., 2010; Krishnan et al., 2013; Shelp et al., 1999; Vijayakumari and Puthur, 2015). Additionally, the GABA shunt is a way to assimilate carbon from glutamate and to generate C:N fluxes that enter the tricarboxylic acid cycle (Bouché and Fromm, 2004)..

Since all germination experiments in this thesis were performed under controlled conditions with temperature as the only variable, I expected higher levels of GABA in seeds imbibed at 35ºC than at 20ºC or 25ºC. Curiously, that was not the case during early imbibition. In fact, GABA levels were higher in seeds imbibed at 20ºC and 25ºC. In agreement with these results, transcript levels of *GAD* (glutamate decarboxylase) were higher in seeds imbibed at 20ºC than at 35ºC. Thus, there is a good correlation between expression of *GAD* and GABA content. The question is what might have caused the observed accumulation of GABA in seeds at 20ºC. Firstly, a myriad of environmental stresses could lead to GABA accumulation. Secondly, it is important to keep in mind that because of the very negative matrix potential of hydrating seeds, initially little or no water is left for uptake by the cells of the embryo (Figure 1). With that in mind it is reasonable to assume, based on their water content, that 6-hour imbibed seeds at 35ºC are more hydrated than 6-hour imbibed seeds at 20ºC and 25ºC, although in both conditions they are not fully hydrated. The practical consequence of this differential hydration state is that seeds imbibed at 20ºC and 25ºC experience a lower matrix potential than seeds imbibed at 35ºC. This reduced hydration state at low temperatures seems to be perceived by the seeds as a drought stress-like condition. This drought stress-like condition is most likely the explanation for GABA accumulation in seeds imbibed at 20ºC and 25ºC as compared to 35°C. There are several reports that show that GABA accumulates in response to drought stress (Bor et al., 2009; Krasensky and Jonak, 2012; Krishnan et al., 2013; Vijayakumari and Puthur,

2015; Wenzel et al., 2014), but none has shown a clear correlation between GABA levels and the water content of the seeds.

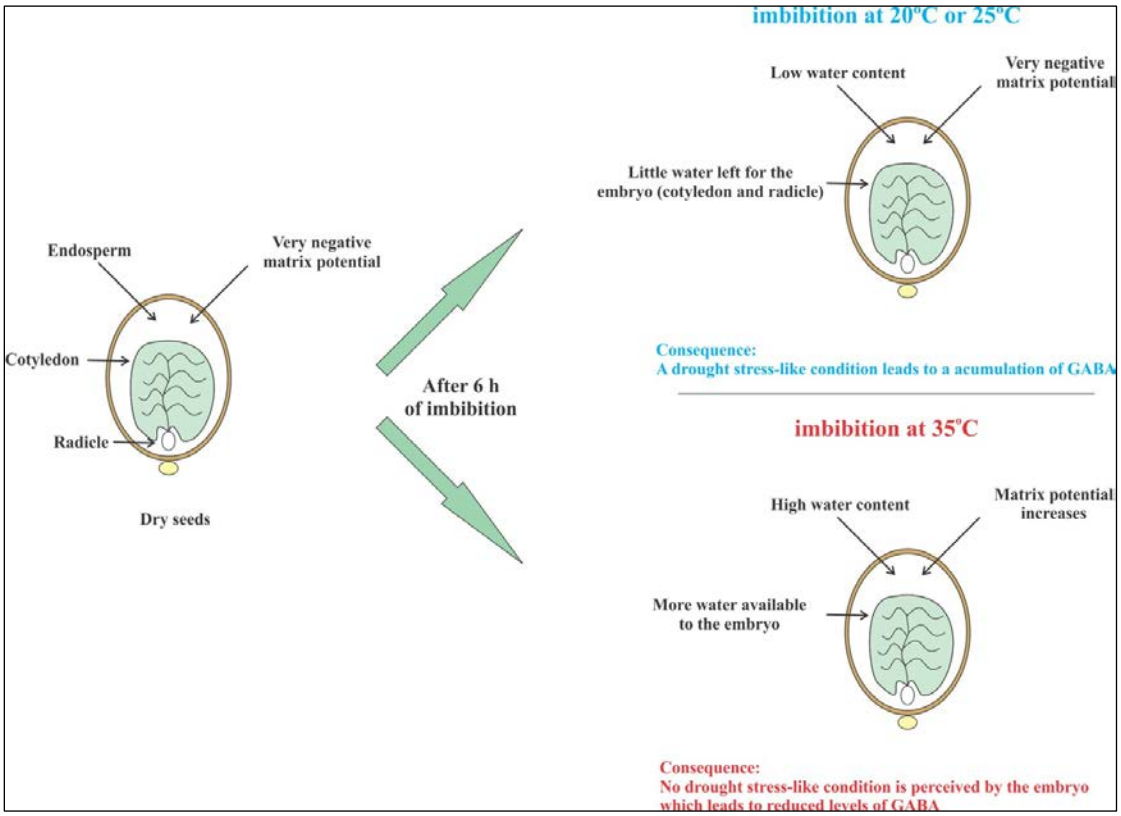

Figure 1. Schematic representation of changes in water content of *R. communis* seeds during early imbibition and relationship with the matrix potential and GABA accumulation in response to temperature.

After radicle protrusion, GABA levels increased in seedlings imbibed at 35ºC as compared with seeds imbibed at 20ºC and 25ºC. This seems to be an early temperature-stress response that is also present in later stages of seedling development, since I showed in Chapter 5 that 14-day-old seedlings grown at 35ºC had higher levels of GABA than 14-day-old seedlings grown at 20ºC. In Chapter 3 I showed that this increase in GABA levels in response to the increasing temperature correlated with higher expression levels of *GAD*. Therefore, during early seedling development GABA accumulation seems to occur as the result of high temperature stress.

GABA may protect biological membranes and balance the decrease in water potential that occurs upon drought stress (Heber et al., 1971; Kinnersley and Turano, 2000). The mechanism by which GABA accumulation could mitigate plant stress relies on the linkage between stress perception and functional physiological responses. Glutamate decarboxylase (GAD) catalyses the formation of GABA from glutamate and is regulated by the cytosolic concentration of H^+ or Ca^{2+} (Baum et al., 1993; Baum et al.,

1996). Increased levels of cytosolic Ca^{2+} lead to the formation of complexes with calmodulin (CaM). Then, the complex Ca^{2+}/CaM activates GAD in the physiological pH range (Baum et al., 1993; Baum et al., 1996). The Ca^{2+}/CaM complex induces the expression of stress responsive genes and may also be involved in acquisition of minerals that activate enzymes in stress-related metabolic pathways (Kinnersley and Turano, 2000). Our results indicate a possible role of GABA during seed imbibition and germination, although more research is needed to fully understand its functional significance.

The perception of heat stress by plants

Higher plants are sessile organisms and, therefore, cannot simply escape from their surroundings in case they experience unfavorable environmental conditions. They must develop a series of biochemical and molecular responses in order to adapt to changing environments (Shao et al., 2007). Tolerance to abiotic stresses is a very complex phenomenon, in part because stress may occur at multiple stages of plant development and often more than one stress simultaneously affects the plant (Chinnusamy et al., 2004). The way in which plants perceive abiotic stresses and switch on adaptive responses is critical to determine their survival and reproduction under unfavorable environmental conditions (Chinnusamy et al., 2004). On the one hand, high temperatures have a profound effect on plant growth and development (Buchwal et al., 2013; Zhang et al., 2013). On the other hand, plants possess a number of adaptive and acclimation mechanisms to cope with high-temperature stress (Černý et al., 2014; Hasanuzzaman et al., 2013; Lipiec et al., 2013; Yamori et al., 2014). Plant responses to high-temperature stress may vary with the degree and duration of exposure to heat, but also is mainly dependent on the plant species. As a result, differences in stress tolerance between genotypes or different developmental stages of a single genotype may arise from differences in signal perception and transduction mechanisms (Chinnusamy et al., 2004). In this context, plant survival under unfavorable environmental conditions will depend on the plant's ability to perceive the stress, generate and transmit the signal, and initiate appropriate physiological and biochemical changes (Hasanuzzaman et al., 2013). For this reason, it is difficult to draw a line to define which temperature is to be considered as a stress condition for plants. I showed that *R. communis* seeds imbibed at 35ºC germinated faster and with a high germination percentage. However, faster germination did not necessarily imply a better performance during seedling establishment. In fact, seedling survival was dramatically reduced at elevated temperatures. If seeds are germinated at 25ºC and then transferred to 35ºC, 100% of the seedlings survive. Thus, we identified a temperature sensitive window during germination in which high temperatures compromise subsequent seedling development.

Metabolic signature of *R. communis* seeds and seedlings in response to temperature

Initial vegetative growth is a very important stage in the establishment of plants and since little is known about biochemical and molecular changes related to temperature adaptation in *R. communis*, I raise the question whether such a highly adaptable species has a metabolic signature in response to temperature. To address this question, I used a multi-omics approach which involved the use of several metabolite profiling techniques employing GC-TOF-MS, GC-MS, and HPLC. Based on the metabolite profiling results, I measured the transcript levels by RT-qPCR of some key genes involved in carbohydrate metabolism.

Carbohydrate and amino acid metabolism appear to be key responsive elements of plasticity and tolerance mechanisms, as suggested by several temperature-responsive metabolite profiling studies to date (Cook et al., 2004; Guy et al., 2008; Kaplan et al., 2004; Obata and Fernie, 2012; Palma et al., 2014; Qi et al., 2011; Rizhsky et al., 2004; Zhang et al., 2013). For example, Rizhsky et al. (2004) assessed the metabolic and molecular responses of Arabidopsis plants to a combination of drought and heat stress. The plants accumulated sucrose and other carbohydrates such as glucose, maltose, melibiose, gulose, and mannitol in response to a combination of drought and heat stress (Rizhsky et al., 2004). A general accumulation of most amino acids and a dramatic decrease in most organic acids and carbohydrates at early time points of low-temperature stress were reported in rice (Zhao et al., 2013). Many studies report that an indistinct and unidirectional increase in the content of most of the identified metabolites seems to be the main response to a variety of environmental stimuli. For example, both heat stress and cold acclimation in Arabidopsis led to a coordinated increase in the content of amino acids, TCA intermediates (fumarate and malate), amine-containing metabolites (β-alanine, GABA, and putrescine) and some carbohydrates, such as maltose, sucrose, raffinose, galactinol and myo-inositol (Gray and Heath, 2005; Kaplan et al., 2004; Kaplan et al., 2007; Rizhsky et al., 2004).

R. communis displayed a high plasticity during initial vegetative growth, which was also reflected in the metabolome of the seeds and seedlings. An increase in temperature did not lead to an indiscriminate accumulation of the identified metabolites. Instead, *R. communis* seedlings seem to have a specific metabolic signature to adjust growth and developmental processes in response to higher temperatures possibly to maintain cellular homeostasis. A shift in the carbon-nitrogen metabolism toward the accumulation of carbohydrates at low temperatures and the accumulation of amino acids at high temperatures is an important biochemical response of *R. communis* seeds and seedlings to temperature. Levels of starch and soluble carbohydrates were up to 20-fold higher in seeds and seedlings grown at 20ºC than at 35ºC, whereas levels of amino acids, such as methionine, tyrosine and tryptophan, were up to 220-

fold higher in seeds and seedlings grown at 35ºC than at 20ºC. This biochemical response was observed during seed imbibition, germination, as well as early seedling growth.

In order to understand the biochemical basis of the changes in the metabolic signature, we investigated whether the higher carbohydrate accumulation in *R. communis* leaves, roots, and seeds, grown at low temperatures results from up-regulation of biosynthetic pathways, from down-regulation of catabolic pathways, or both. For that, I measured transcript levels of genes encoding key enzymes involved in starch biosynthesis, starch catabolism, and gluconeogenesis in seeds and seedlings of *R. communis* grown at 20ºC and 35ºC. Although some genes involved in starch catabolism were up-regulated in leaves of seedlings grown at 20ºC compared with 35ºC, up-regulation of genes involved in starch biosynthesis seems to compensate for this and, therefore, is the likely explanation for higher levels of starch in leaves of seedlings grown at 20ºC (Figure 2). Higher levels of fructose, glucose, and sucrose in leaves of seedlings grown at 20ºC compared with 35ºC may result from a coordinated up-regulation of starch catabolism and gluconeogenesis pathways. In Chapter 5 I inferred that elevated temperatures triggered carbohydrate catabolic pathways in both roots and leaves. Carbohydrates were assumed to be mobilized from the roots, through the hypocotyl, to the aerial parts, leading to preferred growth of the true leaves, at the expense of the roots, since starch degradation and gluconeogenesis seem to be enhanced in the roots of seedlings grown at 35ºC compared with 20ºC (Figure 2).

In seeds, temperature had a greater effect on genes related to starch biosynthesis than on genes related to starch catabolism (Figure 2). Higher expression levels of phosphoglucomutase (*RcPGM*) and starch synthase (*RcSS*) genes is the likely explanation for higher levels of starch in seeds germinated at 20ºC, whereas higher expression levels of phosphoenolpyruvate carboxykinase (*RcPEPCK*) and fructose-1,6-bisphosphatase (*RcFBPase*) genes is the likely explanation for higher levels of fructose, glucose, and sucrose in these seeds. Evidently, post-transcriptional, translational and post-translational control of these genes also cannot be ruled out. For example, the enzyme ADP-glucose pyrophosphorylase (AGP) is known to be allosterically inhibited by inorganic phosphate (Pi) (Kleczkowski, 1999). Since phosphate levels were higher in *R. communis* leaves, roots, and seeds grown at 35ºC than at 20ºC, it seems that not only *AGP* expression, but also AGP activity is reduced at 35ºC as compared with 20ºC. Although the physiological role of the metabolic changes is not fully understood, the results shown in this study provide important insights for the understanding of seed germination and seedling establishment in response to temperature.

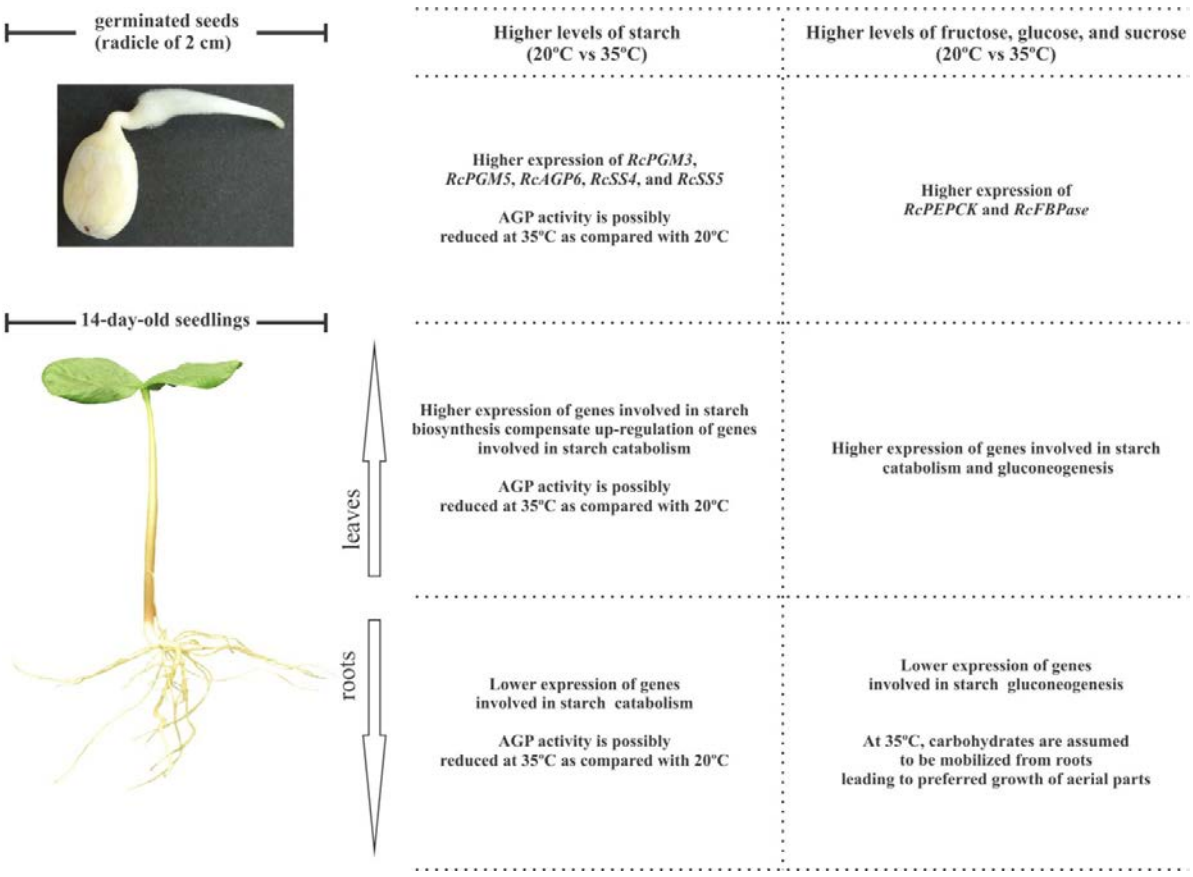

Figure 2. Schematic representation of expression levels of genes encoding key enzymes involved in starch biosynthesis, starch catabolism, and gluconeogenesis and its relation to carbohydrate accumulation at lower temperatures.

Temperature-mediated requirements for successful seedling establishment

Mobilization of seed storage compounds during germination is a crucial process to set proper conditions for seedling establishment (Graham, 2008; Weitbrecht et al., 2011). Initial imbibition is often accompanied by large metabolic changes, followed by an increase in respiratory metabolism and gene expression (Bewley et al., 2013; Botha et al., 1992; Sánchez-Linares et al., 2012; Vicente-Carbajosa and Carbonero, 2005). For example, metabolite profiling of rice embryos during germination showed that one hour after imbibition rapid changes in the metabolism occurred, including increases in hexose phosphates, tricarboxylic acid cycle intermediates, and GABA (Howell et al., 2009). Later changes in carbohydrate-, amino acid-, and cell wall metabolism, appeared to be driven by increases in transcript levels which were observed to increase from 12 hours after imbibition onwards (Howell et al., 2009).

The germination process of an oilseed is characterized by the mobilization of storage lipids from the oil bodies stored in the endosperm (Yaniv et al., 1998). Triacylglycerol (TAG) is a major seed storage

reserve in oilseed plant species which accumulates during seed development. TAG is stored in the seed until germination, after which it is used to fuel initial seedling growth (Graham, 2008). TAGs exist in close proximity of glyoxysomes, the single organelles that house most of the biochemical machinery required to convert fatty acids derived from TAG to 4-carbon compounds. The 4-carbon compounds in turn are converted to soluble carbohydrates that are used to fuel seedling growth (Graham, 2008). In *R. communis*, fatty acids are converted to carbohydrates through gluconeogenesis (Kobr and Beevers, 1971).

In this thesis, I showed that *R. communis* seeds germinate faster and to a higher percentage at 35ºC than at lower temperatures, but this is followed by compromised seedling establishment. Thus, we undertook a functional genomics approach using microarray and RT-qPCR analysis to determine transcriptome changes during seed germination at 20, 25 and 35ºC that could explain this phenotype. The objective of these experiments was to assess the effect of the temperature on important biochemical and molecular mechanisms required for successful seedling establishment.

The results presented in Chapters 3 and 4 demonstrate that temperature has a profound effect on major metabolic processes very early after imbibition has started. It seems that high temperature has a negative effect on important energy-generating pathways, such as the Calvin-Benson-Bassham cycle, gluconeogenesis, starch- and triacylglycerol degradation, and fatty acid oxidation. For example, the initial steps of storage oil breakdown are enhanced at 20ºC, as compared to higher temperatures which is in agreement with the lower levels of FFAs in samples of germinated seeds with a radicle of 2 cm at 20ºC. Phosphoenolpyruvate carboxykinase (*RcPEPCK*) and fructose-1,6-bisphosphatase (*RcFBPase*) are genes involved in gluconeogenesis, which is a metabolic pathway that results in the generation of glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids (Kobr and Beevers, 1971; Leegood and Ap Rees, 1978; Stewart and Beevers, 1967). Gluconeogenesis is an important energy-generating pathway for germinating *R. communis* seeds. In Arabidopsis the energy for germination is derived from stored reserves other than lipids, and seed oil becomes vital for continued growth and seedling establishment after the radicle has emerged (Cernac et al., 2006). The glyoxylate cycle is regarded as essential for seedling establishment of oilseed plants (Eastmond et al., 2000). This corroborate our findings that down-regulation of important energy-generating pathways during seed germination at high temperatures compromises seedling establishment, but not seed germination. Most of these differences in the germination transcriptome occurred between 6 hours of imbibition and radicle protrusion. Interestingly, only three transcripts were down-regulated at elevated temperatures since early imbibition throughout germination: one oxidation-related zinc finger, one F-box and wd40 domain protein, and one DNA binding protein/ MYB-like transcription factor. The Arabidopsis homolog of oxidation-related zinc finger, oxidation-related zinc finger 2 (*AtOZF2*; AT4G29190), is involved in

regulation of transcription and has sequence-specific DNA binding transcription factor activity. *AtOZF2* is involved in ABA and salt stress responses through the ABI2-mediated signaling pathway (Huang et al., 2012). *AtOZF2* and its homolog *AtOZF1* are important regulators for plant tolerance to abiotic stress (Huang et al., 2011; Huang et al., 2012). WD40 proteins play a crucial role in diverse protein-protein interactions by acting as scaffolding molecules and thus assisting in the proper activity of proteins (Mishra et al., 2014; Mishra et al., 2012). Finally, MYB transcription factors are composed of one, two, or three imperfect helix-turn-helix repeats that recognize the major groove of DNA and which are involved in a variety of plant-specific processes, including cell morphogenesis, secondary metabolism, cell differentiation, and stress responses (Shin et al., 2011; Zhang et al., 2011).

Nine transcripts were upregulated at elevated temperatures since early imbibition throughout germination: one BET1P/SFT1P-like protein 14BB, one low-molecular-weight cysteine-rich protein LCR78, one WD-repeat protein, one GAST1 protein, one adenylate kinase 1/P-loop containing nucleoside triphosphate hydrolases superfamily protein, and four conserved hypothetical proteins. One conserved hypothetical protein (29667.m000353) showed high homology to chaperone binding-ATPase activators (AT5G58110). All these transcripts constitute interesting candidate genes for further characterization of temperature-responsive molecular mechanisms in *R. communis*.

Contribution to the field

Compared with major crops, growth and development of *R. communis* is still poorly understood. In this thesis I provide a collection of data that enables a better understanding of the biochemical and physiological aspects of germination and seedling growth in response to temperature.

The approach used in Chapter 2 allowed me to identify reference genes for *R. communis* and it provided important guidelines for RT-qPCR studies in seeds and seedlings of other species (especially in these cases where extensive microarray data is not available). In Chapters 3 and 4, I identified a temperature sensitive window during germination in which high temperatures compromise subsequent seedling development. Additionally, I also provided new insights into the role of GABA during seed germination and added important insights into the molecular requirements for vigorous seedling growth of *R. communis* under different environmental conditions. In Chapters 5 and 6, I demonstrated that *R. communis* showed high plasticity in response to changes of the environmental conditions during initial vegetative growth. *R. communis* seedlings showed a shift in their carbon-nitrogen metabolism as an important biochemical response to adjust growth and developmental processes to higher temperatures likely to maintain cellular homeostasis. It will be interesting to see if this metabolic signature also applies to other crops and if it is causal for plant plasticity in response to temperature. In addition to that, my

results provide important leads for the mechanisms underlying root biomass production in response to varying temperatures that are required to support vigorous seedling growth under high temperature, since greater levels of osmoprotectant molecules and up-regulation of other protective mechanisms might be cause or consequence of higher root growth. In Chapter 7, I provided important information about organ-specific changes in transcript levels of genes related to carbohydrate biosynthesis and catabolism in response to temperature (Figure 2). These results helped me to understand which metabolic pathways are responsible for the specific metabolic signature identified in the previous chapters.

Future perspectives

Based on the transcriptomics data we selected a number of candidate genes that are probably involved in, or required for, proper seed germination and seedling establishment under different temperatures. These genes are now being cloned and used for Agrobacterium-mediated stable transformation of *Arabidopsis thaliana* (floral dip). Overexpression Arabidopsis lines transformed with *R. communis* genes, as well as Arabidopsis T-DNA lines in which homologs of these genes are knocked-out are being generated for further phenotypical analysis. For that, we selected genes for transcription factors, a zinc finger protein, heat-shock proteins, malate synthase, and glycerol kinase which were identified based on the results of this thesis. These overexpression and T-DNA lines will help us to understand the molecular requirements for vigorous seedling growth of *R. communis* under different environmental conditions.

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Summary

The main objective of this thesis was to provide a detailed analysis of physiological, biochemical and molecular-genetic responses of *Ricinus communis* to temperature during seed germination and seedling establishment.

In Chapter 2, I describe the assessment of 17 candidate reference genes across a diverse set of samples, including several tissues, various developmental stages and environmental conditions, encompassing seed germination and seedling growth in *R. communis*. These genes were tested by RT-qPCR and ranked according to the stability of their expression using two different approaches: GeNorm and NormFinder. Both GeNorm and Normfinder indicated that *ACT*, *POB* and *PP2AA1* represent the optimal combination for normalization of gene expression data in inter-tissue studies. I also describe the optimal combination of reference genes for a subset of samples from root, endosperm and cotyledonary tissues. The selection of reference genes was validated by normalizing the expression levels of three target genes involved in energy metabolism with the identified optimal reference genes. This approach allowed me to identify stably expressed genes, and, thus, reference genes for use in RT-qPCR studies in seeds and seedlings of *R. communis*.

In Chapter 3, a thermo-sensitive window is identified during seed germination in which high temperatures compromise subsequent seedling development. I assessed the biochemical and molecular requirements of *R. communis* germination for successful seedling establishment at varying temperatures. For that, I performed metabolite profiling (GC-TOF-MS) and measured transcript levels of key genes involved in several energy-generating pathways such as storage oil mobilization, β-oxidation of fatty acids and gluconeogenesis of seeds germinated at three different temperatures. Transient overexpression of genes encoding for malate synthase (*MLS*) and glycerol kinase (*GK*) resulted in higher starch levels in *N. benthamiana* leaves, which highlights the likely importance of these genes in energy-generating pathways for seedling establishment. Additionally, I showed that γ-aminobutyric acid (GABA), which is a stress-responsive metabolite, accumulated in response to the water content of the seeds during the initial phase of imbibition.

In Chapter 4 I undertook a genomics approach using microarray analysis to determine transcriptome changes in three distinct developmental stages during seed germination at 20, 25 and 35ºC that could explain the thermo-sensitive window that is described in Chapter 3. Most of the differences in the *R. communis* transcriptome occurred between 6 hours of imbibition and the commencement of germination, i.e. radicle protrusion. This coincides with the thermo-sensitive window identified during seed

germination in which high temperatures compromise seedling development. The transcriptome data was used to identify heat-stress responsive genes that might be involved in thermotolerance of *R. communis* during germination. Temperature had a major effect on genes involved in energy generating pathways, such as the Calvin-Benson-Bassham cycle, gluconeogenesis, and starch- and triacylglycerol degradation. Transcripts coding for ATP binding proteins, DNA binding proteins, RNA binding proteins, DNA-directed RNA polymerases I, II, and III, heat shock factor proteins, multiprotein-bridging factor proteins, and zinc finger proteins were also affected by temperature suggesting the whole transcriptional regulatory machinery was altered in response to temperature. Among the downregulated transcripts under high temperature, only three were shared by all three stages: an oxidation-related zinc finger 2, an F-box and wd40 domain protein, and a DNA binding protein/MYB-like transcription factor. Among the upregulated transcripts, nine were shared by all three stages: a BET1P/SFT1P-like protein, 14BB, a low-molecular-weight cysteine-rich protein LCR78, a WD-repeat protein, a GAST1 protein, an adenylate kinase 1/P-loop containing nucleoside triphosphate hydrolases superfamily protein, and four conserved hypothetical proteins. These genes constitute good candidates for further characterization of temperature-responsive genes in *R. communis*.

In Chapter 5, I studied the genetic variation in the effect of temperature on growth of young *R. communis* seedlings and measured primary and secondary metabolites in roots and cotyledons of three *R. communis* genotypes, varying in stress tolerance. Seedling biomass was strongly affected by the temperature, with the lowest total biomass observed at 20ºC. The response in terms of biomass production for the genotype MPA11 was clearly different from the other two studied genotypes: genotype MPA11 produced heavier seedlings at all temperatures but the root biomass of this genotype decreased with increasing temperature, reaching the lowest value at 35ºC. In contrast, root biomass of genotypes MPB01 and IAC80 was not affected by temperature, suggesting that the roots of these genotypes are less sensitive to changes in temperature. A shift in carbon-nitrogen metabolism towards the accumulation of nitrogen-containing compounds seems to be the main biochemical response to support growth at higher temperatures. Carbohydrate content was reduced in response to increasing temperature in both roots and cotyledons, whereas amino acids accumulated to higher levels. The results in this chapter show that a specific balance between amino acids, carbohydrates and organic acids in the cotyledons and roots of genotype MPA11 seems to be an important trait for faster and more efficient growth of this genotype at higher temperatures.

In Chapter 6, I decided to focus on the differential ability of genotypes MPA11 and IAC80 to sustain root biomass production at higher temperatures. Biomass allocation was assessed by measuring dry weight of roots, stems, and cotyledons of seedlings grown at three different temperatures. Seedlings

grown at 25ºC and 35ºC showed greater biomass than seedlings grown at 20ºC. Cotyledon and stem dry weight increased for both genotypes with increasing temperature, whereas root biomass allocation showed a genotype-dependent behaviour. Genotype MPA11 showed a continuous increase in root dry weight with increasing temperature, while genotype IAC80 was not able to sustain further root growth at higher temperatures. Metabolite and gene expression profiles of genotype MPA11 demonstrated an increase in the levels of osmoprotectant molecules, such as galactinol and transcripts of genes encoding antioxidant enzymes and heat shock proteins, to a higher extent than in genotype IAC80.

In Chapter 7 I raised the question whether carbohydrate accumulation in *R. communis* leaves, roots, and seeds, grown at low temperatures, as compared to higher temperatures, results from up-regulation of biosynthetic pathways, from down-regulation of catabolic pathways, or both. To answer this question, transcript levels were measured of genes encoding enzymes involved in starch biosynthesis, starch catabolism, and gluconeogenesis in leaves, roots, and seeds grown at 20ºC and 35ºC. Transcript levels of genes involved in starch catabolism were higher in leaves grown at 20ºC than at 35ºC, but up-regulation of genes involved in starch biosynthesis seems to compensate for this and, therefore, is the likely explanation for higher levels of starch in leaves grown at 20ºC. Higher levels of soluble carbohydrates in leaves grown at 20ºC may have been caused by a coordinated up-regulation of starch catabolism and gluconeogenesis pathways. In roots, starch catabolism and gluconeogenesis seem to be enhanced at elevated temperatures. Higher levels of starch in seeds germinated at low temperatures is associated with higher transcript levels of genes involved in starch biosynthesis. Similarly, higher transcript levels of *RcPEPCK* and *RcFBPase* are most likely causal for fructose and glucose accumulation in seeds germinated at 20ºC.

This thesis provides important insights in the understanding of the plasticity of *R. communis* in response to temperature. The knowledge obtained may apply to other species as well. Additionally, based on the transcriptomics data, we selected several candidate genes that are potentially involved in, or required for, proper seed germination and seedling establishment under different temperatures, such as a number of transcription factors, a zinc finger protein, heat-shock proteins, malate synthase and glycerol kinase. Overexpressing Arabidopsis lines transformed with these *R. communis* genes, as well as Arabidopsis T-DNA lines, in which Arabidopsis homologs of these genes are knocked-out, are being generated for further phenotypical analysis. These overexpression and T-DNA lines should help us to understand the molecular requirements for vigorous seedling growth of *R. communis* under different environmental conditions.

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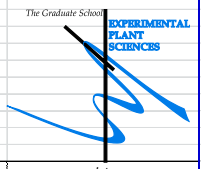
Education Statement of the Graduate School

Experimental Plant Sciences



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1) Start-up phase		*date*
►	**First presentation of your project**	
	Molecular and physiological changes in Ricinus communis seeds and seedlings in response to a changing environment	Mar 17, 2012
►	**Writing or rewriting a project proposal**	
	Molecular and physiological changes in Ricinus communis seeds and seedlings in response to a changing environment	Dec 2011-Feb 2012
►	**Writing a review or book chapter**	
	Chemical constituents and pharmacological activities of the oilseed crop Ricinus communis, Journal of Industrial Crops and Products, to be published	Dec 2014-Jun2015
	Subtotal Start-up Phase	*13.5 credits**
2) Scientific Exposure		*date*
►	**EPS PhD student days**	
	EPS PhD student day, University of Amsterdam	Nov 30, 2012
	EPS PhD student day, Leiden University	Nov 29, 2013
►	**EPS theme symposia**	
	EPS theme 2 'Interactions between Plants and Biotic Agents' Symposium & Willie Commelin Scholten Day, Wageningen University	Feb 10, 2012
	EPS theme 3 'Metabolism and Adaptation', University of Amsterdam	Mar 22, 2013
	EPS theme 3 'Metabolism and Adaptation', Wageningen University	Feb 10, 2015
►	**NWO Lunteren days and other National Platforms**	
	ALW meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 22-23, 2013
	ALW meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 14-15, 2014
►	**Seminars (series), workshops and symposia**	
	- EPS Flying Seminars -	
	Use of resurrection plants as models to understand how plants tolerate extreme water loss: A systems biology approach with applications for making drought tolerant crops - Jill Farrant	Jun 26, 2012
	Tackling natural variance in seed metabolism integrating metabolite profiles via network analysis - Aaron Fait	Dec 04, 2012
	ABA signaling networks in Arabidopsis - Ruth Finkelstein	Dec 14, 2012
	Arabidopsis thaliana as a model system for the study of evolutionary questions - Detlef Weigel	Feb 27, 2013
	Seasonal flowering in annual and perennial plants - George Coupland	Jan 19, 2015
	The evolutionary significance of gene and genome duplications - Yves van de Peer	Feb 03, 2015
	Tomato metabolomics in 2015, the difference a genome makes - Dr. Alisdair Fernie	Mar 11, 2015
	Evolution of floral signals in plants: mechanisms and consequences - Florian Schiestl	Mar 12, 2015
	Inferring species trees given coalescence and reticulation - Michael D. Pirie	Mar 18, 2015
	Long-distance endosome trafficking drives fungal effector production during plant infection - Gero Steinbe	Jun 05, 2015
	- Seminars Biometris -	
	Genotype–phenotype mapping in a post-GWAS world	Sep 17, 2012
	- Other seminars -	
	Strigolactones, new plant hormones. Importance of their stereochemistry for bioactivity as germination stimulant - Yukihiro Sugimoto	Oct 16, 2012
	Plant drug smugglers' about transport of secondary metabolites in plants - Marc Boutry	Mar 13, 2013
	Iridoid glycosides: biochemistry and role in biotic interactions in ribwort plantain - Arjen Biere	Apr 10, 2013
	Towards understanding rice brown spot, a disease induced by physiological stress - Monica Höfte	Feb 06, 2015
	Epigenetic regulation of seed dormancy and germination in Arabidopsis - Yongxiu Liu	Mar 17, 2015
	Structure and evolution of centromeres: lessons learned from plants - Jiming Jiang	Apr 01, 2015
	The uncovering of new transcription factors involved in ripening and post-harvest life of tomato and strawberry fruits - Sonia Osorio	Apr 15, 2015
	Hormone-related Functions of LEC2 in Somatic Embryogenesis Induction in Arabidopsis - Malgorzata	Apr 16, 2015
	Into the Battle between Plants and Viruses, but what about EVEs? - Richard Kormelink	May 12, 2015
	Polyploidy in wild relatives of soybean and other legumes: systematics, comparative and functional genomics, and nodulation - Jeff Doyle	May 12, 2015
►	**International symposia and congresses**	
	9th Annual International Conference of the Metabolomics Society - Scotland	Jul 01-04, 2013
	43rd Annual Meeting of SBBq - Brazil	May 17-20, 2014
	11th International Society for Seed Science - China	Sep 15-19, 2014
►	**Presentations**	
	Untargeted metabolite profiling of Ricinus communis seedlings in response to temperature changes - Lunteren, The Netherlands (Poster)	Apr 22-23, 2013
	Metabolite and gene expression profiles of Ricinus communis seedlings reveal a coordinated shift in carbon-nitrogen metabolism in response to an increase in temperature. - 9th Annual International Conference of the Metabolomics Society - Scotland (Poster)	Jul 01-04, 2013
	Biochemical and molecular requirements for successful seedling establishment in Ricinus communis - Lunteren, The Netherlands (Poster)	Apr 14-15, 2014
	Identification of reference genes for gene expression studies during seed germination and seedling establishment in Ricinus communis L - 43rd Annual Meeting of SBBq - Brazil (Poster)	May 17-20, 2014
	Biochemical responses of Ricinus communis seedlings to high temperature reveal a shift in the carbon-nitrogen balance - 43rd Annual Meeting of SBBq - Brazil (Poster)	May 17-20, 2014
	Biochemical and molecular requirements of Ricinus communis germination for a successful seedling establishment at varying temperatures. - 11th International Society for Seed Science - China (Talk)	Sep 15-19, 2014
	Biochemical and molecular requirements of Ricinus communis germination for a successful seedling establishment at varying temperatures. - 3rd Dutch Seed Symposium (Talk)	Oct 07, 2014
	Subtotal Scientific Exposure	*15.7 credits**
3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Multivariate analysis	Oct 09-11,18-19, 2012
	Systems Biology: statistical analysis of ~omics data'	Dec 10-14, 2012
	Introduction to R for Statistical Analysis	Jun 10-11, 2013
►	**Journal club**	
	Literature discussions in Plant Physiology	2012-2015
	Subtotal In-Depth Studies	*6.6 credits**
4) Personal development		*date*
►	**Skill training courses**	
	Information Literacy including EndNote Introduction	Oct 30-31, 2012
	Career Orientation	Mar 06, 13, 20, 27, 2015
	Techniques for Writing and Presenting a Scientific Paper	Apr 21-24 April 2015
	Subtotal Personal Development	*3.3 credits**
	TOTAL NUMBER OF CREDIT POINTS*	**39.1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS

** A credit represents a normative study load of 28 hours of study.*

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