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FOTOSSÍNTESE C_4 E METABOLISMO ÁCIDO DAS CRASSULÁCEAS (CAM) EM UMA MESMA FOLHA: ELUCIDANDO OS COMPONENTES, A PLASTICIDADE E A SINALIZAÇÃO POR TRÁS DE UMA RARA ADAPTAÇÃO FOTOSSINTÉTICA

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Orientador: Luciano Freschi
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foi por conta do apoio e incentivo constantes de vocês.

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Summary

Resumo

O ciclo C_4 e o metabolismo ácido das Crassuláceas (CAM) são dois mecanismos concentradores de carbono (CCM) prevalentes em plantas vasculares. Apresentam semelhanças bioquímicas, mas representam adaptações ecológicas bem diferentes, resultado de diferenças evolutivas, regulatórias e estruturais. Acreditava-se que seriam incompatíveis entre si, porém o gênero *Portulaca* desafia esta definição, visto que apresenta espécies C_3-C_4 intermediárias e C_4 capazes de alternar para CAM de acordo com a disponibilidade hídrica. Os mecanismos genéticos e regulatórios que possibilitam a ocorrência desta rara adaptação fotossintética são pouco conhecidos. Portanto, o objetivo desta tese de doutorado foi investigar a transição C_4 para CAM em *P. oleracea* do ponto de vista fisiológico e molecular. Após uma introdução geral ao tema, o capítulo I contextualiza e propõe a espécie como modelo de estudo da transição C_4-CAM. O capítulo II caracteriza a maquinaria genética básica envolvida em ambos os CCMs em *P. oleracea* a partir de dados de transcriptoma associados a análises filogenéticas e fisiológicas. O capítulo III explora a plasticidade morfo-fisiológica da espécie, caracterizando o CAM em diferentes subespécies provenientes de diferentes lugares do mundo. O capítulo IV discute as melhores estratégias e protocolos moleculares para explorar *P. oleracea* como um modelo C_4-CAM. Finalmente, o capítulo V caracteriza a sinalização molecular e hormonal durante a transição do C_4 para o CAM e sua reversão, bem como a influência do relógio circadiano sobre o funcionamento de ambos CCMs. No contexto de mudanças climáticas, este trabalho visou ressaltar que C_4 e CAM não representam apenas interessantes adaptações fisiológicas do ponto de vista funcional ou evolutivo, mas também são mecanismos importantes para o desenvolvimento da sociedade, por ocorrerem separadamente em cultivares utilizados na produção de alimentos e biocombustíveis. Desse modo, este trabalho trouxe informações que possibilitam explorar a complexidade de *Portulaca* como um mapa genético para o desenvolvimento futuro via engenharia genética de cultivares que possam intercalar C_4 e CAM em um mesmo organismo, conforme a disponibilidade de recursos no ambiente.

Abstract

The C_4 cycle and crassulacean acid metabolism (CAM) are two common carbon concentrating mechanisms (CCM) in vascular plants. They have biochemical similarities, but represent very different ecological adaptations, as a result of evolutionary, regulatory and structural differences. It was believed they were incompatible to occur in a single organism, but the genus *Portulaca* challenges this assumption, as it presents intermediate C_3-C_4 and C_4 species capable of switching to CAM according to water availability. However, the genetic and regulatory mechanisms that enable this rare photosynthetic adaptation to occur are still poorly understood. Therefore, the aim of this doctoral thesis was to investigate the C_4 to CAM transition in *P. oleracea* from a physiological and molecular point of view. After a brief introduction to the subject, chapter I contextualizes and proposes the species as a model for studying the C_4-CAM transition. Chapter II characterized the central genetic machinery involved in both CCMs in *P. oleracea* using transcriptome data associated with phylogenetic and physiological analyses. Chapter III explored the morphophysiological plasticity of the species, characterizing CAM in different subspecies originated in different parts of the world. Chapter IV discusses the best optimized molecular strategies and protocols enabling the use of *P. oleracea* as a C_4-CAM model. Finally, Chapter V characterizes the molecular and hormonal signaling during the C_4-to-CAM transition and reversion and brings insights into the influence of the circadian clock on both CCMs' functioning. In general, and most importantly in the global context of climate change, this work aimed to emphasize that C_4 and CAM not only represent interesting physiological adaptations from both a functional and evolutionary point of view, but are also important mechanisms for the development of society, as they occur separately in cultivars used in food production and biofuels. Thus, this work provides information that makes it possible to explore the complexity of *Portulaca* as a blueprint for future development via genetic engineering of cultivars that can intercalate C_4 and CAM in a single organism according to the availability of resources in the environment.

Scientific Background

"Botany – the science of the vegetable kingdom, is one of the most attractive, most useful, and most extensive departments of human knowledge. It is, above every other, the science of beauty."

Joseph Paxton

Initial considerations

Six carbon assimilation pathways are currently described across living organisms, but the reductive phosphate pentose cycle (*i.e.* Calvin-Benson Cycle) is the most important in higher plants (BERG, 2011). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) has a major role as the first carboxylation enzyme in this cycle (TABITA et al., 2008), but not without caveats. Rubisco is prone to oxygenase activity since molecular oxygen can interact with the intermediate form of ribulose-1,5-biphosphate (RuBP), that is the acceptor for carbon dioxide (MORONEY et al., 2013). Overall, there is a 25% chance of Rubisco binding oxygen instead of carbon dioxide, what may vary according to the concentration of these compounds in the vicinity of the enzyme (BAUWE; HAGEMANN; FERNIE, 2010; ZABALETA; MARTIN; BRAUN, 2012). Resulting from the binding of RuBP and molecular oxygen is the formation of 3-phosphoglycerate (3PGA) and 2-phosphoglycolate (2PG) molecules, the latter being harmful to the plant since it inhibits photosynthesis (ZABALETA; MARTIN; BRAUN, 2012).

In order to recover CO_2 and eliminate toxicity, 2PG undergoes the photorespiration pathway. This pathway takes place in the presence of light, involving chloroplasts, peroxisomes and mitochondria, and recovering around 75% CO_2 in the form of 3PGA, despite being energetically dispendious (BAUWE; HAGEMANN; FERNIE, 2010; EISENHUT et al., 2008; TCHERKEZ; FARQUHAR; ANDREWS, 2006). To avoid photorespiration, CCMs have evolved in various plant lineages and consist of different strategies to minimize the oxygenase activity of Rubisco (MORONEY et al., 2013).

C_4 photosynthesis and crassulacean acid metabolism (CAM) are the most common CCMs in eudicots (KEELEY; RUNDEL, 2003). As they may require multiple traits to function, both can be referred to as syndromes. Their basic mechanisms are very similar, involving the incorporation of CO_2 molecules into organic acid skeletons that will be decarboxylated close to Rubisco (SAGE, 2002). To do this, the biochemical machinery employed by each CCM is quite similar, involving a common set of enzymes, such as: carbonic anhydrases (CA), phospho*enol*pyruvate carboxylase (PPC), PPC kinase (PPCK), NAD(P)–malate dehydrogenases (MDH), malic enzymes (ME), and phospho*enol*pyruvate carboxykinase (PEPCK), pyruvate, orthophosphate dikinase (PPDK) and PPDK regulatory protein (PPDKPR) (EDWARDS; OGBURN, 2012; KANAI; EDWARDS, 1999; WINTER; SMITH, 1996).

Although their basic biochemistry is similar, C_4 works as a spatial specialization, while CAM relies less on the tissue structure and more on temporal regulation. Nevertheless, due to this crucial difference, different transporters and regulatory steps have evolved for each CCM (SAGE, 2002).

C_4 photosynthesis

A remarkable, but not essential, feature of C_4 plants is the structural arrangement of two types of cells in the mesophyll, named Kranz anatomy, in which mesophyll cells (MCs) are reduced and surround the well-developed bundle sheath cells (BSCs) (SAGE, 2004; VOZNESENSKAYA et al., 2001). In this type of photosynthesis, MCs perform the first carboxylation step by PPC activity and form 4-carbon acids to store CO_2. Acids are then transported to the bundle sheath BSCs to be decarboxylated, redeeming CO_2 to the Calvin Cycle (KANAI; EDWARDS, 1999; SAGE, 2004).

In MCs, there is an increase in levels of CA and PPC in the cytoplasm for CO_2 fixation, and PPDK in the chloroplasts for phosphoenolpyruvate (PEP) regeneration (KANAI; EDWARDS, 1999). On the other hand, Rubisco levels in chloroplasts and glycine decarboxylation enzymes in mitochondria are low. BSCs, in its turn, concentrate Rubisco and glycine decarboxylase (GDC) activities. This assures the decarboxylation step to occur in BSCs and restricts photorespiration to these cells, promoting a CO_2 pump mechanism to enhance Rubisco activity (SAGE, 2004).

After CA equilibrates CO_2 and HCO_3^- in MCs, PPC forms oxaloacetate (OAA), which is converted to a tetracarboxylic acid to be transported to the BSCs. Kanai and Edwards (1999) describe in detail three types of C_4 photosynthesis according to the decarboxylation step in BSCs: NAD-malic (NAD-ME), NADP-malic (NADP-ME) and PEPCK types.

NADP-ME type plants use NADP-malate dehydrogenase to form malate (the remaining OAA forming aspartate), which is then transferred probably via plasmodesmata to the BSC and decarboxylated by NADP-ME feeding CO_2 and NADP to the Calvin Cycle. The product of this reaction is pyruvate, that is transported back to MC, where PPDK regenerates PEP.

NAD-ME type plants may form aspartate by aspartate aminotransferase in MCs, that is transported to the BSC mitochondria do be deaminated by aspartate aminotransferase. They may also form OAA, which is reduced to malate by NAD-malate dehydrogenase and decarboxylated by NAD-ME in BSCs, feeding CO_2 to the Calvin Cycle. The remaining pyruvate is converted to alanine and transported back to MCs to regenerate PEP by alanine aminotransferases.

In PEPCK type plants, aspartate formed via aspartate aminotransferase in MC and is transported to BSCs. Aspartate is deaminated and decarboxylated by PEPCK, redeeming CO_2, and the resulting pyruvate may return to MC as alanine or PEP. The formation of PEP at this step may result in reduced PPDK activity in PEPCK-type plants.

In particular, the NAD(P)-MDH and PPDK enzymes are prone to light regulation (KANAI; EDWARDS, 1999). Kanai and Edwards (1999) also explain that chloroplasts differ

from type to type, having a centrifugal position relative to the vascular bundle and thylakoid with reduced grana in NADP-ME. In NAD-ME and PEPCK types, thylakoids have well-developed grana stacking, but chloroplasts and mitochondria have a centripetal position in NAD-ME type, and chloroplasts are evenly or centrifugally distributed in PEPCK-type.

C_4 representatives are widespread over 19 families of angiosperms, comprising around 8,100 species, of which 80% are monocots (SAGE, 2017). Most representatives of C_4 flora show an herbaceous habit and are distributed in dry regions, where photorespiration would be favored by low stomatal aperture and warm temperatures (SAGE; LI; MONSON, 1999). As reviewed by Sage (2017), the evolution of C_4 lineages probably dates to between 30 and 35 million years ago, a period when CO_2 atmospheric levels decreased and temperatures increased, resulting in intense selective pressure. When it comes to economic use and applicability of C_4 plants, maize, sugarcane and sorghum are among the leading row crops, in addition to forage crops and a large portion of weed and invase species (SAGE, 2017).

In the molecular context, C_4 evolved independently, with the resulting gene product being the same in different plant lineages (MONSON, 1999). The evolution of C_4 involved the recruitment and duplication of C_3 non-photosynthetic genes, besides tuning the regulation of these genes in different mesophyll cell layers (KEELEY; RUNDEL, 2003; MONSON, 1999).

One interesting feature of C_4 photosynthesis was the discovery of single-cell C_4 (Voznesenskaya et al. 2001). Up to date, four members of the family Chenopodiaceae family *(Bienertia cycloptera, B. sinuspersici, B. kavirense and Suaeda aralocaspica)* were found to perform the entire biochemical cycle, commonly divided into two types of cells (MCs and BSCs), in one large, polarized chlorenchyma cell (FREITAG; STICHLER, 2000; LEISNER et al., 2010; PARK; OKITA; EDWARDS, 2009; VOZNESENSKAYA et al., 2002). There is a central, large vacuole that separates the proximal portion of the cell, with few chloroplasts, from a distal part, connected to the vascular bundle and possessing a high density of chloroplasts, and it promotes a high diffusive resistance between these two halves of the cell (VOZNESENSKAYA et al., 2001).

Crassulacean acid metabolism (CAM)

In a nutshell, CAM involves diel fluctuation of acidity in photosynthetic cells, and the whole process can be divided into four phases over the day/night cycle (TING, 1985), as clearly described by Winter and Smith (1996). The first phase takes place at night, when stomata remain open and PPC promotes the assimilation of CO_2 into four-carbon organic acids, that are stored inside MC vacuoles. The second phase starts at dawn, when PPC is deactivated and Rubisco is activated, and there can still be some CO_2 assimilation at this

stage in weak CAM-performing plants. During the third phase, in the light period, stomata remain closed and organic acids are mobilized from the vacuole to the chloroplasts to be decarboxylated, releasing CO_2 in the vicinity of Rubisco. In the fourth phase, at dusk, levels of PPC and Rubisco are inverted again, preceding the nocturnal CO_2 assimilation. Biochemically, there can be NADP-, NAD-ME and PEPCK decarboxylation systems in CAM performing plants as reported for C_4. In addition, as reviewed in Schiller and Bräutigam (2021), CAM plants usually mobilize the phosphorolytic starch degradation pathway instead of the hydrolytic pathway commonly used in C_3 plants.

CAM is usually associated with increased water use efficiency (WUE) when compared to C_3 and C_4 species, being widespread in plants from arid environments (CUSHMAN, 2001), but also occurring in other less obvious habitats, including the epiphytic flora of tropical forests and even certain aquatic plants (WINTER; SMITH, 1996). This syndrome has already been described in at least 16,800 species of 35 plant families (SILVERA et al., 2010). In economic terms, CAM is present in food crops such as pineapple, *Agave* and *Opuntia*, the two latter are also being largely used for biomass production and showing potential as biofuel (YANG et al., 2015).

Ontogenetic and/or environmental features can influence CAM expression, characterizing either the constitutive or facultative forms of CAM (WINTER; SMITH, 1996). Constitutive CAM (also referred to as obligatory or ontogenetic CAM) is developed as the plant matures independently of environmental conditions, and this is usually connected to a strong form of CAM. As such, the intensity of CAM is defined by the total carbon assimilation contribution of dark CO_2 fixation to the overall carbon metabolism of the plant (WINTER, 2019). On the other hand, in facultative CAM plants, the expression of the CAM machinery can be highly variable according to the environmental context, especially with varying light, temperature and water availability conditions (DODD et al., 2002; SILVERA et al., 2010). Among these environmental factors, water availability has been shown to modulate CAM expression in most CAM facultative species analyzed so far. Facultative CAM is usually, but not exclusively, associated with a weak form of CAM, where CO_2 assimilated by PPC corresponds to less than 5% of total carbon gain (WINTER, 2019).

Another subdivision in types of CAM can be defined according to their diel gas exchange and acid accumulation patterns. Plants performing constitutive CAM restrict stomatal opening to the dark period, which results in intense nocturnal acidification and CO_2 assimilation. CAM-cycling and CAM-idling, on the other hand, both show lower nocturnal acidification resulting from reutilizing respiratory CO_2, and both differ in diel CO_2 assimilation pattern. While CAM-cycling is characterized by closed stomata during the dark period and diurnal CO_2 assimilation usually restricted to bursts of assimilation in phase 2 (WINTER; HOLTUM, 2014), plants performing CAM-idling present no net CO_2 exchange

over the entire diel cycle (TING, 1985). CAM-idling is a survival and temporary mechanism when the plant is severely stressed, and occurs as the plant reutilizes respiratory CO_2 to maintain an active but low CAM metabolism (WINTER, 2019).

Signaling events controlling the facultative CAM cycle

In facultative CAM systems, the alternation of C_3 to CAM requires changes in the circadian system, carbohydrate partitioning, intracellular transport of metabolites, osmotic adjustment and the synchronization between two competing carboxylation systems (PPC and Rubisco). These changes occur inside the same cell and are triggered within a time frame that may vary from a few hours to several weeks (FRESCHI; MERCIER, 2012). Consequently, a series of adjustments aiming to keep homeostasis during these changes involve transcriptional, translational, and post-translational regulatory processes (CUSHMAN; BOHNERT, 2000). Therefore, based on the physiological complexity and high responsiveness to environmental stimuli, it is plausible to expect the existence of equally complicated signaling routes controlling C_3-CAM or C_4-CAM transitions.

Distinct lines of evidence indicate that abscisic acid (ABA) acts as one of the major stimulatory signals for CAM expression (TAYBI; CUSHMAN, 2002). Studies conduced on distinct CAM plants, including ice plant (*Mesembryanthemum crystallinum*), *Kalanchöe blossfeldiana* and pineapple (*Ananas comosus*), have demonstrated that exogenous ABA can trigger increases in activity and/or transcript accumulation of CAM-related enzymes either when applied to intact plants or directly supplied to detached leaves. The ABA-induced up-regulation of CAM enzymes is followed by an initiation, or up-regulation, of functional CAM, leading to levels of night-time acid accumulation equivalent to those observed in water-stressed plants (reviewed by Freschi and Mercier 2012).

In parallel to the positive regulation of ABA, which induces CAM expression, the existence of negative endogenous signals that would repress CAM in reversible facultative plants is somewhat expected. Based on available data, cytokinins (CKs) are the best candidates to fulfil a role as negative root-derived signals responsible for inhibiting CAM expression in leaves of well-watered plants (SCHMITT; PIEPENBROCK, 1992). Among the evidence supporting this view, considerable decreases in endogenous CKs have been observed during the water stress-induced up-regulation of CAM in excised ice plant leaves (PETERS et al., 1997) and intact pineapple young plants (FRESCHI et al., 2010). In addition, supplying exogenous CKs has suppressed CAM initiation triggered by stress conditions (DAI et al., 1994; FRESCHI et al., 2010; PETERS et al., 1997). Compared to ABA and CKs, significantly less information is currently available regarding the involvement of other plant hormones in the modulation of CAM expression.

Bioengineering C_4 and CAM into crops to improve drought stress resistance

Climate change is a major concern, possibly causing the aridification of tropical regions, an increase in temperature of temperate areas, and changes in sea levels and ocean currents. When combined, these global changes can result in a loss of arable land and immediately impact agriculture (FAO 2020). Amidst such pessimistic scenarios, food and fuel demands will continue to grow (BORLAND et al., 2009). Therefore, sustainable and green technologies coupled with responsible land management that maximize yield in reduced arable land have never been as crucial as now (FAO 2020).

Another approach in this scenario is to search broadly into the plant's metabolism and physiology, aiming at finding out clever strategies to improving the agricultural performance of crops and other species of interest (FURBANK; QUICK; SIRAULT, 2015; YANG et al., 2015; SCHILLER; BRÄUTIGAM, 2021). In this context, CCMs have evolved as a strategy to reduce carbon loss caused by the oxygenase activity of Rubisco and resulted in plant lineages that express CAM and C_4 photosynthesis, which very frequently colonize habitats characterized by persistent or recurrent abiotic stress events. These two mechanisms have recruited enzymes that already exist in the C_3 metabolism to perform different roles, resulting in a neofunctionalization of the plant primary C_3 metabolism (EDWARDS; OGBURN, 2012; HEYDUK et al., 2019). Hence, although the molecular machinery and regulation of each CCM are still not completely dissected, CCMs represent flexible adaptations, which ensure photosynthetic yield under high irradiance (C_4) and strong tolerance to drought under arid conditions (CAM).

Consequently, it may be promising to investigate the possibility of combining the different aspects of C_4 and CAM into crops (BORLAND et al., 2009; VON CAEMMERER; QUICK; FURBANK, 2012), as an attempt to increase abiotic stress tolerance ensuring plant productivity or its survival when under unfavorable environmental conditions (YANG et al., 2015). Although the journey is long and challenging when it comes to engineering CCMs, two ongoing initiatives stand out: the C_4 Rice project, which has focused on introducing C_4 into a C_3 plant (ERMAKOVA et al., 2020; FURBANK, 2016); and the CAM Biodesign, that seeks to express CAM in Arabidopsis and poplar, also C_3 plants (DEPAOLI et al., 2014; LIU et al., 2018). Even the C_2 metabolism, another variation of CCM which acts as a C_3-C_4 intermediate, has also been discussed as a possible target for bioengineering of crops (LUNDGREN, 2020), but it will not be discussed here. The two mentioned projects deal with CCM-C_3 engineering alone, but another approach would be bioengineering a facultative CAM system into C_4-crop plants.

<u>Portulaca</u> phylogeny and evolution

Multiple plant lineages include both C_4 species and facultative or constitutive CAM representatives, while the occurrence of both C_4 and CAM within a single species is rare, considering each CCM followed distinct and exclusive evolutionary pathways (SAGE, 2002). C_4 evolution favors a well-developed bundle sheath, whereas CAM favors succulence and tight mesophyll packing (SAGE, 2004; SILVERA et al., 2010). The evolution of these contrasting anatomical features is favored by different selective pressures, preconditioning the evolution of either C_4 or CAM, while limiting the chances of both pathways to evolve in a single individual. Hence, C_4 and CAM evolved a differential regulation in enzyme activation of carboxylation and decarboxylation steps, different metabolite transport dynamics, and specialized structural arrangements for each syndrome (SAGE, 2002).

Nevertheless, it may also have been simply a matter of not having enough time to evolve the two syndromes in one single organism. There are a few extreme examples of CCM connectivity, such as the *Portulaca* species in Caryophyllales (SAGE, 2002; WINTER, 2019). In fact, Caryophyllales concentrates many independent and parallel origins of CCMs, being considered a hotspot for CCM evolution (GOOLSBY et al., 2018; EDWARDS; OGBURN, 2012).

Portulacaceae (Caryophyllales) is a monotypic family and comprises ca. 100 species distributed worldwide (APG III, 2009; OCAMPO; COLUMBUS, 2012). The genus probably diverged around 23 million years ago somewhere in the southern hemisphere and became widely distributed probably via long-distance dispersal, but its exact place of origin is still uncertain (OCAMPO; COLUMBUS, 2012). Centers of diversity for the family are South America, Africa, and Australia (OCAMPO; COLUMBUS, 2012). The clade is a member of the Portulacineae (Caryophyllales), which includes well-known CAM lineages such as the cacti and Didiereaceae (SILVERA et al., 2010).

The family is divided into two main lineages: the opposite-leaved clade (OL), comprising fewer species and distributed in Africa, Asia and Australia; and the alternate-leaved clade (AL), more widespread and including most of the species (OCAMPO; COLUMBUS, 2012). Within the AL clade are: *P. cryptopetala*, identified as a C_3-C_4 intermediate (VOZNESENSKAYA et al., 2010), and three groups: the Oleracea, Umbraticola and Pilosa clades (OCAMPO; COLUMBUS, 2012). There still remains considerable uncertainty about species relationships within these groups, and the species that is best known physiologically (*P. oleracea*) is not monophyletic (OCAMPO; COLUMBUS, 2012).

Except for *P. cryptopetala*, all taxa show C_4 leaves and C_3 stems (LAETSCH, 1974; SAGE; CHRISTIN; EDWARDS, 2011; VOZNESENSKAYA et al., 2010, 2016). Different types of Kranz anatomy were reported in representatives of the family, as were NADP-ME and NAD-ME-type species (KU et al., 1981; VOZNESENSKAYA et al., 2010). While Ocampo et al. (2013) preferred the interpretation of a single inferred origin of C_4 at the base

of *Portulaca* with an evolutionary reversion to C_3-C_4, there is an alternative hypothesis of multiple, parallel evolutionary shifts to C_4 (CHRISTIN et al., 2014). There are various lines of evidence to support this. First, each of the three major C_4 clades presents a distinct C_4 leaf anatomical configuration, and second, different clades have recruited different decarboxylating enzymes to release CO_2 in the BSCs (VOZNESENSKAYA et al., 2010). Third, *Portulaca* has recruited a novel, recently duplicated homolog of one gene encoding PPC (*PPC1E1a')* in its C_4 cycle. Codon model tests strongly favored independent adaptive evolution of *PPC1E1a'* in each of the three clades (Oleracea, Umbraticola and Pilosa), with each AL clade presenting a unique suite of amino acid substitutions known to show selection for C_4 function (CHRISTIN et al., 2014). This supports the scenario that selection of *PPC1E1a'* for C_4 function occurred in each lineage separately, after they diverged from their common ancestor.

Moreover, the *Portulaca* genus is remarkable, since all C_4 and C_3-C_4 intermediate species studied up to date undergo CAM induction upon water deficit (GURALNICK; JACKSON, 2001; HOLTUM et al., 2017; KOCH; KENNEDY, 1980, 1982; KRAYBILL; MARTIN, 1996; KU et al., 1981; WINTER et al., 2019). The most studied species, *P. oleracea,* provides us with the blueprint, refined over millions of years of evolution, for developing this group as a new workhorse of CCM-based crop improvement and genetic engineering. Also, studying facultative CAM systems can provide good insight into CAM regulation and signal transduction cascades, since the ontogenetic factor of constitutive CAM species are not present (WINTER; HOLTUM, 2014).

Portulaca oleracea L. as a model system to study C_4-CAM compatibility

Commonly known as purslane, *P. oleracea* is an annual cosmopolite usually found growing in human-disturbed areas as gardens, field crops, lawns and sidewalks, or as a pioneer species (VENGRIS; DUNN; STACEWICZ-SAPUNCAKIS, 1972). It has succulent leaves and stems, solitary flowers, a self-fertilization system and a pronounced seed production (VENGRIS; DUNN; STACEWICZ-SAPUNCAKIS, 1972; ZIMMERMAN, 1977). Adding to the remarkable photosynthetic plasticity of *Portulaca* species, *P. oleracea* also presents extreme tolerance to drought (JIN et al., 2015; REN et al., 2011), salinity (YAZICI et al., 2007), as well as variations in temperature, photoperiod, soil and light intensity (ZIMMERMAN, 1976). When combined, all these factors may help to explain the wide distribution of this species, its weedy propagation in agricultural systems and a wide morphologic diversification (DANIN; DOMINA; RAIMONDO, 2008; OCAMPO; COLUMBUS, 2012; WALTER; VEKSLYARSKA; DOBEŠ, 2015).

Even though it is considered one of the ten most noxious weeds on the planet (SINGH; SINGH, 1967), *P. oleracea* has many uses for different cultures. For example, it

exhibits pharmacological effects and has been used in traditional Chinese medicine for thousands of years (CHAN et al., 2000; CHEN; SHI; LIU, 2003; GONNELLA et al., 2010; HABTEMARIAM; HARVEY; WATERMAN, 1993; KARIMI; HOSSEINZADEH; ETTEHAD, 2004; RADHAKRISHNAN et al., 2001; RASHED; AFIFI; DISI, 2003). It is also considered a neglected crop by the Food and Health Organization, rich in vitamins, antioxidants, and omega 3-fatty acids, and is widely consumed in African and Asian countries either in salads or soups (EGEA-GILABERT et al., 2014; GONNELLA et al., 2010; HERNÁNDEZ-BERMEJO; LEÓN, 1994; LIM; QUAH, 2007; OBIED; MOHAMOUD; MOHAMED, 2003).

However, *P. oleracea* has unresolved taxonomic issues. On the one hand, it is considered an aggregate of subspecies or microspecies (DANIN; BAKER; BAKER, 1978; DANIN; RAUS, 2012), also sometimes referred to as different species (DANIN; DOMINA; RAIMONDO, 2008; DANIN; REYES BETANCORT, 2006; RICCERI; ARRIGONI, 2000). The most recent taxonomic reports list 19 microspecies distinguished according to their seed size and seed coat ornaments (DANIN; RAUS, 2012). On the other hand, *P. oleracea* can be referred to as a complex, being considered a polymorphic species that, due to its cosmopolitan distribution and high adaptability, is expected to present high variability in morphological and physiological traits among populations, even forming a continuum (GORSKE; RHODES; HOPEN, 1979; MATTHEWS; KETRON; ZANE, 1993; WALTER; VEKSLYARSKA; DOBEŠ, 2015). In this sense, seed size and ornamentation are not considered enough to define the different subspecies, as there should be more noticeable morphological differences associated with allopatric populations (MATTHEWS; KETRON; ZANE, 1993; WALTER; VEKSLYARSKA; DOBEŠ, 2015).

The leaves of *P. oleracea* have three types of cells: (1) water storage cells (WSCs), whose contribution to overall carbon gain may be minimal due to their small number of chloroplasts; (2) photosynthetically active cells usually referred to as MCs; and (3) and BSCs that surround the vascular bundles forming the Kranz anatomy (which is atriplicoid type) (VOZNESENSKAYA et al., 2010). This species is a NAD-malic enzyme (NAD-ME) type of C_4 plant (KENNEDY; LAETSCH, 1973; KENNEDY, 1976), accumulating malate and aspartate as a means of transferring assimilated CO_2 from MCs to BSCs for decarboxylation and Rubisco carboxylation (LARA; DRINCOVICH; ANDREO, 2004). Due to the presence of photosynthetically active cells and no apparent Kranz anatomy, *P. oleracea* stems are considered C_3 under well-watered conditions (KOCH; KENNEDY, 1980; MAZEN, 1996; VOZNESENSKAYA et al., 2010).

P. oleracea leaves have been shown to increase nocturnal acidity and perform a low level of CO_2 assimilation in the dark after prolonged periods of water deprivation (KOCH; KENNEDY, 1982a, 1980; KRAYBILL; MARTIN, 1996; LARA et al., 2003; LARA;

DRINCOVICH; ANDREO, 2004; MAZEN, 1996, 2000; WINTER; HOLTUM, 2014). After this discovery, Lara et al. (2004) developed a first hypothetical model of C_4-CAM compatibility in *P. oleracea*, and provided preliminary evidence that PPC may be up-regulated in WSCs when plants were droughted. In this model, malate generated from CAM in WSCs and MCs is shuttled to BSCs for decarboxylation.

A potential breakthrough occurred when Christin et al. (2014) analyzed the transcriptome of two individuals of *P. oleracea* grown under well-watered and droughted conditions and identified two genes possibly responsible for encoding two different isoforms of PPC, each one involved in one photosynthetic behavior. While *PPC1E1c* was highly expressed during the night in droughted plants, *PPC1E1a'* was predominantly expressed during the day and in well-watered samples (CHRISTIN et al., 2014). This finding is correlated with the different properties and activity of PPC in well-watered and droughted *P. oleracea* plants (LARA et al., 2003; MAZEN, 1996, 2000).

Despite the remarkably impressive photosynthetic plasticity observed within *P. oleracea*, very limited information is currently available on the biochemical, physiological and genetic regulation of CAM expression in this fascinating genus. Therefore, *P. oleracea*, whose leaves can present both C_4 and CAM and whose stems can perform either C_3 or CAM, represents a precious model for exploring the molecular, biochemical and signaling mechanisms responsible for allowing the occurrence of these distinct photosynthetic modes within a single individual.

Given such promising system for photosynthetic plasticity studies, *P. oleracea* was chosen as the target species for exploring the connectivity between the C_4 and the CAM cycles in this work. Gene recruiting for either CO_2 concentration mechanisms (CCMs), the plasticity inside the *Portulaca oleracea* complex, and the signaling events controlling the up- and down-regulation of either CCM cycles, were the object of study in this thesis.

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Hypothesis and goals

Three primary hypotheses tested here were:

1. The evolution of a C_4 cycle on top of a CAM-performing organism in *P. oleracea* might have required recruiting distinct genes encoding proteins specifically enrolled in each CCM cycle.

2. Due to its cosmopolite distribution, *P. oleracea* might possess high plasticity in CAM expression, particularly when comparing subspecies that have evolved under contrasting environmental conditions (*e.g.*, hot deserts *versus* cold mesic regions).

3. The alternation between C_4 and CAM might be differently regulated by endogenous signals in *P. oleracea* leaves, possibly involving different arrays of transcription factors and hormonal signals.

The overall goal was to significantly increase the understanding of molecular, biochemical, physiological and regulatory aspects of the photosynthetic plasticity in *P. oleracea*, which can be considered a photosynthetic marvel due to the presence of three photosynthetic pathways in a single organism (*i.e.* C_3 in stems, C_4 in leaves, and CAM in both organs). More specifically, the aims of this thesis were:

A. To utilize RNA-seq and RT-qPCR analysis to generate a global transcriptional profile and identify key components of the C_4 and CAM machineries involved in the complex photosynthetic transitions occurring in leaves and stems of *P. oleracea* under drought stress (to test **hypothesis 1**);

B. To evaluate C_4 and CAM plasticity within *P. oleracea* subspecies complex under well-watered and droughted conditions by using physiological and molecular approaches (to test **hypothesis 2**);

C. To explore the signaling events responsible for the up- and down-regulation of CAM expression in leaf tissues of *P. oleracea* plants subjected to contrasting water availability conditions using transcriptional profiling and network analysis data (to test **hypothesis 3**).

D. As an additional goal, we aimed to develop technical resources for promoting *P. oleracea* as a genetic model in the context of C_4-CAM photosynthesis, including the optimization of conditions for gene expression analysis and a stable genetic transformation protocol.

Final considerations and future perspectives

"Knowledge is like a garden: if it is not cultivated, it cannot be harvested."

African proverb

The overall goal of this Thesis was contributing to elucidating the molecular mechanisms that allow *Portulaca oleracea*, a C_4 weed, to perform the unusual CAM transition when drought-stressed. Our primary motivation was that this species represents a natural blueprint for dissecting how both C_4 and CAM can occur inside a single organism, and such knowlodge may be crucial when seeking to improve stress-resistance in crops, especially in the context of climate change. To pursue this goal, physiological, biochemical, molecular and bioinformatic tools were employed here and yielded a series of significant results.

To provide a global characterization of the gene expression reprogramming required for the C_4-to-CAM transition in *P. oleracea*, we first performed RNA-Seq analysis in well-watered and droughted leaves and stems. We identified shared and exclusive genes in the machinery of each CCM, partially refuting our first hypothesis, since not all genetic components of C_4 were exclusively co-opted for this pathway. Steps as acid decarboxylation and PPCK regulation seem to be shared between the C_4 and CAM cycles. We also generated detailed expression profiles via qPCR for genes involved in each CCM and associated processes (i.e., photorespiration and carbohydrate metabolism), and this revealed the changes in the diel expression of these components in distinct organs (leaves and stems) and conditions (well-watered and drought). In addition, we identified that, although CAM is completely facultative and reversible in leaves, it is also under developmental control in stems. Also, fine adjustments in starch metabolism were shown to be necessary for CAM functioning.

In the second part, we characterized multiple accessions of *P. oleracea* with different geographical origins using morphometric variables, C_4 and CAM marker genes, physiological attributes of CAM (such as acidity and gas exchange), and also monitored chlorophyll *a* fluorescence during water stress treatment. This allowed us to conclude that all accessions performed CAM, and the degree of CAM expression was not directly correlated to the climatic variations of their place of origin and morphometric differences, partially contradicting our second hypothesis. A similar response to drought and rewatering was observed for all CCM genes analyzed, but a continnum of CAM intensity was observed across the genotypes. Therefore, this study identified both morphologically similar genotypes with contrasting degrees of CAM expression and also morphologically distinct accessions performing similar CAM levels, which may be a valuable research material for further studies about the C_4-CAM transition.

In the third part, we compiled a molecular toolkit for *P. oleracea*, including standardization of RNA extraction protocols, selection of reference genes for qPCR under varied conditions, and *in vitro* regeneration and transformation protocols. We believe these

data may facilitate the future development of *P. oleracea* as a model species to the study of the C_4-CAM transition.

Finally, in the last chapter we started elucidating the hormonal and molecular signaling networks controlling the drought-induced transition from C_4 to CAM and its reversion during rewatering. These were the first insights in the literature focused on the signaling mechanisms taking place in this unique system. Abscisic acid was identified as a major signal during CAM induction, whereas exogenous cytokinins were able to recover C_4-related transcripts in droughted plants, possibly playing an antagonistic role on the expression of the CAM machinery. In addition, free-running time-course experiments demonstrated that the circadian expression of specific CCM components (e.g., PPCK) are particularly dependent on the proper functioning of the molecular clock, presumably playing a central role in avoiding futile cycling between the two cycles. In addition, a list of TFs was compiled, and that will be important for functional genomics studies on the species.

When combined, the results from this Thesis lay a strong foundation for future studies on C_4-CAM photosynthesis, since they pinpoint gene modules and signaling events as targets for modulation in bioengineering and synthetic biology approaches. Although combining C_4 and CAM may be yet new and little explored, CCM engineering to produce improved crops has already been the focus of the C_4 Rice and the CAM Biodesign projects. The large-scale 'omics data generation for as many species that express CCMs as possible has been shown to be of utmost importance for both projects, representing one of the early stages of identifying the target genes for manipulation.

This topic is far from being exhausted and the photosynthetic transition in *Portulaca* species, in particular, could still count on analysis of other signaling molecules, such as other classes of plant hormones, nitric oxide (NO) and cytosolic Ca^{2+}, and many other regulatory levels besides the transcriptional regulation of core CCM genes. The findings presented here pave the way for functional genomics studies, which may provide critical information to deepen our current understanding of the C_4/CAM compatibility and its biotechnological potential. One example would be the generation of loss-of-function or overexpression lines for candidate genes identified in chapters 1 and 4 using the standardized molecular biology and transformation protocols for *P. oleracea* described in chapter 3.

Moreover, research seeking to understand the localization of C_4 and CAM inside a single leaf can also benefit from the results produced here. For instance, the production of *P. oleracea* transgenic plants expressing reporter genes fused to the regulatory or coding regions of either C_4- or CAM-marker genes might bring conclusive information on the tissue localization of each CCM, which remains a controversial and poorly understood issue in C_4/CAM hybrid system. In addition, probes can be generated targeting the key C_4-CAM genes described here, which would be helpful to fully explore the spatial configuration of C_4

and CAM cycles within the leaf via *in-situ* hybridization and similar techniques. Yet, another approach that benefits from having the core C_4-CAM genes identified involves microdissection and enzymatic protoplast isolation. These methods combined with monitoring the transcript abundance of the mentioned genes via RT-qPCR could provide key information on the localization of each CCM.

Overall, these approaches are exciting prospects for plant physiologists looking for sustainable and green solutions for tomorrow's problems. In this context, making *P. oleracea* a model C_4-CAM species is an up-to-date and ambitious proposal with yet plenty of room for data generation. It will involve the direct application of cutting-edge technologies and may contribute to generating new tools and methodologies in order to accomplish the goal of expressing multiple or hybrid CCM traits into crops of economic importance.