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"Era lo último que iba quedando de un pasado cuyo aniquilamiento no se consumaba, porque seguía aniquilándose indefinidamente, consumiéndose dentro de sí mismo, acabándose a cada minuto pero sin acabar de acabarse jamás".

Gabriel García Márquez, Cien años de soledad

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Resumo

A fotossíntese consiste na absorção da energia proveniente da radiação solar resultando na síntese de NADPH e ATP, os quais são utilizados na fixação do CO_2 com a consequente formação de carboidratos. Em condições de estresse, pode ocorrer redução na fixação de carbono devido ao fechamento dos estômatos, como também, uma alteração na capacidade de absorção e uso da luz, adequando o metabolismo à nova condição ambiental. O ambiente epifítico é caracterizado por uma disponibilidade reduzida de água e nutrientes, devido à falta de acesso à água e aos nutrientes presentes no solo. O suprimento hídrico acaba dependendo do regime de chuvas, expondo as plantas a uma seca intermitente. Algumas espécies respondem ao déficit hídrico com a indução do metabolismo ácido das crassuláceas (CAM), contribuindo para a manutenção de um balanço positivo do carbono e para uma economia hídrica. Esse metabolismo é caracterizado pela carboxilação noturna e formação de ácidos orgânicos e pela descarboxilação desses ácidos durante o dia. Estudos sugerem uma modulação do uso da energia pelo fotossistema II (PSII) em plantas CAM por meio da disponibilização do CO_2, o qual é fornecido via reação de descarboxilação. Dessa forma, momentos com alta atividade de descarboxilação coincidiriam com um maior uso fotoquímico da energia pelo PSII. Um exemplo de espécie que ocupa o ambiente epifítico e que, em resposta ao déficit hídrico, apresenta indução ao CAM e uma regulação negativa na sua capacidade de absorção da luz é a bromélia *Guzmania monostachia*. Para avaliar a influência da CO_2 proveniente da descarboxilação sobre o uso fotoquímico da energia pelo PSII e investigar possíveis modulações na composição e organização das proteínas do tilacoide com a indução do CAM, plantas de *G. monostachia* foram submetidas a 20 dias de seca e foram avaliados os seguintes parâmetros: 1) atividade da enzima PEPCK e expressão do gene que codifica para essa proteína, 2) eficiência quântica e dissipação não fotoquímica do PSII, 3) conteúdo de ácidos, 4) trocas gasosas, 5) organização dos complexos proteicos do tilacoide e abundância de proteínas do PSII, 6) proporção de clorofilas e carotenoides e 7) expressão de genes envolvidos em mecanismos de regulação transcricional de proteínas dos fotossistemas. Foi observada uma regulação pós-traducional e sugerida uma regulação transcricional da PEPCK

que possivelmente resultou em uma maior atividade de descarboxilação às 12:00h. Essa maior atividade coincidiu com o mesmo momento em que houve um maior uso fotoquímico da energia pelo PSII e menor dissipação não fotoquímica. Esse resultado indica fortemente a influência da disponibilidade de CO_2, pela descarboxilação dos ácidos, sobre o uso da energia pelo PSII já que não havia assimilação de CO_2 atmosférico. Adicionalmente, os resultados indicaram uma maior expressão, principalmente às 12:00h, de genes que codificam para cinases responsáveis pela fosforilação de proteínas do tilacoide. Uma maior fosforilação dessas proteínas pode causar uma mudança na disposição das membranas do tilacoide, o que poderia facilitar o transporte linear de elétrons, favorecendo a fixação de CO_2 em plantas CAM no momento de sua maior disponibilidade. Os resultados também mostraram uma redução na proporção de proteínas do PSII/PSI após 20 dias de seca. Sendo assim, a indução do CAM por déficit hídrico parece ser acompanhada por modificações bioquímicas e moleculares que ajustam a absorção e o uso da energia, auxiliando a sobrevivência de *G. monostachia* no ambiente epifítico, o qual está sujeito a períodos de seca intermitentes.

Palavras chave: PEPCK, ácido málico, tilacoide, fotossíntese, PSII, CAM.

Abstract

Photosynthesis is a light-energy absorption process that results in NADPH and ATP production, which can be used to assimilate CO_2 and produce carbohydrates. Stress conditions can decrease the photosynthetic rate due to stomatal closure limiting the atmospheric carbon assimilation and downregulating the capacity of light absorption. Epiphytic plants normally do not have access to soil moisture and nutrients, which characterizes this habitat as reduced in water and nutrient availability. As a result, the plants depend on rain for their water provisions and are frequently submitted to periods of drought. Some species can induce Crassulacean acid metabolism (CAM) when under water deficit, and this metabolism can conserve water and keep a positive carbon balance. This photosynthetic metabolism is characterized by nocturnal acidification due to transient CO_2 fixation in acid molecules and by a diurnal decarboxylation. Previous studies suggested a photosystem II (PSII) light-energy use modulation in CAM plants through CO_2 availability by the decarboxylation reaction. As a result, it was expected that the major decarboxylation activity would coincide with the time of the highest PSII photochemical efficiency. The bromeliad *Guzmania monostachia* is an example of an epiphytic species that responds to water deficit with CAM induction and downregulation of the light absorption capacity. To test the influence of CO_2 provided by the decarboxylation reaction over the PSII photochemical efficiency and to investigate the adjustment in composition and organization of thylakoid proteins with the CAM induction, we submitted plants of *G. monostachia* to 20 days of drought and evaluated the following parameters: 1) PEPCK enzyme activity and expression of the encoding gene, 2) PSII quantum efficiency and non-photochemical yield, 3) acid content, 4) net CO_2 exchange, 5) the organization of thylakoid protein complexes and amount of photosystem proteins, 6) chlorophyll and carotenoid rate, and 7) expression of genes involved in transcriptional regulation mechanisms of photosystem proteins. The results indicated a post-translational and a transcriptional regulation of PEPCK, which probably resulted in a high decarboxylation activity at 12:00h. This major activity coincided with the highest PSII photochemical efficiency and a minor non-photochemical quenching. These results strongly indicated the influence of CO_2 availability by PEPCK activity over the PSII energy use since the atmospheric

CO_2 uptake was null in water deficit plants. In addition, the results showed an upregulation, especially at 12:00h, of kinases transcript levels responsible for thylakoid protein phosphorylation. The increase in the phosphorylation of these proteins can induce changes in the thylakoid membrane conformation, which facilitates the linear electron transport, consequently powering the CO_2 assimilation in CAM plants, particularly at the time with the major availability of this gas. The results also indicated a reduction in PSII/PSI protein proportion after 20 days of drought. As a result, the CAM induction appears to be followed by biochemical and molecular modulations adjusting the light-energy absorption and use, which could facilitate the survival of this species under stressful conditions.

Key words: PEPCK, malic acid, thylakoid, photosynthesis, PSII, CAM.

Índice

1. Introdução geral

1.1. Fotossíntese: da luz ao açúcar

A fotossíntese é o processo biológico pelo qual a energia luminosa, proveniente da radiação solar, é convertida em energia química e estocada em moléculas de NADPH e ATP. Posteriormente essa energia química poderá ser utilizada para a construção de esqueletos carbônicos, como os açúcares, que serão utilizados para o crescimento e desenvolvimento dos organismos fotossintetizantes e de toda a cadeia alimentar (Junge, 2019). A fotossíntese costuma ser dividida em duas etapas ou fases: a fotoquímica e a bioquímica e ambas acontecem em uma organela denominada de cloroplasto. Essa organela é delimitada por um envoltório externo e um outro interno e no seu interior encontra-se o tilacoide, um extenso sistema de membranas de bicamada lipídica que pode se apresentar empilhado, denominada de lamela granal, formando uma estrutura conhecida como *granum*. O tilacoide pode também estar na forma não empilhada, conhecida como lamela estromal (Mechela *et al.*, 2019). Essa bicamada é responsável por delimitar dois espaços importantes para as reações fotossintéticas, sendo o espaço externo o estroma e o espaço interno conhecido como lume. A seguir serão apresentados detalhes sobre essas duas etapas que constituem a fotossíntese.

1.1.1. Etapa fotoquímica

A etapa fotoquímica consiste na obtenção de energia proveniente da radiação solar que dá início a uma cadeia de sucessivas oxidações e reduções com consequente formação de NADPH e ATP. A absorção de energia é desempenhada principalmente por duas estruturas compostas por proteínas e moléculas de clorofilas e carotenoides, denominadas de complexo antena I ou II (Drop *et al.*, 2014). As proteínas que compõem o complexo antena II (LHCII, sigla em inglês para light-harvesting complex II) podem ser classificadas em dois tipos: proteínas do complexo antena maior e proteínas do complexo antena menor (Dekker & Boekema, 2005). As primeiras são encontradas em maior abundância e na forma de trímeros, resultado de diferentes combinações entre três proteínas, Lhcb1, Lhcb2 e Lhcb3, codificadas pelos genes *Lhcb1*, *Lhcb2* e *Lhcb3* (Jansson, 1994; Gao *et al.*, 2018). Já as proteínas do complexo antena menor,

conhecidas como CP29, CP26 e CP24, codificadas pelos genes *Lhcb4*, *Lhcb5* e *Lhcb6*, ocorrem normalmente como monômeros e apresentam funções de 1) conexão entre os trímeros do complexo antena maior e a estrutura do fotossistema II (PSII, sigla em inglês para photosystem II), 2) regulação na transferência de energia, além de apresentar pequena capacidade de 3) captação da energia proveniente da radiação solar (Dekker & Boekema, 2005; Nicol & Croce, 2018). Após absorvida pelo LHCII, a energia é direcionada até o PSII, uma estrutura dimerizada composta por proteínas, moléculas de clorofila a e de feofitina (Ferreira *et al.*, 2004), levando à excitação de elétrons e ao início da cadeia de transporte de elétrons (Krause & Weis, 1991). O centro catalítico do PSII é composto pelas proteínas D1, D2, CP47 e CP43, codificadas pelos genes *PsbA*, *PsbD*, *PsbB* e *PsbC*. Em conjunto, D1 e D2 compõem o centro de reação e são responsáveis pelo processo de separação de carga e início do transporte de elétrons (Gao *et al.*, 2018). Já as proteínas CP47 e CP43 atuam como antena e na transferência de energia de outras partes do LHCII para o centro de reação.

O complexo antena I (LHCI), de igual modo, é composto por pigmentos (clorofilas a e b e carotenoides) e proteínas. A antena do fotossistema I (PSI) é organizada em dois dímeros, um composto por Lhca1 e Lhca4 e o outro por Lhca2 e Lhca3, codificados por *Lhca1* e *4* e *Lhca2* e *3* (Croce & van Amerongen, 2013; Nicol & Croce, 2018). Já o PSI apresenta uma estrutura menor que o PSII e o centro de reação é composto pelos monômeros PsaA (*PsaA*) e PsaB (*PsaB*), as maiores unidades nas quais a maior parte das clorofilas estão ancoradas, entretanto algumas unidades proteicas menores também coordenam alguns pigmentos (Caffarri *et al.*, 2014). No centro de reação do PSI também ocorre a separação de cargas e, consequentemente, o transporte de elétrons.

O transporte de elétrons que se dá entre o PSII e o PSI tem a participação de alguns outros elementos, como por exemplo as plastoquinonas e o complexo citocromo b_6f (Rochaix, 2011). Após o PSI, ainda se tem a participação, entre outros elementos, da FERREDOXIN-$NADP^+$ REDUCTASE (FNR), resultando no armazenamento de energia em moléculas de NADPH. Uma singularidade marcante que diferencia o PSII do PSI é a presença de um complexo capaz de oxidar água, responsável pela reposição dos elétrons doados à cadeia de transporte de elétrons (Ferreira *et al.*, 2004). Por outro lado, os elétrons doados

pelo PSI são repostos pelos elétrons provenientes do PSII, mantendo a continuidade da cadeia e garantindo a produção de poder redutor.

O transporte de elétrons também é responsável pela formação de um potencial eletroquímico que é utilizado como força motriz pela ATP sintase, um complexo proteico responsável pela produção de ATP. Esse potencial eletroquímico é estabelecido pelo bombeamento de prótons H^+ do estroma para o lume através da ação de plastoquinonas, e pelos prótons provenientes da oxidação da água, causando a acidificação do lume do tilacoide (Allen, 2002). Dessa forma, a intensificação do transporte de elétrons leva à uma redução acentuada do pH o que, por sua vez, induz uma via de dissipação de energia conhecida como ciclo das xantofilas. Na condição de pH ácido ocorre a ativação da enzima VIOLAXANTHIN DE-EPOXIDASE (VDE), responsável por converter a xantofila violaxantina em zeaxantina (Baker, 2008). As zeaxantinas associadas ao PSII apresentam grande capacidade em dissipar energia na forma de calor, protegendo o PSII de possíveis danos devido ao excesso de energia (Bilger & Björkman, 1990). Com o aumento do pH do lume ocorre a desativação da VDE e a ativação da ZEAXANTHIN EPOXIDASE (ZE), responsável por converter zeaxantina em violaxantina, resultando na redução da dissipação de energia (Baker, 2008). Assim, esse mecanismo atua regulando a energia que é utilizada por via não fotoquímica (por exemplo, dissipação de energia na forma de calor) e fotoquímica (transporte de elétrons para produção de NADPH) (Adams & Demmig-Adams, 1992).

1.1.2. Etapa bioquímica

A etapa bioquímica é responsável pela fixação do CO_2, através da atividade carboxilase da enzima RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE (RUBISCO) no ciclo de Calvin-Benson. Esse ciclo é composto por três etapas, sendo a carboxilação a primeira delas, seguido da etapa de redução e por último a de recuperação. Na primeira ocorre a carboxilação da ribulose-1,5-bisfosfato, levando à formação de duas moléculas de 3-fosfoglicerato. Esse produto, então, será convertido a 1,3-bisfosfoglicerato, com o consumo de ATP, que por sua vez será reduzido a gliceraldeído-3-fosfato. Percebe-se, então, que na fase de redução ocorre o consumo de NADPH e ATP e a produção de trioses fosfato. Já a terceira fase é chamada de regeneração,

quando o substrato inicial do ciclo, ribulose-1,5-bisfosfato é regenerado com o consumo de ATP (Gutteridge & Jordan, 2001). Essa etapa ocorre no estroma dos cloroplastos, onde as trioses podem ser convertidas e estocadas na forma de amido ou exportadas para o citosol, onde podem ser convertidas a sacarose e exportadas via floema para outras partes da planta (Eaton-Rye *et al.*, 2012). Dessa forma, é possível notar que as duas etapas da fotossíntese estão interligadas pela redução/oxidação do NADPH e consumo/síntese do ATP.

A reação de carboxilação pode ser regulada por diversos fatores, entre eles, a proporção na disponibilidade de CO_2 e O_2 tem importante papel, já que a RUBISCO apresenta tanto a atividade carboxilase quanto oxigenase. Essa característica é definida pela afinidade do sítio ativo da enzima por ambos os substratos e em condições em que ocorre um aumento na quantidade de O_2 em relação ao CO_2 a atividade oxigenasse é favorecida (Kangasjärvi *et al.*, 2012). A oxigenação da ribulose-1,5-bisfosfato resulta na produção de 3-fosfoglicerato, mas também de 2-fosfoglicolato, que precisará passar por uma série de reações para ser convertido novamente a 3-fosfoglicerato. Essas reações ocorrem em três organelas, sendo cloroplasto, peroxissomo e mitocôndria e em cada uma ocorre a produção de alguns compostos marcantes, como o peróxido de hidrogênio (H_2O_2) através da ação da GLYCOLATE OXIDASE (GLO1) no peroxissomo, o amônio (NH_4^+) e o CO_2 através da mesma reação, realizada pela GLYCINE DECARBOXYLASE (GDP1) na mitocôndria (Busch, 2013). Esse conjunto de reações é conhecido como fotorrespiração e uma das suas características é a perda de carbono através do CO_2, o que é desvantajoso, quando se avalia a produtividade na forma de acúmulo de carbono. Além da perda de carbono, ocorre o consumo de energia para recuperar o 2-fosfoglicolato, para eliminar o H_2O_2 e assimilar o NH_4^+.

Estresses abióticos muitas vezes podem influenciar as etapas fotoquímica e/ou bioquímica. Por exemplo, o fechamento estomático devido à falta de água, resulta na redução da taxa de trocas gasosas entre o mesofilo e a atmosfera, o que pode resultar no aumento da concentração de O_2 em relação ao CO_2 e assim, estimular a fotorrespiração. Outro exemplo de fator abiótico é a exposição excessiva à luz, podendo, por exemplo, afetar a forma como a energia provinda da luz é utilizada na etapa fotoquímica, onde possivelmente mais energia será destinada a via não fotoquímica e uma menor quantidade usada de fato para a

produção de poder redutor (Demmig-Adams & Adams III, 1992). A alta intensidade luminosa pode também regular o sistema de absorção e uso da energia através da modulação da transcrição de componentes da cadeia de transporte de elétrons. A proteína CHLOROPLAST SENSOR KINASE (CSK) apresenta capacidade de inibir a transcrição de genes que codificam para componentes do PSII e PSI. A atividade cinase dessa proteína é regulada pelo estado redox do cloroplasto, sendo inibida pela tiorredoxina reduzida, e consequentemente, removendo o efeito negativo da fosforilação sobre o fator de iniciação SIGMA FACTOR 1 (SIG1), promovendo a ligação do PLASTID-ENCODED RNA POLYMERASE (PEP) e a transcrição dos genes (Puthiyaveetil *et al.*, 2012). Outras proteínas também respondem ao estado redox da célula e desencadeiam respostas de transcrição, como a PROTEIN PLASTID REDOX INSENSITIVE 2 (PRIN2) (Kindgren *et al.*, 2012; Díaz *et al.*, 2018) e a SERINE/THREONINE-PROTEIN KINASE STN7 (STN7) (Wunder *et al.*, 2013). Essas respostas levam a uma adequação do metabolismo a mudanças nas condições do ambiente. Um ambiente em que as plantas estão submetidas a diversos fatores estressantes é o hábitat epifítico, o qual será apresentado no próximo tópico.

1.2. O hábito epifítico e a família Bromeliaceae

O hábito epifítico é caracterizado pelo estabelecimento de um indivíduo vegetal sobre um hospedeiro, estando assim sem acesso a água e nutrientes presentes no solo (Benzing, 2000). Adicionalmente, as plantas epífitas estão submetidas à intermitência na disponibilidade hídrica e, em alguns casos, à exposição sazonal ou até mesmo permanente à radiação solar excessiva (Maxwell *et al.*, 1995). Dessa forma, o ambiente epifítico impõe algumas limitações ao desenvolvimento dos vegetais, sendo comum registrar baixas taxas de crescimento desses indivíduos (Zotz & Hietz, 2001). Mesmo com essas condições adversas, esse habitat é ricamente ocupado, especialmente na região neotropical (Nieder *et al.*, 2001), típica região de ocorrência da família Bromeliaceae, que se destaca pelo alto número de espécies epífitas (Givnish *et al.*, 2011).

Bromélias epífitas apresentam algumas adaptações morfológicas e fisiológicas associadas à obtenção e economia hídrica, que podem estar

relacionadas com o sucesso na ocupação do ambiente epifítico. Essas adaptações podem ser exemplificadas pela abundância de tricomas absortivos, formação do tanque e o metabolismo ácido das crassuláceas (CAM) (Crayn *et al.*, 2004). Os tricomas absortivos estão relacionados principalmente com a função de absorver água e nutrientes. De forma geral, as bromélias apresentam uma redução na proporção entre parte área e parte radicular e as raízes apresentam função majoritária de fixação ao hospedeiro, ao invés de absorção de nutrientes, a qual é desempenhada principalmente pelas folhas (Males, 2016). Nesse ponto os tricomas desempenham papel fundamental no suprimento de água e nutrientes, apresentando inclusive capacidade de absorver a água presente na neblina (Martorell & Ezcurra, 2007). Algumas espécies de bromélias apresentam maior densidade de tricomas na base foliar (Takahashi *et al.*, 2007; Kleingesinds *et al.*, 2018). Já outras espécies podem apresentar as folhas com uma distribuição mais uniforme dos tricomas, como é o caso de algumas espécies do gênero *Tillandsia* (Matiz *et al.*, 2013; Males, 2016). Muitas vezes a maior abundância de tricomas na base das folhas está acompanhada da presença do tanque, o que facilita a absorção de água e nutrientes, já que o tanque propicia o acúmulo de uma solução composta por água da chuva, detritos e excretas de animais (Benzing, 2000; Gonçalves *et al.*, 2016). O tanque pode ser descrito como uma estrutura constituída pela sobreposição da base das folhas, formando uma espécie de reservatório (Zotz *et al.*, 2011). Assim é possível notar que essas duas estratégias estão relacionadas com a aquisição de água pelo vegetal. Por outro lado, o CAM está mais associado a um uso mais eficiente da água já obtida. Mais detalhes serão apresentados a seguir.

1.3. Metabolismo ácido das Crassuláceas

O CAM é um tipo de metabolismo fotossintético caracterizado por uma variação diuturna no conteúdo de ácidos, isto é, uma forma transiente de armazenamento do CO_2. Durante a noite ocorre a obtenção de CO_2 atmosférico, pela abertura dos estômatos e a sua fixação através da ação em sequência das enzimas PHOSPHOENOLPYRUVATE CARBOXYLASE (PEPC) e MALATE DEHYDROGENASE (MDH), resultando na formação de malato que é armazenado no vacúolo na forma de ácido málico (Ranson & Thomas, 1960;

Winter, 2019). A abertura estomática durante a noite, resulta em uma menor perda de água por mol de CO_2 absorvido, já que a umidade relativa do ar normalmente é mais alta nesse período do dia, resultando em um uso mais eficiente da água quando comparado com espécies C_3 e C_4. Durante o período claro, ocorre a redução no conteúdo de ácidos devido a sua descarboxilação, e como os estômatos normalmente estão fechados, o CO_2 é acumulado no sítio ativo da RUBISCO (Niechayev *et al.*, 2019).

Alguns autores sugerem que o CAM surgiu em diversos momentos na história evolutiva das plantas e a ausência da necessidade de enzimas além das já presentes em espécies C_3 provavelmente contribuiu para esse surgimento em diferentes momentos e grupos (Crayn *et al.*, 2004; Mioto *et al.*, 2015). Baseado nisso, foi proposta a existência de um gradiente entre espécies C_3 e CAM onde diferentes níveis de absorção noturna de CO_2 e de acúmulo de ácidos podem ser notados (Winter *et al.*, 2015; Niechayev *et al.*, 2019). Dentro desse gradiente, temos espécies que apresentam um padrão de abertura estomática típico de plantas C_3, ou seja, sem absorção de CO_2 durante a noite, denominadas de CAM cycling. Já um outro grupo, classificado como CAM idling, apresenta os estômatos fechados tanto durante o dia como durante a noite. Em ambos grupos a acidificação noturna é devido a refixação do CO_2 proveniente da respiração e o nível de acúmulo de ácidos normalmente é menor do que o apresentado por plantas CAM clássicas, ou seja, as que apresentam o padrão noturno de abertura estomática.

Algumas espécies ainda apresentam a fotossíntese C_3 quando em boas condições hídrica, de temperatura, nutricional e de luz, porém são induzíveis a algum nível de CAM quando ocorre variação nessas condições (Maxwell *et al.*, 1995; Nievola *et al.*, 2005; Herrera, 2009; Winter & Holtum, 2014). Essas espécies são caracterizadas como CAM facultativas e normalmente retornam ao estado C_3 com o reestabelecimento de boas condições ambientais, propiciando crescimento e desenvolvimento. Nesses casos o CAM é visto como uma estratégia de enfretamento às condições adversas, principalmente o CAM idling, já que é um metabolismo que não promove o crescimento devido ao constante fechamento estomático.

O estudo do CAM em espécies facultativas tem gerado avanços importantes no entendimento sobre esse tipo fotossintético, como dos processos

de sinalização em resposta aos estresses abióticos (Freschi *et al.*, 2010*a*; Mioto & Mercier, 2013; Pereira *et al.*, 2013); efeito na economia hídrica, atividade fotossintética e produtividade durante períodos de seca (Pikart *et al.*, 2018; Silva *et al.*, 2019); relações filogenéticas entre C_3 e CAM (Crayn *et al.*, 2015); e tem sido de essencial importância no entendimento de quais genes são recrutados para o funcionamento do CAM (Cushman *et al.*, 2008*b*). Exemplo disso é a identificação de uma isoforma da PEPC que apresenta transcrição induzida com a transição do C_3 para o CAM (Cushman *et al.*, 1989). Recentemente, pesquisadores têm proposto estratégias para programar o CAM em espécies C_3 de interesse agrícola com o objetivo de aumentar a eficiência no uso da água por essas espécies (Borland *et al.*, 2014; Yang *et al.*, 2015; Winter, 2019). No entanto**,** ainda são necessárias outras investigações visando entender melhor o funcionamento desse metabolismo, assim como compreender o efeito da seca sobre a expressão de genes relacionados ao metabolismo fotossintético. Nesse sentido, espécies com fotossíntese CAM facultativa são excelentes modelos de estudo e podem contribuir para o avanço do conhecimento sobre a expressão do CAM nos vegetais. Um exemplo de espécie CAM facultativa é a *Guzmania monostachia* (L.) Rusby ex Mez (Medina, 1974).

1.4. Guzmania monostachia como modelo de estudo

Guzmania monostachia é uma bromélia epífita que apresenta tanque na sua fase adulta e que é induzida ao CAM idling após sete dias de déficit hídrico (Freschi *et al.*, 2010*b*; Kleingesinds *et al.*, 2018). Pode ser encontrada desde o sul da América do norte, em regiões da América central e norte da América do Sul (Pittendrigh, 1948; Smith *et al.*, 1986; Maxwell *et al.*, 1994). No Brasil é classificada como uma espécie vulnerável e pode ser encontrada principalmente no estado do Ceará nas formações vegetais de floresta ombrófila densa e estacional semidecidual e em brejos de altitude da Caatinga (Martinelli *et al.*, 2008; Martinelli & Moraes, 2013).

Estudos anteriores realizados no laboratório de fisiologia do desenvolvimento vegetal (IB-USP) demonstraram a existência de uma divisão funcional nas folhas dessa bromélia em três regiões distintas (base, mediana e ápice) (Freschi *et al.*, 2010*b*; Mioto & Mercier, 2013; Pereira *et al.*, 2013; Abreu *et al.*, 2018; Kleingesinds *et al.*, 2018; Pikart *et al.*, 2018). Onde a base estaria

mais relacionada à função de absorção de água e nutrientes, atuando como uma porção dreno por apresentar maior quantidade de hidrênquima e de tricomas e uma maior concentração de açúcares solúveis, tais como glicose, frutose e sacarose. Já o ápice é a porção com maior expressão de parâmetros relacionados ao CAM, como atividade da PEPC e MDH e acúmulo noturno de ácidos, quando exposta ao déficit hídrico. Entre as porções, o ápice é a que apresenta a maior exposição à luz, densidade estomática, quantidade de pigmentos fotossintetizantes e de amido, atuando dessa forma como fonte. Ainda para *G. monostachia*, foi demonstrado que o CAM pode contribuir de forma significativa para manter um balanço positivo de carbono, sugerindo que esse tipo fotossintético atua como uma estratégia para manutenção da atividade fotossintética durante períodos de seca (Pikart *et al.*, 2018). Além disso, os resultados desse mesmo estudo sugeriram que a disponibilidade de ácidos poderia influenciar o uso da energia pelo PSII devido a regulação na disponibilidade de CO_2 pela descarboxilação desses ácidos. Entretanto, existem poucas informações a respeito da fase clara do CAM, especificamente sobre a descarboxilação dos ácidos orgânicos, e da influência da seca, a qual essa espécie está submetida em seu ambiente natural, sobre a estrutura e funcionamento do PSII. Além disso, o significado fisiológico das alterações observadas na expressão de genes responsivos à seca, incluindo genes relacionados à fotossíntese, ainda é muito pouco compreendido (Pinheiro & Chaves, 2011). Com base nessas informações, algumas questões foram propostas:

1) Ocorreria modulação na atividade de descarboxilação e, consequentemente, na disponibilidade de CO_2 através de regulação transcricional e pós-traducional da enzima PEPCK?
2) A modulação no uso da energia pelo PSII acompanharia a atividade de descarboxilação dos ácidos?
3) Com a indução do CAM ocorreria um ajuste no conteúdo de pigmentos e proteínas do tilacoide, o que poderia ajustar o aporte de energia para a planta?
4) Esse ajuste nas proteínas estaria sob algum controle transcricional, como o já conhecido controle da cinase CSK sobre certos genes do centro de reação do PSII e PSI?

2. Objetivo

Investigar modulações da atividade de descarboxilação diurna dos ácidos orgânicos, da composição pigmento-proteica das membranas do tilacoide e do uso da energia pelo PSII de plantas de *G. monostachia* induzidas ao CAM como resposta ao déficit hídrico. Para alcançar esse objetivo algumas estratégias foram adotadas:

1) Avaliar a atividade de descarboxilação por meio de ensaio seletivo à forma ativa da enzima PEPCK e a expressão do gene que codifica para essa proteína.
2) Mensurar o uso fotoquímico e não-fotoquímico da energia pelo fotossistema II.
3) Investigar modificações no conteúdo de clorofilas e na proporção entre clorofilas e carotenoides.
4) Avaliar possíveis modulações na composição proteica e arranjo dos complexos proteicos das membranas do tilacoide.
5) Averiguar abundância de proteínas do PSII e PSI e a expressão dos respectivos genes codificantes.
6) Investigar o perfil transcricional de elementos reguladores da transcrição e da fosforilação de componentes do PSII e PSI.

3. Capítulo 1

Diurnal modulation of PEPCK decarboxylation activity impacts photosystem II light-energy use in a drought-induced CAM species

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Abstract

Crassulacean acid metabolism (CAM) is a photosynthetic pathway characterized by nocturnal CO_2 fixation into organic acids followed by its diurnal decarboxylation. Decarboxylation activity is time regulated, presenting normally higher activity in the middle of the day. This process can be performed by PEPCK enzyme resulting in an increase in the CO_2 concentration and may act to suppress photorespiration. It is suggested that the CO_2 provided by the decarboxylation can improve the photochemical use of energy in CAM plants. This photosynthetic pathway also contributes to improve water use efficiency, and, in an interesting way, some species can engage this photosynthesis when in a water deficit condition. *Guzmania monostachia* is a bromeliad that occupies habitats with intermittent water availability and CAM may be induced by water deficit. To check the light-time modulation of decarboxylation activity and light-energy use in *G. monostachia*, we analyzed the PEPCK activity, the photochemical and non-photochemical quenching and content of organic acids at three times of the day. The results showed a diurnal modulation where the highest PEPCK decarboxylation activity coincided with an increase in the photochemical quenching and a decrease in the non-photochemical. In addition,

a reduction in the acid content was observed, strongly indicating the interface between CO_2 generation through acid decarboxylation and its effect on PSII photochemical activity in CAM-induced plants. The same was not observed for well-watered plants. Also, PEPCK activity modulation may be related to transcriptional and post-translational regulation. Moreover, we suggest that the PEPCK is of great importance for the photosynthetic performance in CAM inducible plants and its activity regulation triggered by CAM induction is an interesting subject to be studied.

Keywords
PEPCK; *Guzmania monostachia*; decarboxylation; photosynthesis; drought; malic acid.

Abbreviations: CAM, Crassulacean acid metabolism; WUE, water use efficiency; PEPC, PHOSPHOENOLPYRUVATE CARBOXYLASE; OAA, oxaloacetate; ME, MALIC ENZYME; PEPCK, PHOSPHOENOLPYRUVATE CARBOXYKINASE; RUBISCO, RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE; PEP, phosphoenolpyruvate; H_2O_2, hydrogen peroxide; GLO, GLYCOLATE OXIDASE; PSII, photosystem II; F_v/F_m, maximum quantum efficiency of PSII photochemistry; ϕPSII, operational efficiency of photosystem II; Y(NPQ), quantum yield of non-photochemical; Y(NO), quantum yield of non-regulated energy loss at PSII.

3.1. Introduction

Crassulacean acid metabolism (CAM) is a photosynthetic adaptation that provides better water use efficiency (WUE) due to stomatal closure during the light-time of day, a critical period with the lowest air humidity. In this way, the atmospheric carbon dioxide (CO_2) uptake during the night results in less water loss per mol of assimilated CO_2 (Lüttge, 2004). During this period, the CO_2 can be stored as malic acid in the vacuoles through the combined action of PHOSPHOENOLPYRUVATE CARBOXYLASE (PEPC) and MALATE DEHYDROGENASE activities (carboxylation phase) (Lüttge et al., 1986; Silvera et al., 2010). During the transition of dark to light-time (dawn) there is a decrease in the carboxylation activity of PEPC and an increase in RIBULOSE-1,5-

BISPHOSPHATE CARBOXYLASE/OXYGENASE (RUBISCO) (Matiz *et al.*, 2013). At this time, the decarboxylation activity is still low, and the CO_2 is supplied via stomata. The major decarboxylation activity with closed stomata occurs during the day, allowing an increase in the internal CO_2 concentration (Cushman and Borland, 2002). The CO_2 becomes available through malate or oxaloacetate (OAA) decarboxylation, reactions performed by MALIC ENZYME-NAD/NADP (ME) (Casati *et al.*, 1999) or PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) enzyme (Dittrich *et al.*, 1973; Leegood and Walker, 2003), respectively (decarboxylation phase). Consequently, the CO_2 can be fixed by the RUBISCO in the C_3 cycle. During the transition from day to night (dusk), the stomata start opening, the carboxylation activity by RUBISCO declines, and the malate pool is almost totally consumed. Some species present a decarboxylation activity performed by both ME and PEPCK enzymes; however, PEPCK plays a major role in the total decarboxylation activity (Holtum and Osmond, 1981).

CAM photosynthesis is also recognized as a carbon concentration mechanism that can act in reducing the photorespiration rate and avoiding the overexcitation of the photosynthetic apparatus (Griffiths, 1989; Ehleringer and Monson, 1993). This mechanism is advantageous since it can reduce the production of reactive oxygen species, like hydrogen peroxide (H_2O_2), mostly during the day (Niewiadomska and Borland, 2008). The stomatal closure during the day allows the concentration of CO_2; however, it also promotes an increase in the internal O_2 concentration at the end of the day, up-regulating photorespiration reactions (Luttge, 2002). Furthermore, as the mechanism of CO_2 concentration depends on the vacuolar malic acid, the depletion of this acid could induce H_2O_2 production by GLYCOLATE OXIDASE (GLO), an important enzyme for the photorespiration metabolism (Fock and Krotkov, 1969; Niewiadomska and Borland, 2008). Therefore, the CAM light-time events (*i.e.*, decarboxylation reactions and stomatal closure) may influence not just the WUE but also the light-energy use and photorespiration.

A close relation between the CAM and the photochemical use of energy has been previously described. Based on results showing a decrease of both the acid content and the electron transport rate in the CAM species *Kalanchoe daigremontiana* (Raym. -Hamet & H. Perrier) A. Berger. and *Kalanchoe pinnata* (Lam.) Pers., it was suggested that CO_2 provided by malate decarboxylation

modulated light-energy use in a photochemical way (Griffiths *et al.*, 2008). The same modulation effect was suggested for CAM induced species *Mesembryanthemum crystallinum* L. (Niewiadomska *et al.*, 2011) and *Guzmania monostachia* (L.) Rusby ex Mez (Pikart *et al.*, 2018). However, none of these studies provided a complete analysis providing data about photosystem II (PSII) energy use, acid content and decarboxylation activity during the course of a day. In addition, knowledge about the expression of CAM-genes during the day-night cycle is lacking (Winter, 2019).

Guzmania monostachia features a neotropical distribution occupying an epiphytic habitat (Medina *et al.*, 1977; Maxwell *et al.*, 1999; Martinelli *et al.*, 2008), which is normally characterized by no access to soil moisture, being dependent instead the atmospheric water (*e.g.* rain, air humidity, fog) (Zotz, 2016). Thus, this bromeliad presents a plastic photosynthetic capacity capable of adapting to different light and water conditions, inducing CAM idling under water stress (Medina and Troughton, 1974; Maxwell *et al.*, 1992; Freschi *et al.*, 2010). CAM idling is characterized by stomatal closure during all day and a small variation in the acid content due to the recycling of the respired CO_2 through PEPC activity (Cushman, 2001; Borland *et al.*, 2011). Moreover, the induction of this type of CAM is reported as a result of abiotic stress, such as water shortage (Rayder and Ting, 1983; Herrera *et al.*, 2008; Ceusters *et al.*, 2009), as a metabolic strategy to save water and survive an adverse period (Herrera, 2000).

Since CAM plants show time modulations in the decarboxylation reactions, which, together with the stomatal closure, provide a special control of intracellular CO_2 availability, CAM seems to influence the PSII light energy use and carbohydrate synthesis reactions. Considering that CAM idling is induced under water deficit in *G. monostachia*, we propose that the CO_2 provided by the PEPCK decarboxylation powers the PSII photochemical light energy use instead of the non-photochemical use. More specifically, the OAA decarboxylation provides CO_2 for the carboxylation reaction performed by RUBISCO, consuming NADPH and providing $NADP^+$, inducing the photochemical energy use. Therefore, the aim of this study was to investigate the influence of the light-time dynamic of CAM reactions on the light energy use by the epiphytic C_3-CAM facultative bromeliad, *G. monostachia* submitted to a period of drought by evaluating (1) PEPCK

decarboxylation activity, (2) *PEPCK* transcript abundance, and (3) diurnal dynamic of PSII light energy use.

3.2. Material and Methods

3.2.1. Plant growth and experimental design

Adult tank-forming plants of *Guzmania monostachia* (L.) Rusby ex Mez (Tillandsioideae, Bromeliaceae) (SISGEN record number A279903) with approximately 2.5 years of *ex-vitro* growth were cultivated as described by Rodrigues *et al.* (2016). The plants were acclimated in a growth chamber with a photoperiod of 12h (light-time from 6:00h until 18:00h), 250 µmol of photons m^{-2} s^{-1}, temperature of 27/22°C, humidity of 60/70% (day/night) and watered daily for 30 days before the beginning of the experiment. These same conditions of light, humidity, and temperature were employed during the experiments.

The plants were submitted to two hydric conditions: water deficit (WD), watering withholding for 20 days; and well-watered (WW), daily watered (control). The samples were collected in three different times, 7:00h (dawn), 12:00h (noon), and 17:00h (dusk). The chlorophyll fluorescence and CO_2 gas-exchange measurements were obtained using the 8th youngest developed leaf of seven intact plants. The samples for the relative water content, titratable acidity, PEPCK decarboxylation activity, H_2O_2 content, and relative gene expression were composed only by the apical parts of the leaves from the 8th youngest developed leaf until the 12th node of at least four plants. They were collected and immediately frozen in liquid nitrogen. After that, the foliar samples were ground to a powder and stored at -80°C for the following analyses.

3.2.2. *Relative water content*

The relative water content was determined as described by Pikart *et al.* (2018) using the equation described by Weatherley (1950). The ratio of dry mass (DM)/fresh mass (FM) was used to correct and present the titratable acidity, PEPCK activity, and hydrogen peroxide content per gram of DM.

3.2.3. *Titratable acidity*

The organic acids extraction was performed as described by Matiz *et al.* (2017), and the upper phase was collected and used to perform the titratable

acidity in a microplate using different concentrations of sodium hydroxide (NaOH) per row. The pH indicator used was the phenolphthalein (Merch) prepared in ethanol with a final concentration of 0.03 mM. A NaOH solution was used to neutralize the pH and calculate the amount of H^+.

3.2.4. Chlorophyll fluorescence and gas-exchange analyses

The maximum quantum efficiency of PSII photochemistry (F_v/F_m) and the PSII operating efficiency (ϕPSII) were measured as defined by Baker (2008), the electron transport rate (ETR) calculated as described by Alves *et al.* (2016), and non-photochemical quantum yield [Y(NPQ) and Y(NO)] as described by Klughammer and Schreiber (2008). All these parameters were obtained with a pulse-amplitude modulated chlorophyll fluorometer (PAM 2500, Walz). A 30-min dark-adapting time with manufacturers leaf clip was used to obtain the F_0 and F_m parameters, and a saturating pulse (6000 µmol photons m^{-2} s^{-1}) to obtain the F_m and F_m'.

The net CO_2 exchange was measured using an infrared gas analyzer system (Li-6400, LI-COR) with a partial pressure of CO_2 of 400 ppm and a photosynthetic flux density of 250 µmol photons m^{-2} s^{-1}.

3.2.5. PEPCK decarboxylation activity assay

The assay for decarboxylation activity was based on the method described by Martín et al. (2007) with modifications. For the extraction a buffer was used composed of 100 mM Tris-HCl, pH 8.5; 2 mM EDTA; 5 mM $MgCl_2$; 20% (v/v) glycerol; 1 mM DTT; 7 µM cysteine protease inhibitor E-64; and 1 mM serine protease inhibitor PMSF prepared in 100% ethanol (James, 1978). The two proteases inhibitors were prepared in a stock solution, stored at -20°C, and added to the buffer before the protein extraction.

The extraction was performed with 400 mg FM (fresh mass) of powdered leaves divided into two microtubes with 1 mL of the extraction buffer, mixed and then centrifuged for 10 min (12 000 g, 4°C). The supernatant was filtered in fiberglass with a syringe and desalinized with a Sephadex column G-25 (GE life sciences) at 4°C. The columns were previously washed and conditioned with ultrapure water and extraction buffer before use. The reaction buffer contained Hepes-NaOH 100 mM, pH 8.0; 2 mM $MgCl_2$, and 1 mM $MnCl_2$. Aliquots of ATP

(2 mM), ADP (0.2 mM), NADH (0.1 mM), and OAA (0.5 mM) were prepared in reaction buffer and stored separately. The OAA aliquot was prepared just before the analysis. For this evaluation, two other enzymes were also necessary, the PYRUVATE KINASE, 1 unit; and the LACTATE DEHYDROGENASE, 2.5 units. The reaction was started with the OAA, and the NADH consumption was measured continuously at 340 nm. Since the OAA spontaneously presents decarboxylation when in the reaction medium, it was necessary to measure this decarboxylation without the sample (blank reaction) and subtract this from the assay with the sample, enabling the determination of the actual decarboxylation activity.

3.2.6. *Quantitative PCR analysis*

The total RNA was extracted from 100 mg FM of powdered leaves using TRIzol reagent (Invitrogen) and the PureLink RNA mini kit (Life Technologies) following the manufacturer instructions. The quantity and integrity of the extracted RNA were checked using a nanodrop spectrophotometer and a 1% (w:v) agarose gel electrophoresis. Before cDNA synthesis, DNA traces were removed using DNase I (Invitrogen); it was then synthesized by a SuperScript IV reverse transcriptase (Thermo Fisher Scientific) and treated with RNaseOUT ribonuclease inhibitor (Invitrogen), reaching a final volume of 20 µL. Each sample was diluted to a final concentration of 5 ng reverse-transcribed RNA μL^{-1} to be used for real-time PCR reactions.

The primers were designed for sequences of the genes of interest obtained on the *G. monostachia* transcriptome (Mercier *et al.*, in press). To check the functionality of the sequences obtained, the translation reads of each sequence were analyzed using the SnapGene software (https://www.snapgene.com/) and BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The software PearlPrimer (http://perlprimer.sourceforge.net/) was used to design the primers for the sequences of interest. Then, the quantitative PCR reactions and primer efficiency were performed as described by Matiz *et al.* (2019). The forward and reverse primer sequences are available in Table S1. Gene stability of the reference genes *BAM* and *HSDD* were tested using NormFinder software (Andersen *et al.*, 2004), and the geometric mean was used to calculate the ΔΔCt method (Hellemans *et al.*, 2007) of the target gene. The relative gene expression was calculated using

four biological replicates and the well-watered treatment used as normalizer factor.

3.2.7. Hydrogen peroxide content

The hydrogen peroxide content analysis was performed as described by Carvalho *et al.* (2017).

3.2.8. Statistical analyses

Comparisons between time of day and water conditions were performed using two-way ANOVA and Tukey HSD *post hoc* ($\alpha = 0.05$), comparisons between water conditions were made using ANOVA and Tukey HSD *post hoc* ($\alpha = 0.05$) or ANOVA and T-test *post hoc* ($\alpha = 0.05$) (Table S2) with the statistical software JMP 14 (https://www.jmp.com/en_us/home.html).

3.3. Results

3.3.1. Drought modulates the photosynthetic capacity and the photosynthesis pathway

In order to investigate the effects of a long-time water deficit over the photosynthetic capacity and CAM induction, we evaluated the water status, some photosynthetic parameters and the mRNA abundance of CAM isoform of *PEPC* (*PPC1*). The results showed a decrease of approximately 25% in the water content of the apical part of their leaves (Fig. 1A), which may have resulted in a reduction of atmospheric CO_2 uptake, probably due to stomatal closure (Fig. 1B), and decrease of 25% in the maximum efficiency that PSII can perform photochemistry (F_v/F_m) (Fig. 1C). In addition, the *PPC1* expression was upregulated during the entire light-time period compared to the WW plants (Fig. 1D). However, after seven days of rehydration, the plants recovered their water content (Fig. 1A) as well as photosynthetic activity (Fig. 1B and 3C), and the abundance of *PPC1* transcripts was equivalent to a C_3 plant (Fig. 1D). Knowing that *G. monostachia* can recover from this long drought treatment, we investigated the diurnal CAM activity after 20 days of water deficit.

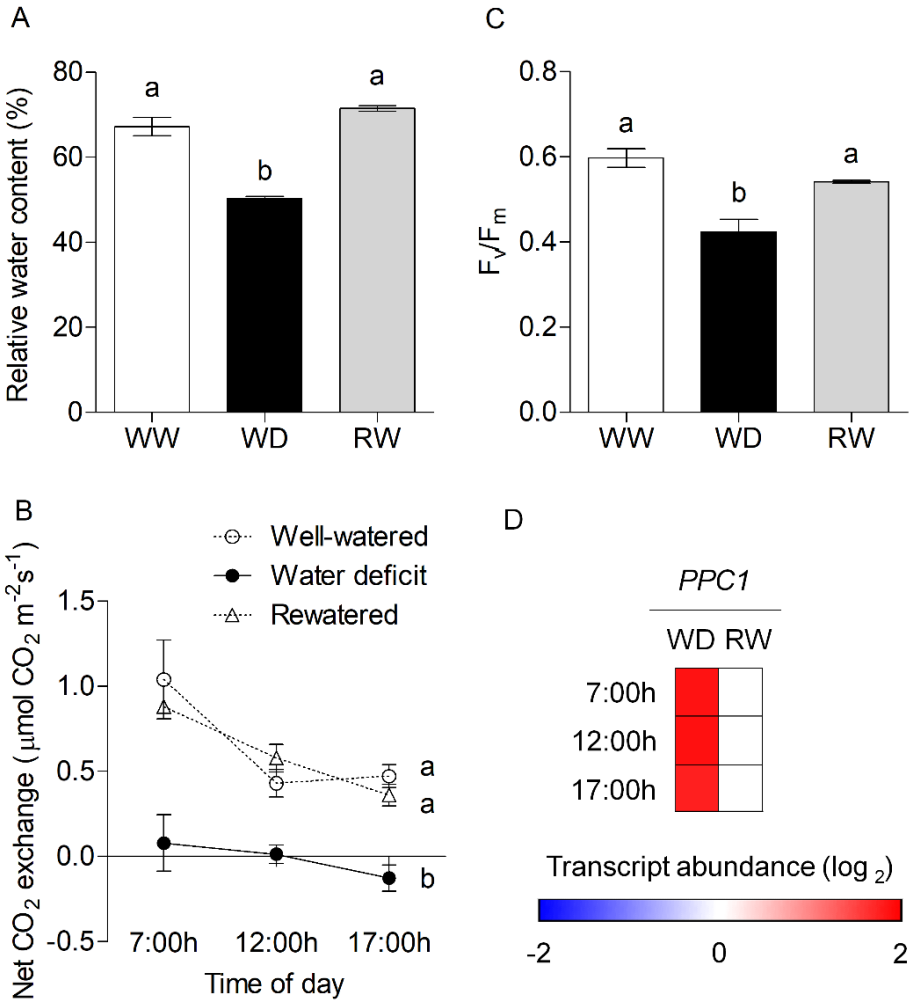

Figure 1. (A) Relative water content, (B) net CO_2 exchange, (C) maximum quantum efficiency of PSII photochemistry (F_v/F_m), and (D) *PHOSPHOENOLPYRUVATE CARBOXYLASE* (*PPC1*) transcript abundance of *G. monostachia* plants well-watered (WW), under 20 days of water deficit (WD) and rewatered (RW) for seven days after the WD treatment. Different letters indicate a statistical difference between means of four independent plants in (A) and (C) (ANOVA, Tukey *post hoc* test, α = 0.05) and between water conditions (WW, WD, and RW) in (B) (ANOVA two-factors, Tukey HSD *post hoc* test, α = 0.05). Error bar indicates the standard error (SE). Only statistically different *PPC1* transcript abundance is presented (ANOVA, T-test *post hoc*, α = 0.05).

3.3.2. Diurnal decarboxylation activity of CAM-drought-induced plants

Besides the *PPC1* mRNA levels, the CAM induction was also verified by the titratable acidity levels analyzed (Fig. 2A), and it is possible to note that the WD plants presented a three-fold increase in acid accumulation, considering accumulation as the difference in acid content between dawn and dusk. Moreover, at dawn, the WD plants showed a value of acidity 37% higher than WW plants. The acids were continuously consumed along the day, reaching the same level of WW plants at dusk, indicating the decarboxylation of these acids. In order to investigate the diurnal dynamic of decarboxylation reactions in this inducible CAM bromeliad, we evaluated the abundance of *PEPCK* transcripts (Fig. 2B) and the decarboxylation activity of non-phosphorylated PEPCK (Fig. 2C). The results showed that the *PEPCK* was differentially regulated in the WD plants compared with the WW condition. The *PEPCK* mRNA level was higher at dawn when compared with watered plants. On the other hand, the decarboxylation activity of WD plants showed the highest activity at noon, being 1.5-fold higher when compared with the dawn and dusk. However, diurnal differences in the PEPCK activity were not observed in WW bromeliads.

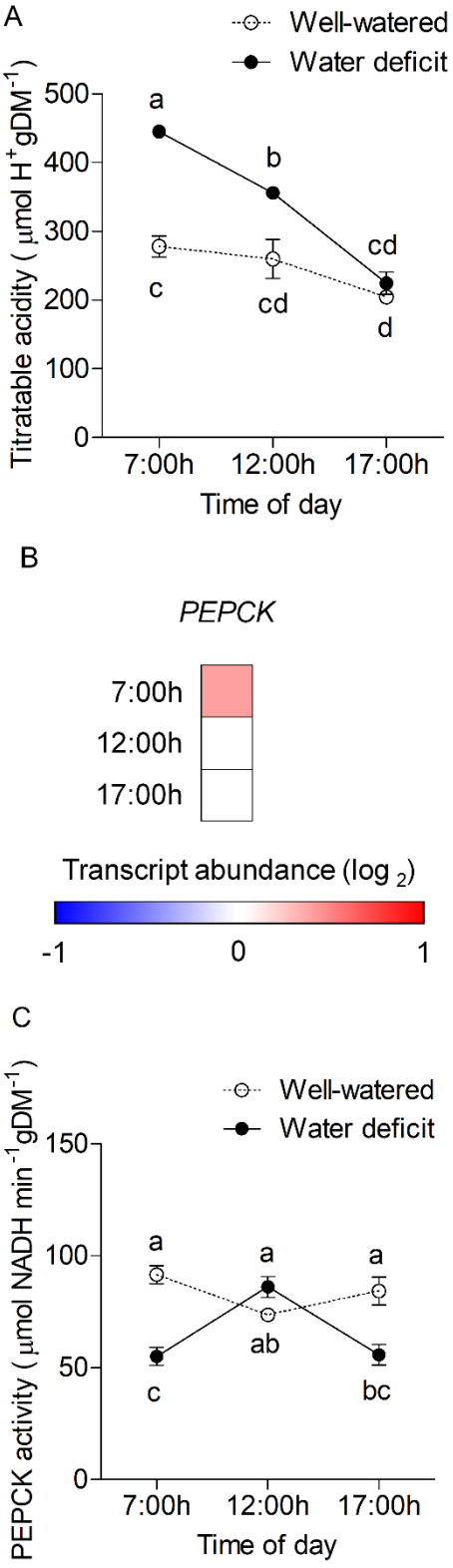

Figure 2. (A) Titratable acidity, (B) *PHOSPHOENOLPYRUVATE CARBOXYKINASE* (*PEPCK*) transcript abundance, and (C) PEPCK decarboxylation activity of *G. monostachia* plants under 20 days of water deficit and well-watered conditions. The decarboxylation assay was performed in a selective medium evaluating the activity of the dephosphorylated PEPCK *(*Walker *et al., 2002)*. Different letters indicate a statistical difference between means of four independent plants (ANOVA two-factors, Tukey HSD *post hoc* test, α = 0.05). Error bar indicates the standard error (SE). Only statistically different transcript abundance is presented (ANOVA two-factors, Tukey HSD *post hoc* test, α = 0.05).

3.3.3. PSII light-energy use of a CAM bromeliad

Since the uptake of CO_2 was almost null in WD plants, the source of this gas was the decarboxylation of acids. In this way, to verify if the light-energy use was regulated by CO_2 availability, we analyzed the photochemical (ϕPSII and ETR) and non-photochemical [Y(NPQ) and Y(NO)] use of this energy at three different times (7:00h, 12:00h, and 17:00h). The photochemical use was overall reduced in WD bromeliads (Fig. 3A and 3B), registering up to three-fold decreases, especially at dawn and dusk (7:00h and 17:00h). Interestingly, a diurnal modulation of these parameters was noted, where the highest use of light-energy to reduce electron acceptors and perform electron transport was registered at 12:00h. In contrast, the lowest regulated non-photochemical quenching yield [Y(NPQ)] rate was noted at noon in WD plants (Fig. 3C). Meanwhile, the watered plants presented no modulation of these parameters. Additionally, the quantum yield of non-regulated energy loss at PSII [Y(NO)] presented values higher than the WW plants with no time modulation in either water condition (Fig. 3D).

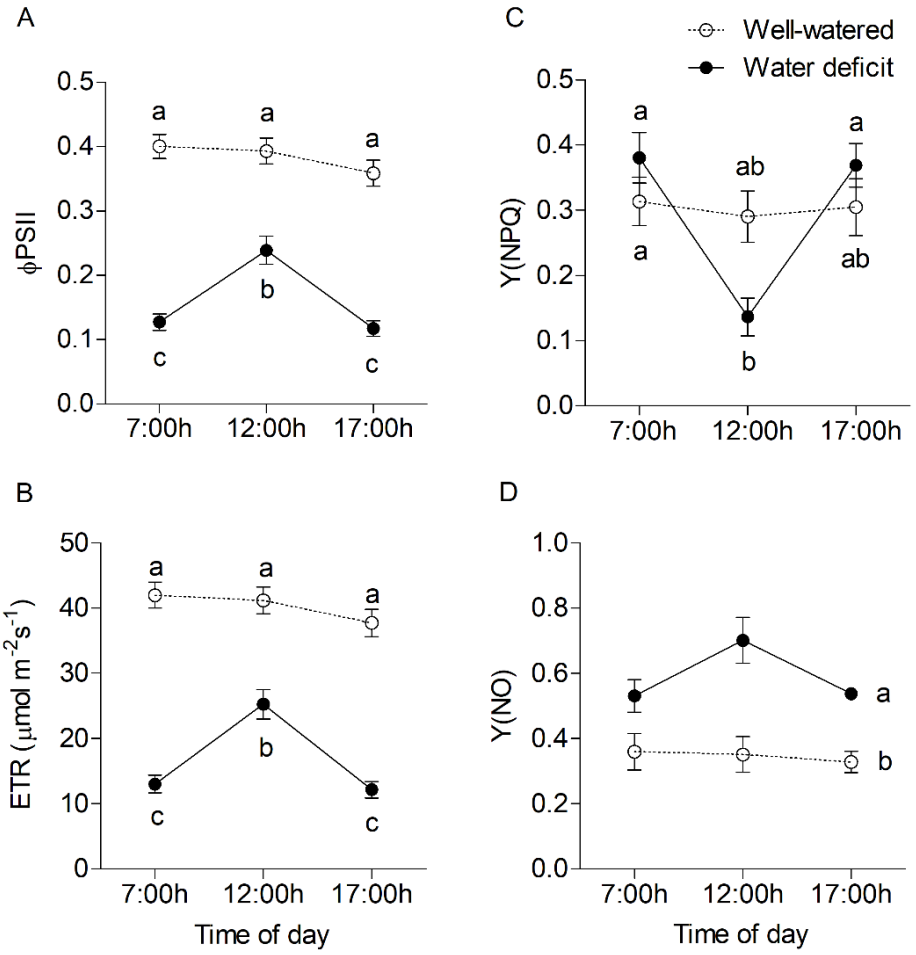

Figure 3. (A) PSII operational efficiency (ϕPSII), (B) electron transport rate (ETR), (C) non-photochemical quantum yield [Y(NPQ)], and (D) quantum yield of non-regulated non-photochemical quenching [Y(NO)] of *G. monostachia* plants under 20 days of water deficit and well-watered conditions. Different letters indicate a statistical difference between means of seven independent plants (ANOVA two-factors, Tukey *post hoc* test, α = 0.05). In (D) different letters indicate a difference between water conditions (ANOVA two-factors, Tukey HSD *post hoc* test, α = 0.05). Error bar indicates the standard error (SE).

3.4. Discussion

3.4.1. Guzmania monostachia is CAM-induced under long-term drought

In the epiphytic habitat, *G. monostachia* faces intermittent water availability (Maxwell *et al.*, 1994). As a strategy to deal with the drought stress condition, this species presents the induction of CAM pathway (Medina and Troughton, 1974), already reported for different times of exposure, such as four days (Beltrán *et al.*,

2013; Pikart *et al.*, 2018) or a week of water withholding (Freschi *et al.*, 2010; Mioto and Mercier, 2013). Considering this, we submitted this bromeliad to a drought treatment for 20 days followed by a recovery period of seven days of rewatering. The extended drought condition caused a decrease in the water status of *G. monostachia* plants (Fig. 1A) and may also have induced the decrease in the PSII light use capacity (Fig. 1C), which was not observed in a short period (4 and 8 days) of water restriction (Abreu *et al.*, 2018; Pikart *et al.*, 2018). Likewise, when *Arabidopsis thaliana* (L.) Heynh was submitted to progressive drought treatment, a reduction in the F_v/F_m was observed only after 12 days (Chen *et al.*, 2016). However, this C_3 species lost approximately 74% of its water content, which indicates the utmost importance of CAM induction as a water-saving mechanism for *G. monostachia*. Interestingly, after a two-week period of drought, North *et al.* (2019) observed that *G. monostachia* recovered leaf hydraulic conductance four days after rehydration. Moreover, after 20 days of drought, this plant was able to recover from the effects on the water status, atmospheric CO_2 uptake, and F_v/F_m after seven days of rewatering, demonstrating the reestablishing capacity from a stress condition (Fig. 1). In this investigation we demonstrated an upregulation of *PPC1* expression (Fig. 1D) and consequently an increase in the acidity levels (Fig. 2A), indicating that even a long-term treatment can induce the CAM photosynthesis in this species. These results indicate the metabolic plasticity of this bromeliad, and, therefore, the capacity to tolerate the condition of water intermittence may determine its success in occupying the canopies.

3.4.2. CAM diurnal reactions and PSII light-energy use

After the WD treatment, the decarboxylation reactions and modulations on PSII light-energy use were investigated in CAM drought-induced plants of *G. monostachia*. At dawn, the CAM plants normally present a reduction in the PEPC carboxylation and an increase in the carboxylation by RUBISCO (Nimmo, 2000; Griffiths *et al.*, 2002). Additionally, the stomata remain open, and the decarboxylation activity is low; consequently, both carboxylation enzymes are supplied by atmospheric CO_2 (Matiz *et al.*, 2013). The CO_2 can be used by RUBISCO to produce carbohydrates consuming NADPH and producing $NADP^+$, which can be reduced again at the end of the thylakoid electron transport chain.

As classically described for CAM plants (Ting, 1985), the bromeliad *G. monostachia* also showed a high content of organic acids and low decarboxylation activity at dawn (Fig. 2). However, the atmospheric CO_2 uptake was nearly zero (Fig. 1B), which could result in a low CO_2 supply, reducing the NADPH consumption by RUBISCO and influencing the PSII photochemical efficiency (Genty *et al.*, 1989; Baker, 2008). In fact, the results at 7:00h and 17:00h clearly showed a low use of energy for photochemical reaction (ϕPSII) and a higher dissipation of energy, noted by the Y(NPQ) (Fig. 3A and 3C). A reduced ϕPSII was also observed in CAM state *M. crystallinum* at the start of light-period (Niewiadomska *et al.*, 2011), and this downregulation of photochemical energy use could be due to low RUBISCO activity (Griffiths *et al.*, 2002). In addition to this plethora of events that take place at dawn, we presented data showing *PEPCK* transcript abundance of a CAM-inducible species (Fig. 2B). Indeed, it was the only time during light-time that WD plants showed a difference in *PEPCK* mRNA levels compared to WW plants. As a matter of fact, the CAM-related genes remain poorly studied (*e.g. PEPCK*), with more research devoted to the expression variations during the day-night cycle of these genes (Winter, 2019).

During the day, the malic acid is transported from the vacuole to the cytosol as malate, later converted in OAA, which can be decarboxylated by PEPCK (Dittrich *et al.*, 1973; Holtum *et al.*, 2005). The present data showed a reduction in the content of acids accompanied by an increase in the decarboxylation activity in WD plants of *G. monostachia* (Fig. 2A and 2C). Likewise, *K. daigremontiana* presented a stable cytosolic pH at dawn, followed by acidification of the cytosol at noon (Hafke et al., 2001). In addition, the authors also observed a decrease in the vacuolar malic acid content, indicating a high efflux of acids from vacuole at this time of day. The increase in *PEPCK* transcript levels may have contributed to the regulation of decarboxylation through an increase in the amount of protein in WD plants (Fig. 2B). In drought-induced CAM species, *Clusia aripoensis* Britton and *Clusia minor* L., an increase in PEPCK protein level and its activity was noted after 10 days of water deficit, but a diurnal dynamic was not explored (Borland *et al.*, 1998). On the other hand, a modulation in the PEPCK protein level in the day-night cycle of the CAM bromeliad *Tillandsia fasciculata* Sw. was not observed (Walker and Leegood, 1996). However, Walker and Leegood also observed a

diurnal oscillation in the phosphorylated form of PEPCK, indicating that this protein is post-translationally regulated by phosphorylation in CAM plants. In this study we investigated the PEPCK decarboxylation activity of the active form of this protein by using a selective assay (Walker *et al.*, 2002). In this way, the increase in the decarboxylation activity at 12:00h could be due to the dephosphorylation of PEPCK (Fig. 2C), increasing the affinity to OAA and, consequently, increasing the decarboxylation activity (Walker *et al.*, 2002). A similar result was observed in *Ananas comosus* (L.) Merr. when the decarboxylation activity was investigated in a selective assay condition, where suppression of the activity was observed during the night and an increase during the day, reaching the maximum decarboxylation rate at 12:00h (Leegood and Walker, 2003). This post-translational regulation seems to be very important in CAM plants, avoiding a futile cycle of OAA synthesis by PEPC and OAA decarboxylation by PEPCK (Ku *et al.*, 1980; Walker and Leegood, 1996). Interestingly, only the WD bromeliads showed a modulation in the PEPCK activity, which could avoid this futile cycle in CAM-induced plants. This indicates that the CAM induction in *G. monostachia* is accompanied by a regulation of a protein that acts as PEPCK kinase, controlling the PEPCK phosphorylation and modulating its activity.

Since the acid decarboxylation increases the internal CO_2, it can be used in the Benson-Calvin cycle, driving the NADPH oxidation. In fact, the WD *G. monostachia* plants at 12:00h presented the highest value of ϕPSII compared with the other times of the day and the lowest Y(NPQ) (Fig. 3A and 3C), indicating the use of light energy to transport electrons (Fig. 3B), probably driving $NAPD^+$ reduction and a minor necessity to dissipate energy (*e.g.* heat). Otherwise, the WW plants do not show fluctuations in the photochemical and non-photochemical parameters, possibly because they were well provided by atmospheric CO_2 (Fig. 1B). Therefore, the data suggest that CAM syndrome, through the CO_2 provided by the organic acid decarboxylation, may act to regulate the PSII efficiency, at the same time improving the production of NADPH and powering the Calvin-Benson cycle.

3.4.3. CAM diurnal reactions: importance and perspectives

The results showed here call attention to the great importance of decarboxylation reaction to the photochemical activity of this CAM-induced bromeliad. In this way, researchers demonstrated using computational analyses that CAM plants can achieve the same productivity as a C_3 crop through an increase in the carboxylase/oxygenase ratio of the RUBISCO (Shameer *et al.*, 2018). Moreover, CAM plants that use PEPCK for the decarboxylation presented best-predicted productivity over species that use MALIC ENZYME. In this context, *G. monostachia*, a PEPCK-CAM species that showed differential expression and activity of this protein, can provide important knowledge about the regulation of essential processes that take place in the light-time period. Furthermore, the upregulation of both pathways, carboxylation through PEPC and decarboxylation through PEPCK could improve the plant biomass productivity in CAM or even in C_3-CAM bioengineered plants. The increase in the malate synthesis/decarboxylation process could reduce the photorespiration effects increasing the RUBISCO carboxylation/oxygenation rate and improving the PSII photochemical light energy, enhancing the NADPH production.

Investigating some photorespiration indicators, we observed a downregulation of the *GLO1* expression in WD plants at dawn and dusk (Fig. S1A), and only at dusk was an increase in the H_2O_2 observed (Fig. S1B). These results may indicate that WD plants present some photorespiration suppression; however detailed investigations regarding this subject should be made. We suggest that projects aiming at CAM manipulation (*e.g.* genes involved in CAM diurnal decarboxylation) should also consider evaluating changes over the metabolism yield.

3.4.4. Final remarks

In conclusion, the exposure of *G. monostachia* plants to 20 days of water deficit resulted in CAM up-regulation without causing severe damage that put the survival of the plant at risk since the plants were able to recover after seven days of rewatering. The drought treatment also regulated transcriptionally and post-translationally the PEPCK protein, resulting in diurnal modulation in the decarboxylation activity of this protein, with higher activity in the middle of the day. Moreover, this study provides more evidence about the CAM photosynthesis

regulation regarding the light energy use by PSII, indicated by the reduction in the organic acid content and Y(NPQ) at the same time of the day in which an increase in the decarboxylation activity and ϕPSII occurred. Additionally, the results call attention to PEPCK that are involved in the coordination of these light-time CAM process; the mechanism that controls its phosphorylation and consequently its activity should be considered for future studies. A graphical abstract illustrating the results of CAM up-regulated plants can be found in figure 4.

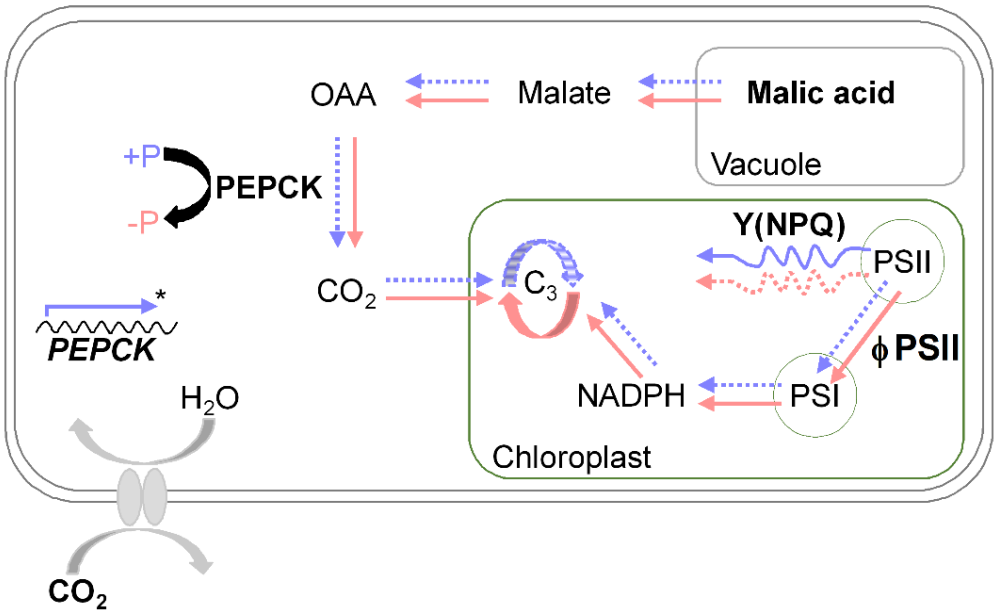

Figure 4. Diurnal modulation on PEPCK activity and PSII light energy in CAM-induced *G. monostachia*. In bold, physiological, biochemical and molecular parameters analyzed in this work. In blue, measurements made at 7:00h and 17:00h and in red, at 12:00h. Dashed lines and continuously lines indicate a process that was downregulated or upregulated, respectively. The asterisk indicates that the *PEPCK* transcription was upregulated only at 7:00h.

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References

Abreu ME, Carvalho V, Mercier H. 2018. Antioxidant capacity along the leaf blade of the C_3-CAM facultative bromeliad *Guzmania monostachia* under water deficit conditions. Functional Plant Biology 45, 1–10.

Alves FRR, de Melo HC, Crispim-Filho AJ, Costa AC, Nascimento KJT, Carvalho RF. 2016. Physiological and biochemical responses of photomorphogenic tomato mutants (cv. Micro-Tom) under water withholding. Acta Physiologiae Plantarum 38, 1–14.

Andersen CL, Jensen JL, Ørntoft TF. 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64, 5245–5250.

Baker NR. 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annual review of plant biology 59, 89–113.

Beltrán JD, Lasso E, Madriñán S, Virgo A, Winter K. 2013. Juvenile tank-bromeliads lacking tanks: Do they engage in CAM photosynthesis? Photosynthetica 51, 55–62.

Borland AM, Barrera Zambrano VA, Ceusters J, Shorrock K. 2011. The photosynthetic plasticity of Crassulacean acid metabolism: an evolutionary innovation for sustainable productivity in a changing world. New Phytologist 191, 619–633.

Borland AM, Técsi LI, Leegood RC, Walker RP. 1998. Inducibility of crassulacean acid metabolism (CAM) in *Clusia* species; physiological/biochemical characterisation and intercellular localization of carboxylation and

decarboxylation processes in three species which exhibit different degrees of CAM. Planta 205, 342–351.

Carvalho V, Abreu ME, Mercier H, Nievola CC. 2017. Adjustments in CAM and enzymatic scavenging of H_2O_2 in juvenile plants of the epiphytic bromeliad Guzmania monostachia as affected by drought and rewatering. Plant Physiology and Biochemistry 113, 32–39.

Casati P, Drincovich MF, Edwards GE, Andreo CS. 1999. Malate metabolism by NADP-malic enzyme in plant defense. Photosynthesis Research 61, 99–105.

Ceusters J, Borland AM, Londers E, Verdoodt V, Godts C, De Proft MP. 2009. Differential usage of storage carbohydrates in the CAM bromeliad *Aechmea* “Maya” during acclimation to drought and recovery from dehydration. Physiologia plantarum 135, 174–84.

Chen Y-E, Liu W-J, Su Y-Q, Cui J-M, Zhang Z-W, Yuan M, Zhang H-Y, Yuan S. 2016. Different response of photosystem II to short and long term drought stress in *Arabidopsis thaliana*. Physiologia plantarum, 225–235.

Cushman JC. 2001. Crassulacean Acid Metabolism. A plastic photosynthetic adaptation to arid environments. Plant Physiology 127, 1439–1448.

Cushman JC, Borland AM. 2002. Induction of Crassulacean acid metabolism by water limitation. Plant, Cell and Environment 25, 295–310.

Dittrich P, Campbell WH, Black CC. 1973. PHOSPHOENOLPYRUVATE CARBOXYKINASE in plants exhibiting Crassulacean acid metabolism. Plant Physiology 52, 357–361.

Ehleringer JR, Monson RK. 1993. Evolutionary and Ecological Aspects of Photosynthetic Pathway Variation. Annual Review of Ecology and Systematics 24, 411–439.

Fock H, Krotkov G. 1969. Relation between photorespiration and GLYCOLATE OXIDASE activity in sunflower and red kidney bean leaves. Canadian Journal of Botany 47, 237–240.

Freschi L, Takahashi CA, Cambui CA, Semprebom TR, Cruz AB, Mioto PT, Versieux LM, Calvente A, Latansio-Aidar SR, Aidar MPM, Mercier H. 2010. Specific leaf areas of the tank bromeliad *Guzmania monostachia* perform distinct functions in response to water shortage. Journal of Plant Physiology 167, 526–533.

Genty B, Briantais J-M, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta (BBA) - General Subjects 990, 87–92.

Griffiths H. 1989. Carbon dioxide concentrating mechanisms and the evolution of CAM in vascular epiphytes. In: Vascular plants as epiphytes: evolution and ecophysiology. Berlin: Springer, 42–86.

Griffiths H, Helliker B, Roberts A, Haslam RP, Girnus J, Robe WE, Borland AM, Maxwell K. 2002. Regulation of Rubisco activity in Crassulacean acid metabolism plants: better late than never. Functional Plant Biology 29, 689–696.

Griffiths H, Robe WE, Girnus J, Maxwell K. 2008. Leaf succulence determines the interplay between carboxylase systems and light use during Crassulacean acid metabolism in *Kalanchoë* species*. Journal of Experimental Botany 59, 1851–1861.

Hafke JB, Neff R, Hiitt MT, Liittge U, Thiel G. 2001. Day-to-night variations of cytoplasmic pH in a Crassulacean acid metabolism plant. Protoplasma 216, 164–170.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8, R19.

Herrera A. 2000. Effects of Drought on CAM and Water Relations in Plants of *Peperomia carnevalii*. Annals of Botany 86, 511–517.

Herrera A, Ballestrini C, Tezara W. 2008. Nocturnal sap flow in the C_3-CAM species, *Clusia minor*. Trees 22, 491–497.

Holtum J, Osmond C. 1981. The gluconeogenic metabolism of pyruvate during deacidification in plants with Crassulacean acid metabolism. Functional Plant Biology 8, 31.

Holtum JAM, Smith JAC, Neuhaus HE. 2005. Intracellular transport and pathways of carbon flow in plants with Crassulacean acid metabolism. Functional Plant Biology 32, 429–449.

James GT. 1978. Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. Analytical Biochemistry 86, 574–579.

Klughammer C, Schreiber U. 2008. Complementary PS II quantum yields calculated from simple fluorescence parameters measured by PAM fluorometry and the Saturation Pulse method. PAM Application Notes 1, 27–35.

Ku MS., Spalding MH, Edwards GE. 1980. Intracellular localization of PHOSPHOENOLPYRUVATE CARBOXYKINASE in leaves of C_4 and CAM plants. Plant Science Letters 19, 1–8.

Leegood RC, Walker RP. 2003. Regulation and roles of PHOSPHOENOLPYRUVATE CARBOXYKINASE in plants. Archives of Biochemistry and Biophysics 414, 204–210.

Luttge U. 2002. CO_2-concentrating: consequences in Crassulacean acid metabolism. Journal of Experimental Botany 53, 2131–2142.

Lüttge U. 2004. Ecophysiology of Crassulacean acid metabolism (CAM). Annals of Botany 93, 629–652.

Lüttge U, Stimmel H, Smith JAC, Griffiths H. 1986. Comparative ecophysiology of CAM and C_3 bromeliads. I. The ecology of the Bromeliaceae in Trinidad. Plant, Cell and Environment 9, 377–383.

Martín M, Plaxton WC, Podestá FE. 2007. Activity and concentration of non-proteolyzed PHOSPHOENOLPYRUVATE CARBOXYKINASE in the endosperm of germinating castor oil seeds: Effects of anoxia on its activity. Physiologia Plantarum 130, 484–494.

Martinelli G, Vieira CM, Gonzalez M, Leitman P, Piratininga A, Costa AF da, Forzza RC. 2008. Bromeliaceae da Mata Atlântica brasileira: lista de espécies, distribuição e conservação. Rodriguésia 59, 209–258.

Matiz A, Cambuí CA, Richet N, Mioto PT, Gomes F, Pikart FC, Chaumont F, Gaspar M, Mercier H. 2019. Involvement of aquaporins on nitrogen-acquisition strategies of juvenile and adult plants of an epiphytic tank-forming bromeliad. Planta 250, 319–332.

Matiz A, Mioto PT, Aidar MPM, Mercier H. 2017. Utilization of urea by leaves of bromeliad *Vriesea gigantea* under water deficit: much more than a nitrogen source. Biologia Plantarum 61, 751–762.

Matiz A, Tamaso P, Yepes A, Freschi L, Mercier H. 2013. CAM photosynthesis in bromeliads and agaves: What can we learn from these plants? Photosynthesis. InTech, 91–134.

Maxwell C, Griffiths H, Borland AM, Broadmeadow MSJ, McDavid CR. 1992. Photoinhibitory responses of the epiphytic bromeliad *Guzmania monostachia* during the dry season in Trinidad maintain photochemical integrity under adverse conditions. Plant, Cell and Environment 15, 37–47.

Maxwell C, Griffiths H, Young AJ. 1994. Photosynthetic acclimation to light regime and water stress by the C_3-CAM epiphyte *Guzmania monostachia*: Gas-exchange characteristics, photochemical efficiency and the xanthophyll cycle. Functional Ecology 8, 746–754.

Maxwell K, Marrison JL, Leech RM, Griffiths H, Horton P. 1999. Chloroplast acclimation in leaves of *Guzmania monostachia* in response to high light. Plant physiology 121, 89–96.

Medina E, Delgado M, Troughton JH, Medina JD. 1977. Physiological ecology of CO_2 fixation in Bromeliaceae. Flora 166, 137–152.

Medina E, Troughton JH. 1974. Dark CO_2 fixation and the carbon isotope ratio in Bromeliaceae. Plant Science Letters 2, 357–362.

Mercier, H., Rodrigues, M.A., Andrade, S.C.S., Coutinho, L.L., Gobara, B.N.K., Matiz, A., Mioto, P.T., Gonçalves, A.Z. Transcriptional foliar profile of the C_3-CAM bromeliad *Guzmania monostachia*. In press.

Mioto PT, Mercier H. 2013. Abscisic acid and nitric oxide signaling in two different portions of detached leaves of *Guzmania monostachia* with CAM up-regulated by drought. Journal of Plant Physiology 170, 996–1002.

Niewiadomska E, Bilger W, Gruca M, Mulisch M, Miszalski Z, Krupinska K. 2011. CAM-related changes in chloroplastic metabolism of *Mesembryanthemum crystallinum* L. Planta 233, 275–285.

Niewiadomska E, Borland AM. 2008. Crassulacean acid metabolism: a cause or consequence of oxidative stress in planta? Progress in Botany.247–266.

Nimmo HG. 2000. The regulation of PHOSPHOENOLPYRUVATE CARBOXYLASE in CAM plants. Trends in Plant Science 5, 75–80.

North GB, Brinton EK, Browne MG, Gillman MG, Roddy AB, Kho TL, Wang E, Fung VA, Brodersen CR. 2019. Hydraulic conductance, resistance, and resilience: how leaves of a tropical epiphyte respond to drought. American Journal of Botany 106, 943–957.

Pikart FC, Marabesi MA, Mioto PT, Gonçalves AZ, Matiz A, Alves FRR, Mercier H, Aidar MPM. 2018. The contribution of weak CAM to the photosynthetic

metabolic activities of a bromeliad species under water deficit. Plant Physiology and Biochemistry 123, 297–303.

Ranson SL, Thomas M. 1960. Crassulacean acid metabolism. Annual Review of Plant Physiology 11, 81–110.

Rayder L, Ting IP. 1983. CAM-idling in *Hoya carnosa* (Asclepiadaceae). Photosynthesis Research 4, 203–211.

Rodrigues MA, Hamachi L, Mioto PT, Purgatto E, Mercier H. 2016. Implications of leaf ontogeny on drought-induced gradients of CAM expression and ABA levels in rosettes of the epiphytic tank bromeliad *Guzmania monostachia*. Plant Physiology and Biochemistry 108, 400–411.

Shameer S, Baghalian K, Cheung CYM, Ratcliffe RG, Sweetlove LJ. 2018. Computational analysis of the productivity potential of CAM. Nature Plants 4, 165–171.

Silvera K, Neubig KM, Whitten WM, Williams NH, Winter K, Cushman JC. 2010. Evolution along the Crassulacean acid metabolism continuum. Functional Plant Biology 37, 995–1010.

Ting IP. 1985. Crassulacean acid metabolism. Annual reviews Plant Physiol 960, 596–622.

Walker RP, Chen Z, Acheson RM, Leegood RC. 2002. Effects of phosphorylation on PHOSPHOENOLPYRUVATE CARBOXYKINASE from the C_4 plant guinea grass. Plant physiology 128, 165–172.

Walker RP, Leegood RC. 1996. Phosphorylation of PHOSPHOENOLPYRUVATE CARBOXYKINASE in plants. Studies in plants with C_4 photosynthesis and Crassulacean acid metabolism and in germinating seeds. Biochemical Journal 317, 653–658.

Weatherley PE. 1950. Studies in the water relations of the cotton plant. I. The field measurement of water deficits in leaves. New Phytologist 49, 81–97.

Winter K. 2019. Ecophysiology of constitutive and facultative CAM photosynthesis. Journal of experimental botany, 1–14.

Zotz G. 2016. Plants on plants – The biology of vascular epiphytes. Springer.

Supplementary material

Table S1. Forward (F) and reverse (R) sequence primers and their respective melting temperatures used in the quantitative real-time PCR analysis.

Gene	Primers 5'-3'
PEPCK	F: ATGTGAGAGGAAATGTTGTTGG
	R: TTGAGAATGACAGCAGAGCA
PPC1	F: GAACGTCTTTGCTTTAGCACAC
	R: CCTGCCATATTCTAGTTCAGGTG
GLO1	F: GCATCATCGTGTCCAACCA
	R: GCCAAAGAGTACAGCACAG
BAM	F: CTTTCTGGATGCGAAGGGTATC
	R: TGAAGGAAGGAGGAACACG
HSDD	F: CCTCAAGTCTCACTAACGAAG
	R: GATGTAGCAGCCGAATCAAC

Table S2. Analysis of variance (ANOVA) of net CO_2 exchange, *PHOSPHOENOLPYRUVATE CARBOXYLASE* (*PPC1*) transcript abundance, titratable acidity, *PHOSPHOENOLPYRUVATE CARBOXYKINASE* (*PEPCK*) transcript abundance, PEPCK decarboxylation activity, PSII operating efficiency (ϕPSII), electron transport rate (ETR), non-photochemical quantum yield [Y(NPQ) and Y(NO)], *GLYCOLATE PEROXIDASE* (*GLO1*) transcript abundance, hydrogen peroxide content (H_2O_2), relative water content (RWC), and maximum quantum efficiency of PSII photochemistry (F_v/F_m) of plants well-watered (WW), under 20 days of water deficit (WD), or rewatered (RW) during seven days after the WD treatment.

ANOVA (α = 0.05)									
	Time of the day			Water conditions			Time vs. water conditions		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
Net CO_2 exchange	2	10.4109	0.0002	2	29.1811	<0.0001	4	1.6089	0.1866
PPC1	2	0.0275	0.9729	2	45.1839	<0.0001	4	0.1309	0.9696
Titratable acidity	2	43.9949	<0.0001	1	53.9971	<0.0001	2	10.7530	0.0002
PEPCK	2	10.0198	0.0023	1	24.9557	0.0023	2	10.0198	0.0023
PEPCK activity	2	2.3754	0.1136	1	23.4131	<0.0001	2	16.8925	<0.0001
ϕPSII	2	34.7090	<0.0001	1	163.2756	<0.0001	2	27.4842	<0.0001
ETR	2	10.2936	0.0003	1	236.7131	<0.0001	2	6.5146	0.0038
Y(NPQ)	2	6.2256	0.0058	1	0.8247	0.3716	2	3.6987	0.0376
Y(NO)	2	1.8387	0.1750	1	31.0057	<0.0001	2	1.6575	0.2061
GLO1	2	0.3127	0.7361	1	16.7429	0.0010	2	0.3127	0.7361
H_2O_2	2	5.5608	0.0195	1	19.4887	0.0008	2	0.7378	0.4987
Well-watered vs. Water deficit vs. Rewatered									
	d.f.	F	P						
RWC	2	71.8993	<0.0001						
F_v/F_m	2	15.7428	0.0002						
Well-watered vs. Water deficit									
	7:00h			12:00h			17:00h		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
PPC1	1	7.8592	0.0310	1	533.1583	<0.0001	1	50.4567	0.0004
GLO1	1	37.8126	0.0008	1	1.5740	0.2779	1	7.7662	0.0386
H_2O_2	1	1.7604	0.2552	1	6.0639	0.0695	1	36.6632	0.0038
Well-watered vs. Rewatered									
	7:00h			12:00h			17:00h		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
PPC1	1	5.0985	0.0647	1	3.1053	0.1528	1	5.6662	0.0547

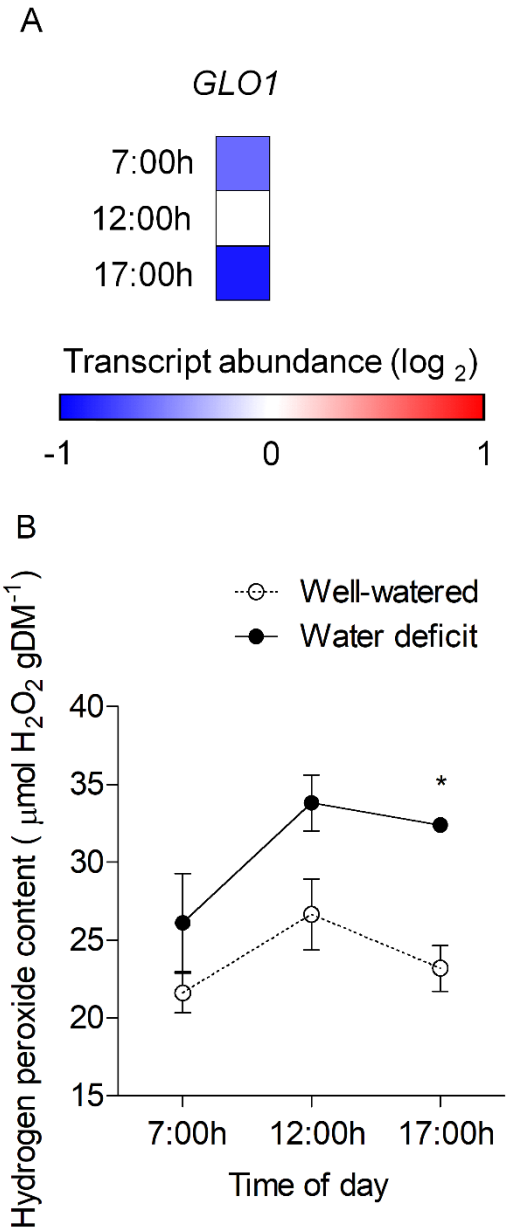

Figure S1. (A) *GLYCOLATE OXIDASE 1* (*GLO1*) transcript abundance and (B) hydrogen peroxide (H_2O_2) content of the bromeliad *G. monostachia* under 20 days of water deficit and well-watered conditions. Only statistical different *GLO1* transcript abundance was presented (ANOVA, T-test *post hoc*, α = 0.05). The asterisk indicates statistical difference between water conditions compared at the same time of day (ANOVA, T-test *post hoc*, α = 0.05). Error bar indicates the standard error (SE).

4. Capítulo 2

CAM induction is associated with modification in the protein composition of thylakoid membranes in a bromeliad species under drought

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Abstract

The photosynthesis process can be divided in two steps: 1) light absorption by pigments and proteins located at the thylakoid membranes, and 2) carbon fixation at the stroma. The chloroplast can regulate the machinery of light-energy use through gene expression modulation triggered by the redox state, promoting acclimation to the environmental changes. *Guzmania monostachia* is an epiphytic bromeliad capable of acclimating the light-harvesting capacity and carbon fixation metabolism. The induction of the Crassulacean acid metabolism (CAM) by water deficit contributes to recycling CO_2, saving water and balancing the metabolism during drought seasons in this species. Therefore, we investigated the thylakoid membrane composition and the transcriptional modulation of PSII and PSI proteins of the drought-induced CAM *G. monostachia*. CAM-induced plants showed modulation in three aspects of thylakoid membrane composition: 1) an increase in the ATP synthesis capacity indicated by an increment in the amount of the ATP synthase subunit gamma; 2) a decrease in the PSII/PSI ratio triggered by transcriptional and post-transcriptional regulators, which downregulated the photosynthetic capacity; and 3) pH luminal sensibility by an increase in the PsbS abundance. These traits may contribute to the photosynthetic acclimation mechanism of this species.

Keywords
Bromeliaceae, epiphytic habitat, drought, *Guzmania monostachia*, photosynthesis, photosystem stoichiometry, protein kinase.

Abbreviations: PSII, photosystem II; PSI, photosystem I; LHC, light-harvesting complex; PQ, plastoquinone; Cyt b_6f, cytochrome b_6f complex; TRX, thioredoxin; NPQ, non-photochemical quenching; CAM, Crassulacean acid metabolism; PEPC, PHOSPHOENOLPYRUVATE CARBOXYLASE; RUBISCO, RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE; WD, water deficit; WW, well-watered; RWC, relative water content; FM, fresh mass; TM, turgid mass; DM, dry mass; F_v/F_m, maximum quantum efficiency of PSII photochemistry; α-DM, α-dodecyl-D-maltoside; SDS, sodium dodecyl sulfate; BN gel, blue native gel.

4.1. Introduction

Photosynthesis is a biochemical process of carbon dioxide (CO_2) incorporation and oxygen (O_2) production using light-energy. The reactions responsible for absorbing energy from sunlight and converting it in chemical energy take place in the thylakoid membranes of chloroplasts and depends on an electron transport chain performed by specialized proteins. This system is composed of two main complexes, photosystem II (PSII) and photosystem I (PSI), and associated with them are the light-harvesting complexes (LHC), pigment-protein structures responsible for the input of energy through light absorption (Nicol and Croce, 2018). The electron transport chain starts at the PSII, performing a successive sequence of oxidation/reduction reactions, passing through plastoquinone (PQ), cytochrome b_6f complex (Cyt b_6f), plastocyanin, and finally reaching the PSI. At this place, the electrons can reduce the ferredoxin and subsequently the FERREDOXIN $NADP^+$ OXIDOREDUCTASE, which transfers the electron to $NADP^+$, storing energy in NADPH (Fukuyama, 2004). The Cyt b_6f also performs proton transport into the internal space of the thylakoid membrane (lumen), and, in addition with the protons released by the water oxidation at PSII, promotes an acidification of the lumen, and a transmembrane electrochemical gradient (Stroebel *et al.*, 2003; Lubitz *et al.*, 2019). This gradient act as the motive force for the ATP synthesis by the ATP synthase complex (Armbruster *et al.*,

2017), and the NADPH and ATP can be used in metabolic processes, like carbon fixation in the Calvin-Benson cycle. The thylakoid membranes are organized into two main configurations, the grana and the stroma lamellae, differentiated by the appressed and non-appressed membrane organization, respectively (Mustárdy and Garab, 2003). This membrane organization regulates the distribution of photosystems and ATP synthase complex, where the PSII is commonly localized in the grana, and the PSI and ATP synthase in the non-appressed region (*i.e.*, stroma lamellae and grana end-margin membrane) (Shimoni *et al.*, 2005; Rochaix, 2014).

As sessile organisms, plants developed several systems to adapt to different environmental conditions (*e.g.*, variations in light quality and water availability) presenting fast responses, such as stomatal closure in order to avoid excessive water loss and non-photochemical quenching (NPQ), as well as, long-term responses (*e.g., de novo* RNA and protein synthesis) (Croce, 2015; Kollist *et al.*, 2019). The NPQ is regulated by the luminal pH and the PsbS protein act like an acidity sensor, since the protonation of this protein triggers a mechanism of excessive light-energy dissipation at the antenna, protecting the PSII (Li *et al.*, 2000; Croce, 2015). Concerning the long-term acclimation mechanism, the chloroplast itself can regulate the machinery of light-energy use through gene expression modulation of its constitutive components triggered by the redox state (Pfannschmidt *et al.*, 2001; Bhattacharjee, 2019). Therefore, chloroplast metabolism can regulate the nuclear and chloroplast gene expression (*e.g.,* chlorophyll a/b binding proteins, PSII and PSI subunits) by the redox status of cellular components, like PQ and thioredoxin (TRX), modulating the plant metabolism according to environmental variations (Pfannschmidt, 2003; Saibo *et al.*, 2009; Belin *et al.*, 2015).

Despite modulations in the light-energy input, some species are capable of modulating their photosynthetic pathway as a response to abiotic stress. This can be noted in species that can change from C_3 to Crassulacean acid metabolism (CAM) due to these factors (Matiz *et al.*, 2013; Winter, 2019). This photosynthetic metabolism is mainly characterized by nocturnal acidification due to organic acid accumulation in the vacuole promoted by the phosphoenolpyruvate carboxylation via PHOSPHOENOLPYRUVATE CARBOXYLASE (PEPC) (Niechayev *et al.*, 2019). During the day, the organic

acids can be decarboxylated, increasing the internal concentration of CO_2, which is in turn available for use by RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE (RUBISCO) (Cushman, 2001; Berry *et al.*, 2013). Normally during the light phase of day, CAM plants showed closed stomata, resulting in transpiration rates lower than C_3 plants, which provides a more efficient use of the water (Winter *et al.*, 2005). In addition, the abiotic stress can trigger CAM induction, and the suspension of the stress, in some cases, can reestablish the C_3 pathway (Herrera *et al.*, 1991; Freschi *et al.*, 2010; Winter, 2019). In this case the species can be considered as a facultative CAM (C_3-CAM), with this characteristic being connected principally with strategies to save water, reduce the loss of respiratory CO_2 and photoprotection during periods not favorable to the growth and development of this species (Herrera *et al.*, 1991; Maxwell, 2002; Winter *et al.*, 2005; Pikart *et al.*, 2018). Not surprisingly, water deficit is one of these abiotic stressors that can induce CAM, and some species can engage a severe form of this photosynthesis characterized by a day-night stomatal closure associated with a recycling of CO_2 respiration by PEPC, known as CAM idling (Cushman, 2001). Even CAM plants can engage CAM idling, which has an ecological importance since through idling metabolism both C_3-CAM and CAM species can survive dry seasons until the next rain, when they retake the original photosynthesis pathway as well as carbon gain and growth (Rayder and Ting, 1983; Ting, 1985; Herrera, 2009; Freschi *et al.*, 2010).

Epiphytic habitat is characterized by low water availability since the epiphytic plants normally do not have access to the water present in the soil, instead being dependent on the rainfall and the air humidity for the water supply (Smith *et al.*, 1985; Zotz, 2016). This habitat in the neotropics is richly occupied by bromeliads, and one in particular, *Guzmania monostachia* (L.) Rusby ex Mez, is capable of reaching CAM idling when faced with drought. (Medina, 1974; Freschi *et al.*, 2010). The capacity of this species to survive in a stressful environment has been related to the photosynthetic plasticity shown by 1) the acclimation light reaction, 2) a PSII down-regulation, and 3) CAM expression (Maxwell *et al.*, 1994, 1995; Pikart *et al.*, 2018). The study of facultative CAM plants can provide knowledge about physiological mechanisms that may contribute to the success of the epiphytic species in an environment with low water availability. Furthermore, with the actual scenario of intensification of

drought, to understand how these plants overcome this unfavorable condition could be an important tool to make C_3 crops more productive using less water (Yang *et al.*, 2015; Winter, 2019). However, there is little information about the processes concerning the chloroplasts of bromeliads under water deficit. Since the thylakoid proteins are involved in the most important process of energy acquisition, to understand thylakoid composition could be very useful to engineer CAM into C_3 plants. Considering this, the facultative CAM plants have become a very interesting model to study these proteins and metabolic processes when they are affected by the transition from C_3 to CAM caused by drought. Given the importance of studying facultative CAM species and the lack of knowledge about their thylakoid composition, the main aim of this investigation was to identify possible modulation in the thylakoid membrane composition triggered by the CAM state of *G. monostachia* induced by drought. In addition, the transcriptional modulation of PSII and PSI proteins was studied and a discussion of the daily dynamic of the CAM-reactions and its interconnection with that possible chloroplast modulation is presented.

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4.2. Material and Methods

4.2.1. Material and experimental design

Plants of *Guzmania monostachia* (L.) Rusby ex Mez (SISGEN record number A279903) with approximately 2.5 years of *ex-vitro* cultivation were acclimated in a growth chamber under 250 µmol m^{-2} s^{-1}, 12h of photoperiod (6:00h to 18:00h), temperature of 27/22°C, and humidity of 60/70% (day/night). For the experiments, plants were submitted to two different water regimes: (1) well-watered (WW), plants receiving water regularly, and (2) water deficit (WD), with watering suspended for 20 days. After the 20 days of water deficit, measurements of the chlorophyll fluorescence were performed in the 8^{th} mature leaves of three different plants from both treatments. For the relative water content, thylakoid isolation and total RNA extraction, leaves were collected from the 8^{th} to the 12^{th} node of at least three different plants and immediately used for the analyses. These measurements were made after one hour of the beginning of the light-time period (7:00h). For the titratable acidity, leaves were collected from the 8^{th} to the 12^{th} node of three different plants at 7:00h and 17:00h, and for total RNA extraction leaves were also collected at 12:00h. The samples for

titratable acidity and total RNA extraction were immediately frozen in liquid nitrogen and stored at -20ºC and -80ºC, respectively, until the analyses.

4.2.2. Relative water content

To calculate the relative water content (RWC), the fresh mass (FM) of fragments of the leaves was obtained immediately after the sample collection. Then, the same fragments were immersed in distilled water for 24 h to obtain the turgid mass (TM). After that, the samples were dried for 48 h at 65°C to assess the dry mass (DM). The RWC was calculated using the equation described by Weatherley (1950): RWC = [(FM - DM)/(TM - DM)]×100.

4.2.3. *Titratable acidity and nocturnal acid accumulation*

The acid extraction was performed as described by Matiz *et al.* (2017) and titratable acidity calculated using NaOH to reach neutral pH, using phenolphthalein (Merch) as pH indicator. The nocturnal accumulation of H^+ was calculated using the amount of H^+ measured at 7:00h minus the measurement made at 17:00h. The standard error was calculated as described by Popp *et al.* (2003).

4.2.4. Chlorophyll fluorescence analyses

The maximum quantum efficiency of PSII photochemistry (F_v/F_m) and non-photochemical quenching (NPQ) were measured using a Dual-PAM 100 (Walz) with 1000 µE actinic light, a saturating light of 2000 µE (120 ms) and a measuring light of 3 µE. The plants were dark-adapted for 30 min before the NPQ measurements and taken in two cycles of light followed by dark.

4.2.5. Thylakoid isolation

The thylakoid isolation was performed in leaves that were cut in small pieces and blended with 50 mL of grinding buffer (150 mM Hepes-KOH, pH 8.0; 5 mM $MgCl_2$; 0.4 M sorbitol; 5 mM EDTA; 0.2 mM benzamidine; 1 mM caproic acid and aminocaproic acid, proteases inhibitors) to obtain a homogeneous mixture. Then, the mixture was blended six times for eight seconds and filtered in eight layers of a cheese cloth followed by centrifugation (10 min; 1,400 g at 4°C). The supernatant was discarded being careful to not lose the pellet. Then,

the pellet was gently resuspended using a paintbrush in 20 mL of wash buffer (50 mM Hepes-KOH, pH 8.0; 5 mM $MgCl_2$; 0.15 M sorbitol; 2.5 mM EDTA; 0.2mM benzamidine; 1 mM caproic acid and aminocaproic acid), followed by a second centrifugation (10 min; 4,000 g at 4°C), making sure to save the pellet. The pellet was resuspended using a paintbrush in 20 mL of the lysis buffer (50 mM Hepes-KOH, pH 8.0; 5 mM $MgCl_2$; 15 mM NaCl) and then centrifugated to take off the supernatant once again (10 min; 6,000 g at 4°C). The last resuspension was made in 200 µL of resuspension buffer (50 mM Hepes, pH 8.0; 5 mM MgCl2; 15 mM NaCl; 0.4 M sorbitol). This entire process was performed under low-light and low temperature (4°C). After the isolation, the pigments were extracted using acetone 80% (Porra *et al.*, 1989) and the concentration measured (Croce *et al.*, 2002).

4.2.6. Blue native gel casting, sample preparation, and electrophoresis

The gel was prepared based on Järvi *et al.* (2011) with the modifications described by Bielczynski *et al.* (2016). The thylakoid aliquots were defrosted at low temperature (0 to 4ºC) and dark to be solubilized in α-dodecyl-D-maltoside (α-DM). The sample preparation consisted of centrifugation (10 min; 17,000 g) to remove the supernatant, followed by a gentle resuspension of the thylakoids using a pipette in sample buffer 25BTH20G (0.25 M Bis Tris, pH 7.0; 20% glycerol). Then, a second volume of the same sample buffer 25BTH20G was added plus the detergent α-DM (2% of final concentration) to reach a final concentration of 1 µg of chlorophyll μL^{-1} of sample. The protein solubilization was made for 10 min, with a brief and gentle mixing every two minutes. Then, the chlorophyll concentration after solubilization was measured, the loading buffer (1:10; v:v) added (100 mM Bis Tris, pH 7.0; 0.5M amino-n-caproic acid; 30% sucrose, w:v; 50 mg mL^{-1} serva blue G), and each well loaded with 8 µg of chlorophyll.

Thylakoids isolated from *Arabidopsis thaliana* (L.) Heynh were solubilized in α-DM (1% final concentration) and loaded (8 µg of chlorophyll) into the gel as a control. The BN electrophoresis was performed as described by Järvi *et al.* (2011), and after two hours the cathode buffer was replaced with a new one without serva blue G (15 mM Bis Tris-HCl, pH 7.0; 50 mM tricine).

4.2.7. SDS second dimension gel casting and electrophoresis

For the second-dimension gel, hand-cast SDS (sodium dodecyl sulfate) gels were necessary prepared in two steps, separation gel and stacking gel. The first was prepared with 40% acrylamide/bisacrylamide, ultrapure water, separation gel buffer (1.5 M Tris-HCl, pH 8.83; 0.4% SDS; ultrapure water), 10% APS, and 0.1% Tetramethylethylenediamine (TEMED). After polymerization, the stacking gel solution was added, prepared with 40% acrylamide/bisacrylamide, ultrapure water, stacking gel buffer (0.5 M Tris-HCl, pH 6.8; 0.4% SDS; ultrapure water), 10% APS, and 0.1% TEMED.

Gel stripes from the BN gel were carefully cut and loaded into SDS gels. The electrophoresis was performed in a setup for hand-casted gels using a running buffer (25 mM Trizma, pH 8.3; 0.19 M glycine; 0.1% SDS). After the electrophoresis, the gel was stained using Coomassie solution and then incubated in a destaining solution (Brunelle and Green, 2014). The gel images were obtained using an ImageQuant (LAS 4000, GE Healthcare).

4.2.8. Immunoblot analyses

For the immunoblot analyses, isolated thylakoids (1 µg of chlorophyll) was used, and the proteins were separated using SDS commercial gels (4 to 12% Bis-Tris) (Invitrogen). The proteins were transferred to a nitrocellulose membrane using a Trans blot system (BioRad) and incubated in 10% milk TBST solution for 1 h (Tris base 0.2M, pH 7.4; NaCl 1.5M; Tween 0.1%). After that, the membranes were incubated overnight with different primary antibodies at 4ºC, and washed for 10 min, 4 times. Then, the secondary antibody (HRP conjugated) incubation was performed (1 h), followed by a wash with TBST solution (6 times, 10 min). The antibody against the proteins of interest (D1, CP43, CP29, Lhcb1, PsbS, PsaA, Lhca1, and ATP synthase subunit gamma, Agrisera) was detected using Super Signal West Pico (Thermo Scientific), membranes were stained using Ponceau S, and the gel images were obtained using an ImageQuant (LAS 4000, GE Healthcare).

4.2.9. Sucrose gradient and band analyses

The sucrose gradient was prepared in a transparent plastic tube filled with a sucrose solution (10 mM Hepes, pH 7.5; 0.5 M sucrose; 0.06% α-DM) and

frozen overnight (-60ºC), followed by thawing at 4ºC for 3 h. The thylakoid samples were solubilized as described for the BN gel, and 350 µg of chlorophyll were loaded in the top of the sucrose gradient column. Then, the tubes were centrifuged (14 h; 160,000 g; 4ºC), and seven bands were collected using a syringe. These bands were submitted to analyses at room temperature (RT) and at 77K of absorption spectra (350nm to 750nm) using a spectrophotometer (Cary 4000, Agilent), and fluorescence was performed in a spectrofluorometer (Fluorolog, Horiba). For the fluorescence, the samples were diluted to reach an OD<0.05, and the measurements started from 600 nm to 800 nm using 440 nm as excitation light (0.5 nm slit).

Bands two, three, four, six and seven of *G. monostachia* were analyzed using SDS commercial gels (4 to 12% Bis-Tris) (Invitrogen). After the electrophoresis, the gel was stained using Coomassie solution and then incubated in a destaining solution (Brunelle and Green, 2014). The gel images were obtained using an ImageQuant (LAS 4000, GE Healthcare).

4.2.10. *Quantitative RT-PCR analysis*

Total RNA extraction was performed as described by Pereira *et al.* (2018). The cDNA synthesis was performed using SuperScript IV reverse transcriptase (Thermo Fisher Scientific), and samples were treated with RNaseOUT ribonuclease inhibitor (Invitrogen) to a final volume of 20 µL (5 ng reverse-transcribed RNA μL^{-1}). The quantitative RT-PCR reactions and primer efficiency calculations were made as described by Matiz *et al.* (2019). Primers were designed based on sequences obtained from *G. monostachia* transcriptome (Mercier *et al.*, in press). These sequences were selected through similarity analyses with sequences of *Ananas comosus* (L.) Merril., *Zea mays* L., *Oryza sativa* L. or *A. thaliana*. Selected sequences were translated to check their functionality using the SnapGene Viewer software (https://www.snapgene.com/) and the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The software PearlPrimer (http://perlprimer.sourceforge.net/) was used to design the primers (Table S1). Transcript abundance was normalized using the geometric mean of *BAM* and *HSDD* as reference genes, and their stability was tested using NormFinder software (Andersen *et al.*, 2004). Data are presented as relative

gene expression of four biological replicates calculated by the ΔΔCt method (Hellemans *et al.*, 2007), using the well-watered condition as normalizer factor.

4.2.11. Statistical analyses

Comparisons between water conditions were performed using ANOVA and T-test *post hoc* (α = 0.05), and comparisons involving two sources of variations were made using two-way ANOVA with a Tukey's test *post hoc* (α = 0.05) (Table S2). The analyses were performed using the statistical software JMP 14 (https://www.jmp.com/en_us/home.html).

4.3. Results

4.3.1. Drought induces responses in the photosynthesis pathway and photosynthetic parameters

To trigger the CAM photosynthesis in *G. monostachia*, we used a drought treatment, and it was possible to note that the WD plants showed a reduction of approximately 21% in their water status (Fig. 1A). Additionally, the induction of the CAM photosynthesis was verified by an increase in the nocturnal accumulation of acids (Fig. 1B). This drought treatment may also have induced changes in photosynthetic parameters, like the maximum quantum efficiency of PSII photochemistry (F_v/F_m) (Fig. 1C), which showed a reduction of 21% after WD treatment. Conversely, the NPQ was lower in this same condition compared to WW plants (Fig. 1D). Moreover, the WD plants showed a decrease in the chlorophyll a/b content [WW: average (AV), 3.0697; standard error (SE), 0.0471. WD: AV, 2.9021; SE, 0.0133. T-test, P = 0.0108]; however, the same trend was not observed with the chlorophyll/carotenoid content (WW: AV, 3.6497; SE, 0.1353. WD: AV, 3.4636; SE, 0.0063. T-test, P = 0.1618).

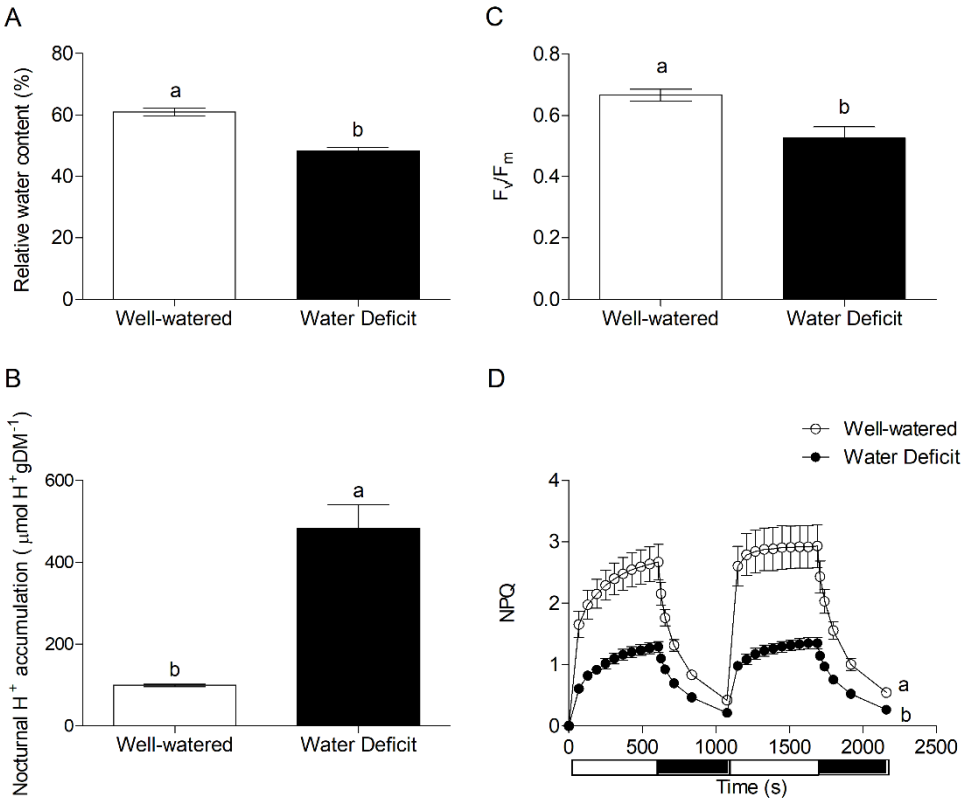

Figure 2. (A) Relative water content, (B) nocturnal H^+ accumulation, (C) maximum quantum efficiency of PSII (F_v/F_m), and (D) non-photochemical quenching (NPQ) of well-watered plants or plants under 20 days of water deficit. Different letters indicate a statistical difference between water conditions [(A), (B) and (C), one-way ANOVA, *post hoc* T-test, α = 0.05. (D), two-way ANOVA, *post hoc* T-test, α = 0.05]. Error bars represent the standard error, and white and black bars represent the actinic light on or off, respectively.

4.3.2. Modulation of protein thylakoid composition in CAM-induced bromeliad

In order to verify possible modulation in the thylakoid protein composition of CAM induced plants, we performed an analysis of thylakoids isolated from WD bromeliads in blue native gels (BN). The results revealed slight differences between WD and WW plants, with a weaker PSII supercomplex band (*i.e.*, PSII associated with its antenna, LHCII) and a stronger (PSI, PSII dimer, and ATP synthase) band noted in CAM-induced plants (Fig. 2A). Furthermore, when we analyzed the subunits of these separated complexes in an SDS second dimension gel (Fig. 2B), the results indicated differences that can be related to proteins comprising the PSII and LHCII.

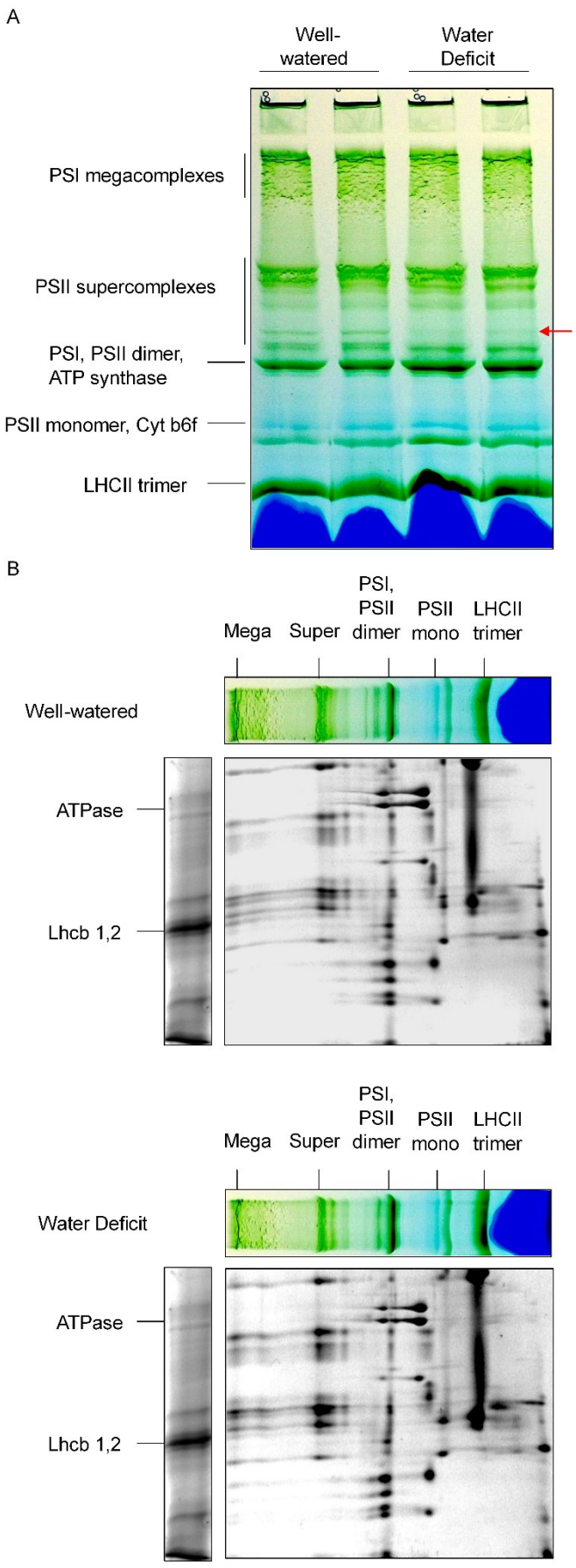

Figure 2. Thylakoid protein composition investigated in (A) blue native gel loaded with thylakoids isolated from plants of *G. monostachia* well-watered or submitted to 20 days of water deficit. The arrow indicates a weaker band in gel loaded with thylakoids isolated from WD bromeliads compared with WW. (B) Second dimension gels loaded with blue native gel stripes from well-watered and water deficit plants.

The sucrose gradient was also used to separate the thylakoid complexes (Fig. 3A), resulting in seven isolated bands of thylakoids from *G. monostachia* in each water condition. The protein composition (Fig. S1) of the bands was investigated, as well as the spectroscopy properties of absorption (Fig. 3B and S2) and fluorescence at room temperature (RT) (Fig. S3) and at 77K (Fig. S4), using the properties of isolated bands from thylakoids of *A. thaliana* as a control. Band one (B1) corresponds to free pigments; band two (B2), lhcb monomer; band three (B3), LHCII trimer; band four (B4), CP29-CP24-LHCII; band six (B6), PSI-lhca; and band seven (B7), PSII supercomplexes. Band four was not present in *A. thaliana*, and band five in *G. monostachia* was almost not present; therefore, it was not possible to perform the protein composition and spectroscopy analyses. Regarding the pattern of proteins from isolated bands, no differences were observed between water treatments (Fig. S1). The analyses of the spectroscopy properties demonstrated slight differences between water treatments, only for the B1 absorption spectrum (Fig. 3B), which indicates difference in the pigments amount, noted in the reduction of chlorophyll a/b.

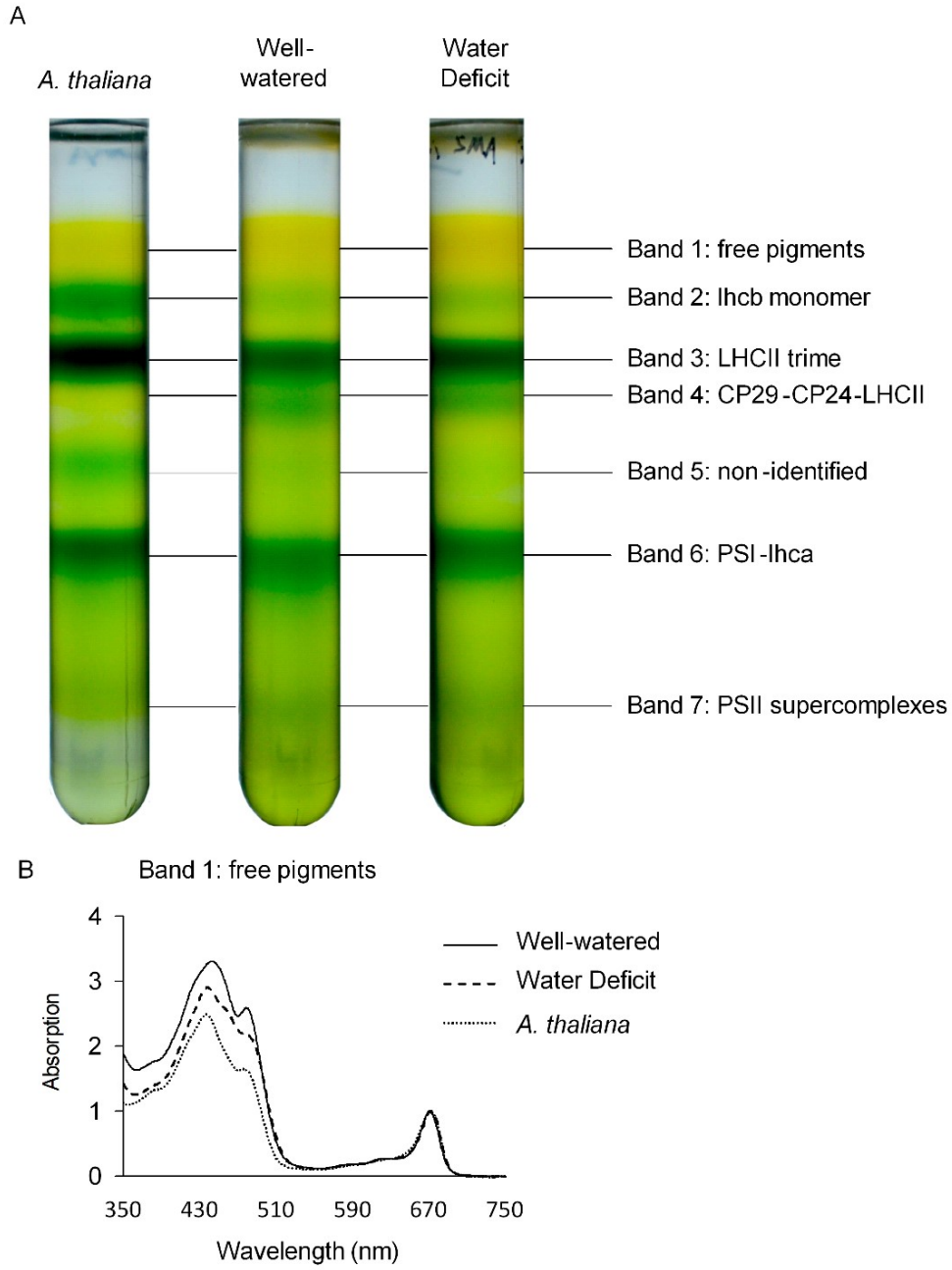

Figure 3. (A) Sucrose gradient of thylakoid isolated from leaves of *G. monostachia* under two water conditions, well-watered and water deficit. Thylakoids isolated from well-watered *A. thaliana* plants was loaded as a control. Each sucrose gradient was loaded with 350 µg of chlorophyll, and seven bands were isolated. Band one (B1), free pigments; band two, Ihcb monomer; band three, LHCII trimer; band four, CP29-CP24-LHCII; band six (B6), PSI-Ihca; and band seven (B7), PSII supercomplexes. Band four was absent in *A. thaliana*. (B) Absorption spectrum of band one, free pigments, of *G. monostachia* under two water conditions, well-watered and water deficit.

To investigate the possible differences in the proteins comprising the PSII supercomplex and PSI, PSII dimer, and ATP synthase band, an immunoblot analysis of some thylakoid proteins was used. The results indicated a reduction in the content of PSII core (D1 and CP43) and LHCII proteins (CP29 and Lhcb1) (Fig. 4). Instead, an increase in the PsbS (luminal pH sensor protein), PSI core (PsaA), LHC of PSI – LHCI – (Lhca1) and ATP synthase subunit gamma (ATPC) was detected when the plants were submitted to drought (Fig. 4).

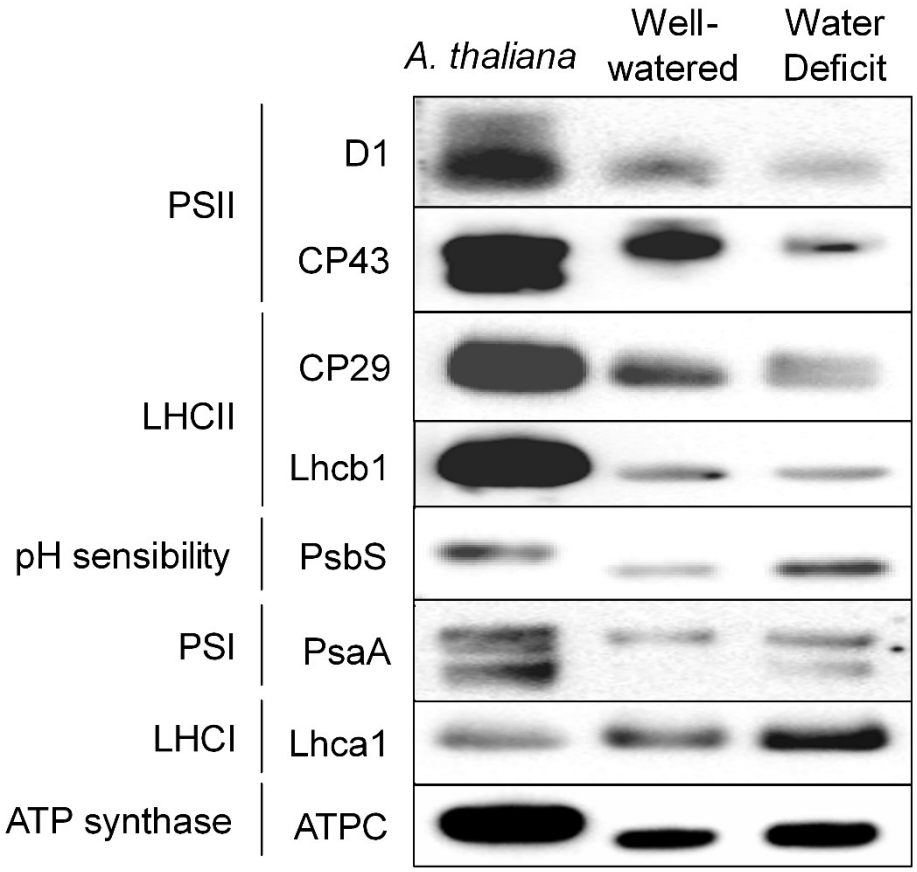

Figure 4. Immunoblot of PSII (D1 and CP43), LHCII (CP29 and Lhcb1), acidity sensor (PsbS), PSI (PsaA), LHCI (Lhca1), and ATP synthase complex (subunit gamma, ATPC) proteins of *G. monostachia* plants well-watered and under 20 days of water deficit. *Arabidopsis thaliana* was used as antibody control, and chemiluminescent signal was registered.

In order to investigate the transcriptional control of these thylakoid proteins, the mRNA levels of the genes that encode for these proteins were analyzed. Additionally, we also investigated the expression of encoding genes of

proteins that transcriptionally and post-translationally regulate some PSII and PSI components. The drought condition altered the transcription abundance of all thylakoid genes analyzed (Fig. 5A). The *PsbA, PsbC, PsaA*, which encode for D1, CP43, and PsaA proteins, respectively, were upregulated and the *Lhcb4*, *PsbS*, *Lhca1*, and *ATPC*, which code for CP29, PsbS, Lhca1, and ATPC proteins, respectively, were downregulated at 7:00h in WD bromeliads (Fig. 5A). Some proteins are involved in transcriptional and post-transcriptional regulation of thylakoid proteins, such as CSK (TYROSINE-PROTEIN KINASE CSK), PRIN2 (PLASTID REDOX INSENSITIVE 2), STN7 (SERINE/THREONINE-PROTEIN KINASE STN7), and STN8 (SERINE/THREONINE-PROTEIN KINASE STN8). The mRNA levels of the encoded genes were analyzed daily, and the *CSK*, *PRIN2*, and *STN8* were upregulated for almost all day, with the exception of *STN7*, which was upregulated only at 12:00h (Fig. 5B).

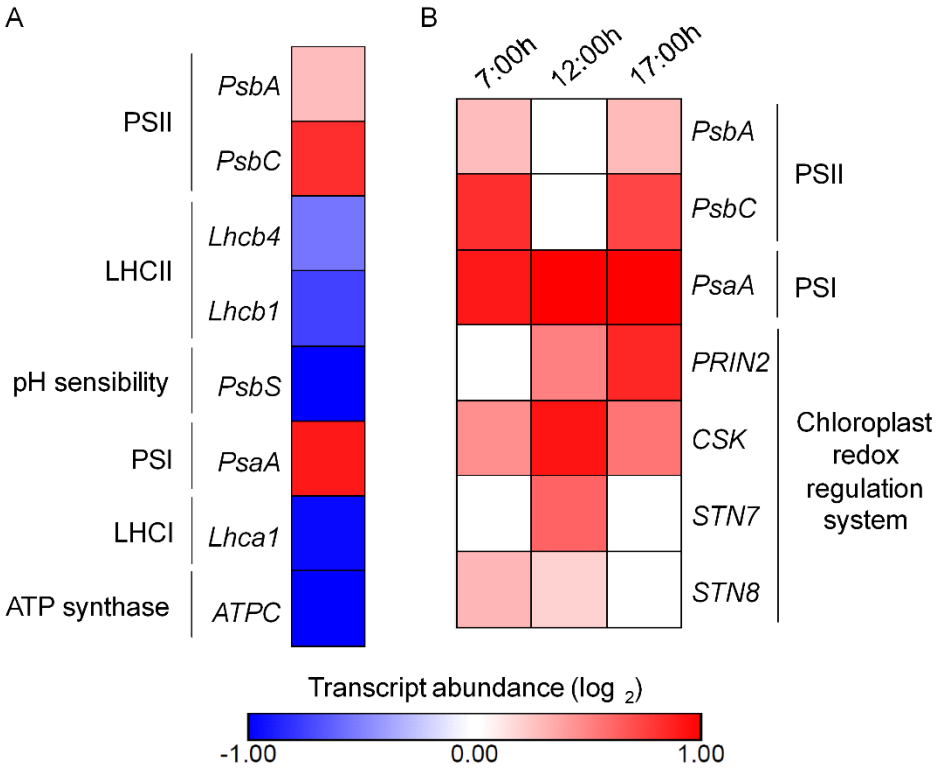

Figure 5. Heatmap representing the transcription abundance (log_2) of (A) thylakoid proteins at 7:00h and (B) chloroplast regulatory proteins (*PRIN2*, *CSK*, *STN7*, and *STN8*) with some regulation targets (*PsbA*, *PsbC*, and *PsaA*) in three different times of day of *G. monostachia* plants under drought treatment for 20

days. Only transcript abundance statistically different compared with the well-watered condition are presented (one-way ANOVA, *post hoc* T-test, α = 0.05).

4.4. Discussion

The presented data showed that in *G. monostachia* the CAM expression after 20 days of water deficit is accompanied by modulation in some thylakoid proteins, affecting the ATP synthase capacity, photosystem stoichiometry, and pH sensibility. The results also suggested a transcriptional and post-translational regulation of the PSII/PSI proportion and a rearrangement of the thylakoid membranes in conformation with the diurnal CAM reactions.

4.4.1. CAM upregulation and photosynthesis downregulation: tradeoff between water saving and carbon gain

Plants of *G. monostachia* under 20 days of water deficit showed a reduction in their water content (Fig. 1A) and an induction of the CAM photosynthesis observed by the increase in the nocturnal acid accumulation (Fig. 1B). This photosynthetic pathway is known as a water-saving mechanism, an important metabolic strategy to survive a period of water shortage (Herrera, 2009; Borland *et al.*, 2014). In addition, a reduction in the photosynthetic light energy use capacity was observed demonstrated by a decrease in the F_v/F_m (Fig. 1C). This result corroborates previous studies in which the authors observed not only CAM upregulation but also a reduction in the net CO_2 exchange in plants exposed to water deficit (Freschi *et al.*, 2010; Pikart *et al.*, 2018; Chapter 1). With stomatal closure, this plant depends on nocturnal PEPC activity to refix the CO_2 provided by the cellular respiration, limiting the growing index during the dry season. A common feature shared by C_3 (Chen *et al.*, 2016; Lima Neto *et al.*, 2017), C_3-CAM (Pieters *et al.*, 2003), and CAM (Ceusters *et al.*, 2019) species is an increase in the energy dissipation in a non-photochemical way when plants are submitted to a long-term water deficit (more than nine days). However, the NPQ decreased in WD plants, indicating a reduction in the non-photochemical light energy use capacity (Fig. 1D). The C_4 species *Sorghum bicolor* (L.) Moench (cv Samsorg 40) also presented a similar result but after five days under water restriction (10% soil water content) (Ogbaga *et al.*, 2014). In addition, the reduction in the chlorophyll a/b could be an indication of the reorganization of the

photosynthetic apparatus decreasing the reaction center complexes as a response to the water restriction condition (Ogbaga *et al.*, 2014). Therefore, the downregulation in the photosynthetic machinery seems to be reasonable, equating the energy input and demand, reducing an over energization status.

4.4.2. Thylakoid protein composition of a C_3-CAM species

The organization and composition of the thylakoid photosynthetic apparatus of *G. monostachia* were investigated using BN-gel, and the drought treatment resulted in differences in the protein complexes (Fig. 2A). These complexes were evaluated using second dimension gels, and a lack in proteins of PSII supercomplexes was noted (Fig. 2B). Therefore, the possible differences in the thylakoid protein composition in drought plants were investigated further.

The immunoblot analyses indicated a rearrangement of the thylakoid membrane proteins in three aspects (Fig. 4): (1) CAM ATP demand, (2) PSII/PSI stoichiometry, and (3) pH luminal sensibility. In the first aspect, computational analysis demonstrated that the CAM requires 1.2-fold more ATP during the day when compared with the C_3 (Shameer *et al.*, 2018). Indeed, the results indicated an increase in the subunit gamma of the ATP synthase complex, which shows an increase in the ATP synthesis capacity to supply this demand. In the second aspect, when photosystem proteins were evaluated in two C_3 species, *A. thaliana* and *Triticum aestivum* L., under 15 and 10 days of water deficit, respectively, it was not possible to detect changes in representative proteins of PSII, PSI and ATP synthase (Tambussi *et al.*, 2000; Chen *et al.*, 2016). However, in this study a reduction in the PSII/PSI ratio of WD plants occurred since as smaller amount of PSII proteins, such as D1 and CP43, was presented. Besides that, more PSI protein (PsaA) was noted. In addition, the results showed an increase in Lhca1 and a decrease in the CP29 and Lhcb1, indicating that the LHCI and LHCII composition were also regulated in WD plants. In a similar way, the C_4 *S. bicolor* under five days of water restriction presented a decrease in the PSII/PSI ratio (Ogbaga *et al.*, 2014). Additionally, *A. thaliana* and *S. bicolor* suffered a reduction of approximately 74% and 60% in water content and 35% and 40% in F_v/F_m, respectively, when exposed to drought (Ogbaga *et al.*, 2014; Chen *et al.*, 2016). However, the CAM *Phalaenopsis* “Edessa” only decreased 20% in water status and presented no reduction in F_v/F_m. As a result, the CAM performed by *G.*

monostachia may aid water conservation (Fig. 1A) and protect the thylakoid proteins (Fig. 1C). Furthermore, the change in the PSII/PSI stoichiometry could indicate a reduction in the granal thylakoid and an increase in the lamellar conformation of the membranes (Fig. 4). This rearrangement of the photosynthetic machinery could be driven by a regulation of gene expression as a response to a stress condition. In the third aspect, an increase in the PsbS abundance it occurred, which is associated with the NPQ (Li *et al.*, 2000). It has been suggested for *S. bicolor* that the PSII protein reduction resulted in less excitation pressure and subsequently less necessity to dissipate energy in a non-photochemical way (Ogbaga *et al.*, 2014). Therefore, the thylakoid rearrangement (including reduction in PSII core and antenna proteins) may have resulted in a downregulation of the NPQ capacity of this bromeliad (Fig. 1D). Moreover, *G. monostachia* occupies an epiphytic habitat susceptible to drought and high light stress since in some periods of the year trees can lose their leaves, exposing this bromeliad to constant light (Maxwell *et al.*, 1992). Early studies have shown an effective xanthophyll cycle (Ruban *et al.*, 1993; Maxwell *et al.*, 1994) and even the maintenance of zeaxanthin overnight in this bromeliad, suggesting a “primed” metabolism to downregulate the PSII during the light period (Maxwell *et al.*, 1995). Thus, the increase in the PsbS amount (Fig. 4) could be part of this “primed” metabolism providing the species with one more mechanism to deal with the harsh epiphytic environment.

4.4.3. CAM induction and differential gene expression regulation may alter the photosynthetic machinery

The changes in the proteins were investigated at a transcriptional level (Fig. 5A). After the water deficit, four proteins showed a direct relationship with regulation of transcription (*Lhcb4*, *Lhcb1*, *PsaA*, and *PsbS*) and modulation of the protein abundance (CP29, Lhcb1, PsaA, and PsbS). However, other proteins seem to be regulated post-transcriptionally, for example, the Lhca1 (*Lhca1*) and ATPC (*ATPC*) by mRNA stability (Monde *et al.*, 2000), and D1 (*PsbA*) and CP43 (*PsbC*) through interference in the translational process (Nishiyama *et al.*, 2001). Moreover, the mechanisms that control the chloroplast protein homeostasis involve an intricate system of signals between chloroplast and nucleus; however, the mechanism of how the metabolism responds to internal and external factors

remains elusive (Zoschke and Bock, 2018; Woodson, 2019). Likely, the most advanced knowledge about photosynthetic membrane proteins regards the regulation of photosystem stoichiometry. This regulation is based on the redox state of the chloroplast, which is very sensitive to environment changes, *e.g.,* light intensity. Consequently, the control of the redox state over transcriptional and translational processes has been described as a kind of acclimation mechanism helping the organism to adapt and survive (Pfannschmidt *et al.*, 2001).

4.4.4. Daily regulation of the thylakoid composition by CSK, PRIN2, and STN7

In CAM plants the light-energy use seems to be regulated by the decarboxylation event (Rascher and Lüttge, 2002; Pikart *et al.*, 2018; Chapter 1), and the CAM may also be the organizer of the light absorption system through the chloroplast redox state, which is dependent upon CO_2 availability. The electron transport chain can deliver energy for the carbon assimilation reaction, to the antioxidant systems, and to the redox regulatory systems (Dietz and Pfannschmidt, 2011). Therefore, when the CO_2 availability is low, the consumption of NADPH by the Calvin-Benson cycle is reduced, increasing the number of electrons that can reduce the TRX. Conversely, with the increase in the CO_2 availability powering the carboxylation reactions, the $NADP^+$ acts as an electron acceptor, changing the redox status of the TRX system. In the middle of the day, with an increase in CO_2 availability, there is also an increase in the demand for NADPH, consequently reducing electron availability for the TRX reduction.

CSK and PRIN2 proteins play an important role in regulate gene expression triggered by chloroplast redox signals (Puthiyaveetil *et al.*, 2008; Kindgren *et al.*, 2012; Bhattacharjee, 2019). PRIN2 can regulate gene expression through a protein-protein interaction with reduced TRX, which causes the monomerization of PRIN2 and triggers the PLASTID-ENCODED RNA POLYMERASE (PEP) activity, increasing the transcription of *Psa* (PSI) and *Psb* (PSII) genes (Díaz *et al.*, 2018). Moreover, the CSK is inactivated by the reduced TRX, releasing the inhibitory effect of phosphorylation driven by this enzyme over PEP activity and also inducing transcription of *Psa* and *Psb* genes (Puthiyaveetil *et al.*, 2012).

The drought and CAM induction condition resulted in a diurnal upregulation of *PRIN2* and *CSK*, except for the first gene at 7:00h (Fig. 5B), indicating an increase in the redox responsive system and a greater ability to respond to the environment. Therefore, the daily variations in the redox state triggered by the decarboxylation reaction, probably regulated the expression of the photosynthetic related genes. This could explain the upregulation of *PsbA*, *PsbC*, and *PsaA* observed at the beginning and at the end of the day in WD plants (Fig. 5B). Another protein that is involved in transcription control of photosystem components is the STN7, but its activity is modulated by PQ redox state (Wunder *et al.*, 2013) and can upregulate the PsaAB operon expression when PQ is reduced (Pesaresi *et al.*, 2009). The *STN7* was regulated daily, and we were able to note an increase in the expression of *STN7* and *PsaA* in the middle of the day, moment with the highest electron transport rate in CAM-induced plants (Chapter 1).

4.4.5. CAM induction and thylakoid membrane organization

In addition to water deficit, the CAM can also be induced by the high light intensity in *G. monostachia* (Maxwell *et al.*, 1994). In a study of chloroplast acclimation of this bromeliad to high light (650 μmol m^{-2} s^{-1}), a reduction was noted in the appressed thylakoid membrane, as well as in the amounts of LHCII, oxygen-evolving complex of PSII, and LHCI which were evaluated by immunolocalization, when compared with the low light treatment (50 μmol m^{-2} s^{-1}) (Maxwell *et al.*, 1999*b*)**.** In the same way, the CAM-induced *Mesembryanthemum crystallinum* L. presented an increase of non-stacking thylakoid, especially in the middle of the day, indicating a change in the thylakoid membrane architecture as an adaptation to the CAM pathway (Niewiadomska *et al.*, 2011). However, for *A. thaliana* a reduction in the appressed thylakoid after water deficit was also described, accompanied by a reduction in the PSII core phosphorylation, which indicates a disassembly of this complex (Chen *et al.*, 2016). Also, the results indicated that the phosphorylation of thylakoid complexes was not suppressed in *G. monostachia* since the kinases *STN7* and *STN8* expression was upregulated in the middle of the day (Fig. 5B). Since STN7 and STN8 are essential for thylakoid membrane adjustment in response to chloroplast redox changes (Schönberg *et al.*, 2017), they could have resulted in an increase

in the phosphorylation of PSII core and LHCII proteins, allowing thylakoid conformational changes at 12:00h (Fristedt *et al.*, 2009; Wood *et al.*, 2018).

The stacking reduction enables the linear electron flux producing NADPH, which is necessary for the Calvin-Benson cycle, and the stacked conformation allows a cyclic electron flux (Wood *et al.*, 2018). Therefore, when CO_2 availability increases through acid decarboxylation (middle of the day), the membrane changes to perform more linear electron flux, providing NADPH. On the other hand, when the CO_2 availability is low due to lack of decarboxylation (beginning of the day) or the vacuolar malate content is low (end of the day), the membrane is adapted to produce less NADPH through cyclic electron flux.

4.4.6. Final remarks

The epiphytic habitat can trigger abiotic stresses due to limitations in nutritional sources, intermittence in the water supply or seasonal over-light excitation. Chloroplasts can act as a sensor of external signals, adapting the metabolism and configuring acclimation to the environmental changes (Pfannschmidt, 2003). *Guzmania monostachia* is a bromeliad which occupies the epiphytic niche of tropical forests with a plastic photosynthesis, capable of acclimating the light-harvesting capacity and carbon fixation metabolism (Maxwell *et al.*, 1994, 1995). The most remarkable trait is the CAM pathway, which can be induced by water deficit and contributes to CO_2 recycling, water conservation and metabolism balance during periods of drought (Maxwell *et al.*, 1992; Pikart *et al.*, 2018).

Here, we provide interesting data showing modulation of the photosynthetic machinery of *G. monostachia* under water deficit. The results showed changes in the stoichiometry between PSII and PSI triggered by transcriptional and likely post-transcriptional regulators, which downregulated the photosynthetic capacity. Additionally, the results indicated an increase in the ATP synthesis capacity of WD plants by: 1) the increase in amount of the subunit gamma of ATP synthase; 2) the change in the PSII/PSI ratio; and 3) the suggested change in the thylakoid conformation that facilitate ATP synthesis (Wood *et al.*, 2018). Indeed, a higher demand for ATP is described for CAM photosynthesis compared to C_3 pathway (Shameer *et al.*, 2018), and the results indicated an adjustment of the ATP synthesis system in CAM-induced plants. All

these traits may also contribute to the photosynthetic acclimation mechanism and to the successful adaptation to the harsh epiphytic habitat. A hypothetical model of diurnal thylakoid membrane organization in CAM plants is presented in figure 6.

As a result of this study, we also provide a method to isolate and maintain the integrity of thylakoids of a CAM-induced species, which can be used in further studies of CAM plant chloroplasts. From the perspective of engineering CAM pathway into C_3 species, this study provides greater knowledge about proteins that are differentially regulated when the plants are CAM-induced. Thus, the regulatory system of these proteins triggered by the CAM induction should be investigated carefully, with the goal of understanding how the metabolism is adjusted to achieve proper functioning of the CAM pathway.

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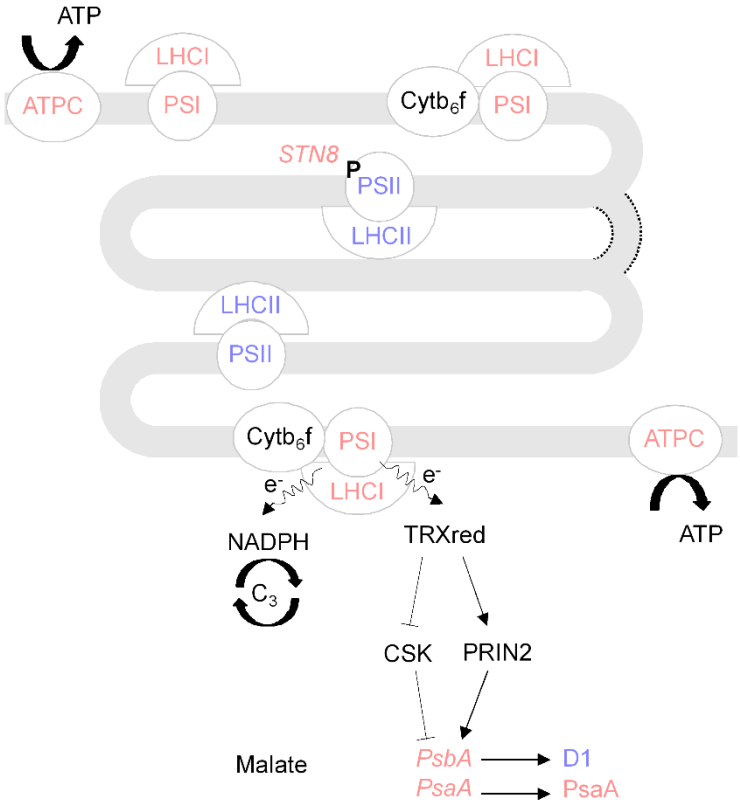
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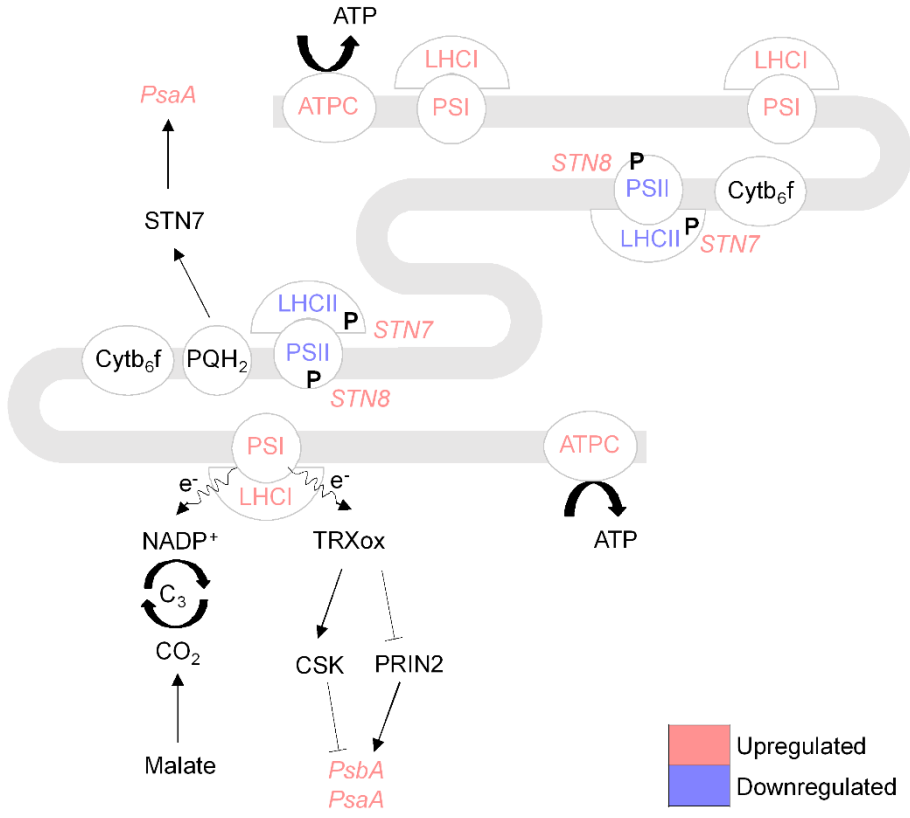
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ATPC
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LHCI
e-
NADP+
TRXox
ATP
C3
CO2
CSK
PRIN2
Malate
PsbA
PsaA
Upregulated
Downregulated

Figure 6. Hypothetical model of diurnal thylakoid membrane organization in CAM plants. In (A) the thylakoid membrane is more appressed, facilitating the cyclic electron flow (Wood *et al.*, 2018). At the beginning of the day the consumption of NADPH by RUBISCO is reduced (Maxwell *et al.*, 1999*a*), increasing the TRX reduced pool and triggering the *PsbA* and *PsaA* expression. The photosystem stoichiometry was affected, presenting a reduction in the PSII/PSI proportion. The dashed region indicated the "membrane bridge" proposed by Chuartzman *et al.* (2008), a region that becomes unstable and allows the membrane de-stacking movement with the phosphorylation of the PSII center and LHCII. In (B) the PSII and LHCII were phosphorylated by STN8 and STN7, respectively, driving the rearrangement of the thylakoid membrane. The non-appressed conformation facilitates the linear electron flow (Wood *et al.*, 2018), and the malate decarboxylation provides CO_2, both powering the Calvin-Benson Cycle. The reduced plastoquinone pool (PQH_2) induces the STN7 activity, triggering the *PsaA* transcription (Wunder *et al.*, 2013).

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References

Andersen CL, Jensen JL, Ørntoft TF. 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64, 5245–5250.

Armbruster U, Correa Galvis V, Kunz HH, Strand DD. 2017. The regulation of the chloroplast proton motive force plays a key role for photosynthesis in fluctuating light. Current Opinion in Plant Biology 37, 56–62.

Belin C, Bashandy T, Cela J, Delorme-Hinoux V, Riondet C, Reichheld JP. 2015. A comprehensive study of thiol reduction gene expression under stress

conditions in *Arabidopsis thaliana*. Plant, Cell and Environment 38, 299–314.

Berry JO, Yerramsetty P, Zielinski AM, Mure CM. 2013. Photosynthetic gene expression in higher plants. Photosynthesis Research, 1–30.

Bhattacharjee S. 2019. ROS and Regulation of photosynthesis. Reactive Oxygen Species in Plant Biology. New Delhi: Springer India, 107–125.

Bielczynski LW, Schansker G, Croce R. 2016. Effect of light acclimation on the organization of photosystem ii super- and sub-complexes in *Arabidopsis thaliana*. Frontiers in Plant Science 7, 105.

Borland AM, Hartwell J, Weston DJ, Schlauch KA, Tschaplinski TJ, Tuskan GA, Yang X, Cushman JC. 2014. Engineering crassulacean acid metabolism to improve water-use efficiency. Trends in Plant Science 19, 327–338.

Brunelle JL, Green R. 2014. Coomassie blue staining. Methods in Enzymology. Elsevier Inc., 161–167.

Ceusters N, Valcke R, Frans M, Claes JE, Van den Ende W, Ceusters J. 2019. Performance index and PSII connectivity under drought and contrasting light regimes in the CAM orchid *Phalaenopsis*. Frontiers in Plant Science 10, 1–15.

Chen Y-E, Liu W-J, Su Y-Q, Cui J-M, Zhang Z-W, Yuan M, Zhang H-Y, Yuan S. 2016. Different response of photosystem II to short and long term drought stress in *Arabidopsis thaliana*. Physiologia plantarum, 225–235.

Chuartzman SG, Nevo R, Shimoni E, Charuvi D, Kiss V, Ohad I, Brumfeld V, Reich Z. 2008. Thylakoid membrane remodeling during state transitions in *Arabidopsis*. The Plant Cell 20, 1029–1039.

Croce R. 2015. PsbS is the plants' pick for sun protection. Nature Structural and Molecular Biology 22, 650–652.

Croce R, Canino G, Ros F, Bassi R. 2002. Chromophore organization in the higher-plant photosystem II antenna protein CP26. Biochemistry 41, 7334–7343.

Cushman JC. 2001a. Crassulacean acid metabolism. A plastic photosynthetic adaptation to arid environments. Plant Physiology 127, 1439–1448.

Díaz MG, Hernández-Verdeja T, Kremnev D, Crawford T, Dubreuil C, Strand Å. 2018. Redox regulation of PEP activity during seedling establishment in *Arabidopsis thaliana*. Nature Communications 9, 1–12.

Dietz K-J, Pfannschmidt T. 2011. Novel regulators in photosynthetic redox control of plant metabolism and gene expression. Plant Physiology 155, 1477–1485.

Freschi L, Takahashi CA, Cambui CA, Semprebom TR, Cruz AB, Mioto PT, Versieux LM, Calvente A, Latansio-Aidar SR, Aidar MPM, Mercier H. 2010. Specific leaf areas of the tank bromeliad *Guzmania monostachia* perform distinct functions in response to water shortage. Journal of Plant Physiology 167, 526–533.

Fristedt R, Willig A, Granath P, Crèvecoeur M, Rochaix JD, Vener A V. 2009. Phosphorylation of photosystem II controls functional macroscopic folding of photosynthetic membranes in *Arabidopsis*. Plant Cell 21, 3950–3964.

Fukuyama K. 2004. Structure and function of plant-type ferredoxins. Photosynthesis Research 81, 289–301.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8, R19.

Herrera A. 2009. Crassulacean acid metabolism and fitness under water deficit stress: If not for carbon gain, what is facultative CAM good for? Annals of Botany 103, 645–653.

Herrera A, Delgado J, Paraguatey I. 1991. Occurrence of inducible crassulacean acid metabolism in leaves of *Talimum triangulare* (Portulacaceae). Journal of Experimental Botany 42, 493–499.

Järvi S, Suorsa M, Paakkarinen V, Aro E-M. 2011. Optimized native gel systems for separation of thylakoid protein complexes: novel super- and mega-complexes. The Biochemical journal 439, 207–14.

Kindgren P, Kremnev D, Blanco NE, De Dios Barajas López J, Fernández AP, Tellgren-Roth C, Small I, Strand Å. 2012. The PLASTID REDOX INSENSITIVE 2 mutant of *Arabidopsis* is impaired in PEP activity and high light-dependent plastid redox signalling to the nucleus. Plant Journal 70, 279–291.

Kollist H, Zandalinas SI, Sengupta S, Nuhkat M, Kangasjärvi J, Mittler R. 2019. Rapid responses to abiotic stress: priming the landscape for the signal transduction network. Trends in Plant Science 24, 25–37.

Li X, Björkman O, Shih C, Grossman AR, Rosenquist M, Jansson S, Niyogi KK. 2000. A pigment-binding protein essential for regulation of photosynthetic light harvesting. Nature 403, 391–395.

Lima Neto MC, Silveira JAG, Cerqueira JVA, Cunha JR. 2017. Regulation of the photosynthetic electron transport and specific photoprotective mechanisms in *Ricinus communis* under drought and recovery. Acta Physiologiae Plantarum 39.

Lubitz W, Chrysina M, Cox N. 2019. Water oxidation in photosystem II. Photosynthesis Research 142, 105–125.

Matiz A, Cambuí CA, Richet N, Mioto PT, Gomes F, Pikart FC, Chaumont F, Gaspar M, Mercier H. 2019. Involvement of aquaporins on nitrogen-acquisition strategies of juvenile and adult plants of an epiphytic tank-forming bromeliad. Planta 250, 319–332.

Matiz A, Mioto PT, Aidar MPM, Mercier H. 2017. Utilization of urea by leaves of bromeliad *Vriesea gigantea* under water deficit: much more than a nitrogen source. Biologia Plantarum 61, 751–762.

Matiz A, Tamaso P, Yepes A, Freschi L, Mercier H. 2013. CAM photosynthesis in bromeliads and agaves: what can we learn from these plants? Photosynthesis. InTech, 91–134.

Maxwell K. 2002. Resistance is useful: diurnal patterns of photosynthesis in C_3 and Crassulacean acid metabolism epiphytic bromeliads. Functional Plant Biology 29, 679–687.

Maxwell K, Borland AM, Haslam RP, Helliker BR, Roberts A, Griffiths H. 1999a. Modulation of RUBISCO activity during the diurnal phases of the Crassulacean acid metabolism plant *Kalanchoë daigremontiana*. Plant Physiology 121, 849–856.

Maxwell C, Griffiths H, Borland AM, Broadmeadow MSJ, McDavid CR. 1992. Photoinhibitory responses of the epiphytic bromeliad *Guzmania monostachia* during the dry season in Trinidad maintain photochemical integrity under adverse conditions. Plant, Cell and Environment 15, 37–47.

Maxwell C, Griffiths H, Borland A, Young A, Broadmeadow M, Fordham M. 1995. Short-term photosynthetic responses of the C_3-CAM epiphyte *Guzmania monostachia* var. monostachia to tropical seasonal transitions under field conditions. Functional Plant Biology 22, 771–781.

Maxwell C, Griffiths H, Young AJ. 1994. Photosynthetic acclimation to light regime and water stress by the C_3-CAM epiphyte *Guzmania monostachia*: gas-exchange characteristics, photochemical efficiency and the xanthophyll cycle. Functional Ecology 8, 746–754.

Maxwell K, Marrison JL, Leech RM, Griffiths H, Horton P. 1999b. Chloroplast acclimation in leaves of *Guzmania monostachia* in response to high light. Plant physiology 121, 89–96.

Medina E. 1974. Dark CO_2 fixation, habitat preference and evolution within the Bromeliaceae. Evolution 28, 677–686.

Mercier H, Rodrigues MA, Andrade SC da S, Lehmann L, Gobara BNK, Matiz A, Mioto PT, Gonçalves AZ. Transcriptional foliar profile of the C_3-CAM bromeliad *Guzmania monostachia*. PLoS ONE, In press.

Monde RA, Schuster G, Stern DB. 2000. Processing and degradation of chloroplast mRNA. Biochimie 82, 573–582.

Mustárdy L, Garab G. 2003. Granum revisited. A three-dimensional model - where things fall into place. Trends in Plant Science 8, 117–122.

Nicol L, Croce R. 2018. Light harvesting in higher plants and green algae. In: Light Harvesting in Photosynthesis. CRC Press, 59–76.

Niechayev NA, Pereira PN, Cushman JC. 2019. Understanding trait diversity associated with Crassulacean acid metabolism (CAM). Current Opinion in Plant Biology 49, 74–85.

Niewiadomska E, Bilger W, Gruca M, Mulisch M, Miszalski Z, Krupinska K. 2011. CAM-related changes in chloroplastic metabolism *of Mesembryanthemum crystallinum* L. Planta 233, 275–285.

Nishiyama Y, Yamamoto H, Allakhverdiev SI, Inaba M, Yokota A, Murata N. 2001. Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. EMBO Journal 20, 5587–5594.

Ogbaga CC, Stepien P, Johnson GN. 2014. Sorghum (*Sorghum bicolor*) varieties adopt strongly contrasting strategies in response to drought. Physiologia Plantarum 152, 389–401.

Pereira PN, Gaspar M, Smith JAC, Mercier H. 2018. Ammonium intensifies CAM photosynthesis and counteracts drought effects by increasing malate transport and antioxidant capacity in *Guzmania monostachia*. Journal of Experimental Botany 69, 1993–2003.

Pesaresi P, Hertle A, Pribil M, et al. 2009. *Arabidopsis* STN7 kinase provides a link between short- and long-term photosynthetic acclimation. the Plant Cell Online 21, 2402–2423.

Pfannschmidt T. 2003. Chloroplast redox signals: How photosynthesis controls its own genes. Trends in Plant Science 8, 33–41.

Pfannschmidt T, Allen JF, Oelmüller R. 2001. Principles of redox control in photosynthesis gene expression. Physiologia Plantarum 112, 1–9.

Pieters AJ, Tezara W, Herrera A. 2003. Operation of the xanthophyll cycle and degradation of D1 protein in the inducible CAM plant, *Talinum triangulare*, under water deficit. Annals of Botany 92, 393–399.

Pikart FC, Marabesi MA, Mioto PT, Gonçalves AZ, Matiz A, Alves FRR, Mercier H, Aidar MPM. 2018. The contribution of weak CAM to the photosynthetic metabolic activities of a bromeliad species under water deficit. Plant Physiology and Biochemistry 123, 297–303.

Popp M, Janett HP, Lüttge U, Medina E. 2003. Metabolite gradients and carbohydrate translocation in rosette leaves of CAM and C_3 bromeliads. New Phytologist 157, 649–656.

Porra RJ, Thompson WA, Kriedemann PE. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta (BBA) - Bioenergetics 975, 384–394.

Puthiyaveetil S, Ibrahim IM, Allen JF. 2012. Oxidation-reduction signalling components in regulatory pathways of state transitions and photosystem stoichiometry adjustment in chloroplasts. Plant, Cell and Environment 35, 347–359.

Puthiyaveetil S, Kavanagh TA, Cain P, Sullivan JA, Newell CA, Gray JC, Robinson C, van der Giezen M, Rogers MB, Allen JF. 2008. The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chloroplasts. Proceedings of the National Academy of Sciences 105, 10061–10066.

Rascher U, Lüttge U. 2002. High-resolution chlorophyll fluorescence imaging serves as a non-invasive indicator to monitor the spatio-temporal variations of metabolism during the day-night cycle and during the endogenous rhythm

in continuous light in the CAM plant *Kalanchoë daigremontiana*. Plant Biology 4, 671–681.

Rayder L, Ting IP. 1983. CAM-idling in *Hoya carnosa* (Asclepiadaceae). Photosynthesis Research 4, 203–211.

Rochaix J-D. 2014. Regulation and Dynamics of the Light-Harvesting System. Annual Review of Plant Biology 65, 287–309.

Ruban A V, Young AJ, Horton P. 1993. Induction of non-photochemical energy dissipation and absorbance changes in leaves evidence for changes in the state of the light-harvesting system of photosystem II *in vivo*. Plant physiology 102, 741–750.

Saibo NJM, Lourenço T, Oliveira MM. 2009. Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Annals of Botany 103, 609–623.

Schönberg A, Rödiger A, Mehwald W, Galonska J, Christ G, Helm S, Thieme D, Majovsky P, Hoehenwarter W, Baginsky S. 2017. Identification of STN7/STN8 kinase targets reveals connections between electron transport, metabolism and gene expression. Plant Journal 90, 1176–1186.

Shameer S, Baghalian K, Cheung CYM, Ratcliffe RG, Sweetlove LJ. 2018. Computational analysis of the productivity potential of CAM. Nature Plants 4, 165–171.

Shimoni E, Rav-Hon O, Ohad I, Brumfeld V, Reich Z. 2005. Three-dimensional organization of higher-plant chloroplast thylakoid membranes revealed by electron tomography. Plant Cell 17, 2580–2586.

Smith JAC, Griffiths H, Bassett M, Griffiths NM. 1985. Day-night changes in the leaf water relations of epiphytic bromeliads in the rain forests of Trinidad. Oecologia 67, 475–485.

Stroebel D, Choquet Y, Popot JL, Picot D. 2003. An atypical haem in the cytochrome b6f complex. Nature 426, 413–418.

Tambussi EA, Bartoli CG, Beltrano J, Guiamet JJ, Araus JL. 2000. Oxidative damage to thylakoid proteins in water-stressed leaves of wheat (*Triticum aestivum*). Physiologia Plantarum 108, 398–404.

Ting IP. 1985. Crassulacean acid metabolism. Annual reviews Plant Physiology 960, 596–622.

Weatherley PE. 1950. Studies in the water relations of the cotton plant. I. The field measurement of water deficits in leaves. New Phytologist 49, 81–97.

Winter K. 2019. Ecophysiology of constitutive and facultative CAM photosynthesis. Journal of experimental botany, 1–14.

Winter K, Aranda J, Holtum J. 2005. Carbon isotope composition and water-use efficiency in plants with Crassulacean acid metabolism. Functional Plant Biology, 381–388.

Wood WHJ, MacGregor-Chatwin C, Barnett SFH, Mayneord GE, Huang X, Hobbs JK, Hunter CN, Johnson MP. 2018. Dynamic thylakoid stacking regulates the balance between linear and cyclic photosynthetic electron transfer. Nature Plants 4, 116–127.

Woodson JD. 2019. Chloroplast stress signals: regulation of cellular degradation and chloroplast turnover. Current Opinion in Plant Biology 52, 30–37.

Wunder T, Liu Q, Aseeva E, Bonardi V, Leister D, Pribil M. 2013. Control of STN7 transcript abundance and transient STN7 dimerisation are involved in the regulation of STN7 activity. Planta 237, 541–558.

Yang X, Cushman JC, Borland AM, et al. 2015. A roadmap for research on Crassulacean acid metabolism (CAM) to enhance sustainable food and bioenergy production in a hotter, drier world. New Phytologist 207, 491–504.

Zoschke R, Bock R. 2018. chloroplast translation: structural and functional organization, operational control, and regulation. The Plant cell 30, 745–770.

Zotz G. 2016. Plants on plants – The biology of vascular epiphytes. Springer.

Supplementary material

Table S1. Forward (F) and reverse (R) sequence primers and their respective melting temperatures used in the quantitative real-time PCR analysis.

Gene	Primers 5'-3'	Melting temperature (°C)
PsbA	F: GTTTCTTCCTCTTGACCG	60
	R: GGCTCCCTATTCAGTGCT	
PsbC	F: CGTACCAGAGAAACCCA	60
	R: GATAAATACCGCCAAAGCC	
Lhcb4	F: GGACGCTGGAAAGGTTGAG	60
	R: CGATGTAGCCGATGACCAC	
Lhcb1	F: TAGGATTGGCTGATGACCC	60
	R: GAAGAACCCGAACATTGAG	
PsbS	F: CTCCTCCTCTTCTTCATCC	60
	R: AACCTTCCGACGAAAAGC	
PsaA	F: CTTTGTGATAATGGAACC	60
	R: TTGAATGGTGATGTGGGC	
Lhca1	F: CCATCCCGAACACTTCCTC	62.5
	R: TCAGATTCCTTGTACCTCTC	
ATPC	F: ACTTCACCAGCGACACGA	60
	R: GGGCAACTCCTACTTCCAG	
STN7	F: GGTGGAGATATGGATGAACGA	60
	R: TTGCTTTGAATAAGACCCGC	
STN8	F: GGCTATTCCCACCTTGAG	60
	R: CCAAATCCCATCCTCTACCAG	
PRIN2	F: ACCCATCGCTTCTTTCCA	60
	R: GATTCTTCTCCGTCAGCCTC	
CSK	F: AAGCACAGTTCAACAAATGGTC	60
	R: GCATCTCTATGTCCTTTCTATCTG	
BAM	F: CTTTCTGGATGCGAAGGGTATC	60
	R: TGAAGGAAGGAGGAACACG	
HSDD	F: CCTCAAGTCTCACTAACGAAG	60
	R: GATGTAGCAGCCGAATCAAC	

Table S2. Analysis of variance (ANOVA) of non-photochemical quenching (NPQ), relative water content (RWC), and maximum quantum efficiency of PSII photochemistry (F_v/F_m), nocturnal H^+ accumulation (acidity), and transcript abundance of some thylakoid proteins (*PsbA*, *PsbC*, *Lhcb4*, *Lhcb1*, *PsbS*, *PsaA*, *Lhca1*, and *ATPC*) and chloroplast regulatory proteins (*STN7*, *STN8*, *CSK*, and *PRIN2*) of plants well-watered (WW) and under 20 days of water deficit (WD), or rewatered (RW) during seven days after the WD treatment.

ANOVA (α = 0.05)									
	Time (s)			Water conditions			Time vs. water conditions		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
NPQ	30	19.8844	<0.0001	1	581.6211	<0.0001	30	3.2762	<0.0001
Well-watered vs. Water deficit									
	d.f.	F	P						
RWC	1	60.8160	0.0002						
F_v/F_m	1	33.2932	0.0002						
Acidity	1	12476.5	<0.0001						
Well-watered vs. Water deficit									
	7:00h			12:00h			17:00h		
	d.f.	F	P	d.f.	F	P	d.f.	F	P
PsbA	1	8.4174	0.0337	1	0.1687	0.6955	1	16.7748	0.0094
PsbC	1	42.0098	0.0029	1	1.1488	0.3250	1	46.2189	0.0005
Lhcb4	1	10.2206	0.0241	1	1.6374	0.2699	1	0.4146	0.5480
Lhcb1	1	7.2830	0.0356	1	3.0053	0.1580	1	1.2833	0.3005
PsbS	1	17.5756	0.0057	1	51.8221	0.0020	1	33.5310	0.0012
PsaA	1	11.8235	0.0185	1	160.0984	0.0002	1	49.6898	0.0009
Lhca1	1	16.8900	0.0093	1	23.1459	0.0086	1	1.4591	0.2725
ATPC	1	14.2515	0.0130	1	4.8789	0.0917	1	3.9108	0.0953
STN7	1	5.7730	0.0741	1	33.2680	0.0022	1	0.8183	0.4005
STN8	1	7.2628	0.0358	1	10.3376	0.0324	1	0.0617	0.8137
CSK	1	6.7609	0.0424	1	48.8816	0.0009	1	39.2079	0.0008
PRIN2	1	1.9298	0.2141	1	21.6620	0.0096	1	14.6883	0.0086

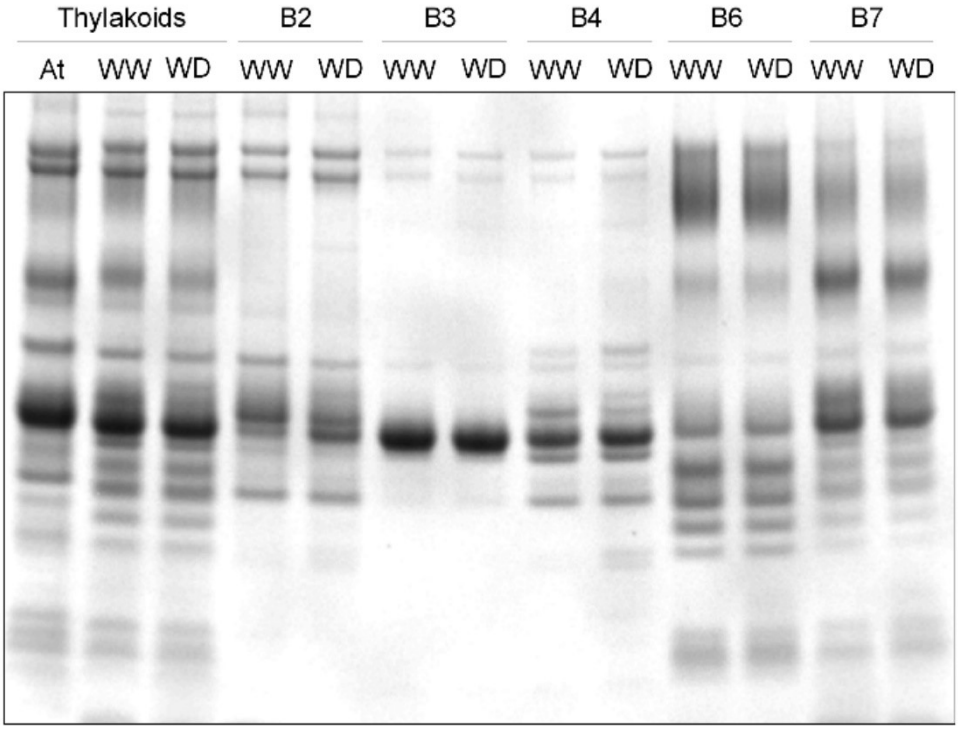

Figure S1. Protein pattern of thylakoids isolated from *G. monostachia* under two water conditions, well-watered (WW) and water deficit (WD); and from *A. thaliana* (At). Bands two (lhcb monomer), three (LHCII trimer), four (CP29-CP24-LHCII), six (PSI-lhca), and seven (PSII supercomplexes) were isolated from thylakoids of *G. monostachia* WW and under WD using a sucrose gradient. SDS gels were stained with Coomassie after electrophoresis.

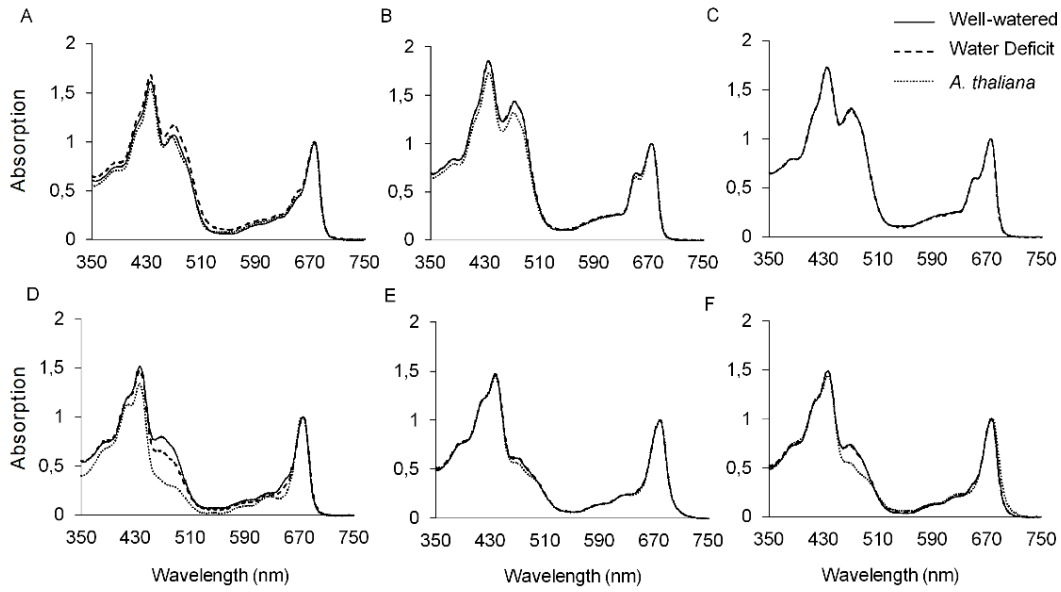

Figure S2. Absorption spectrum of (A) band two, lhcb monomer; (B) band three, LHCII trimer; (C) band four, CP29-CP24-LHCII (not present in *A. thaliana*); (D) band five, non-identified; (E) band six, PSI-lhca; and (F) band seven, PSII supercomplexes; separated using a sucrose gradient of *G. monostachia* under two water conditions, well-watered and water deficit for 20 days. *Arabidopsis thaliana* was used as a control.

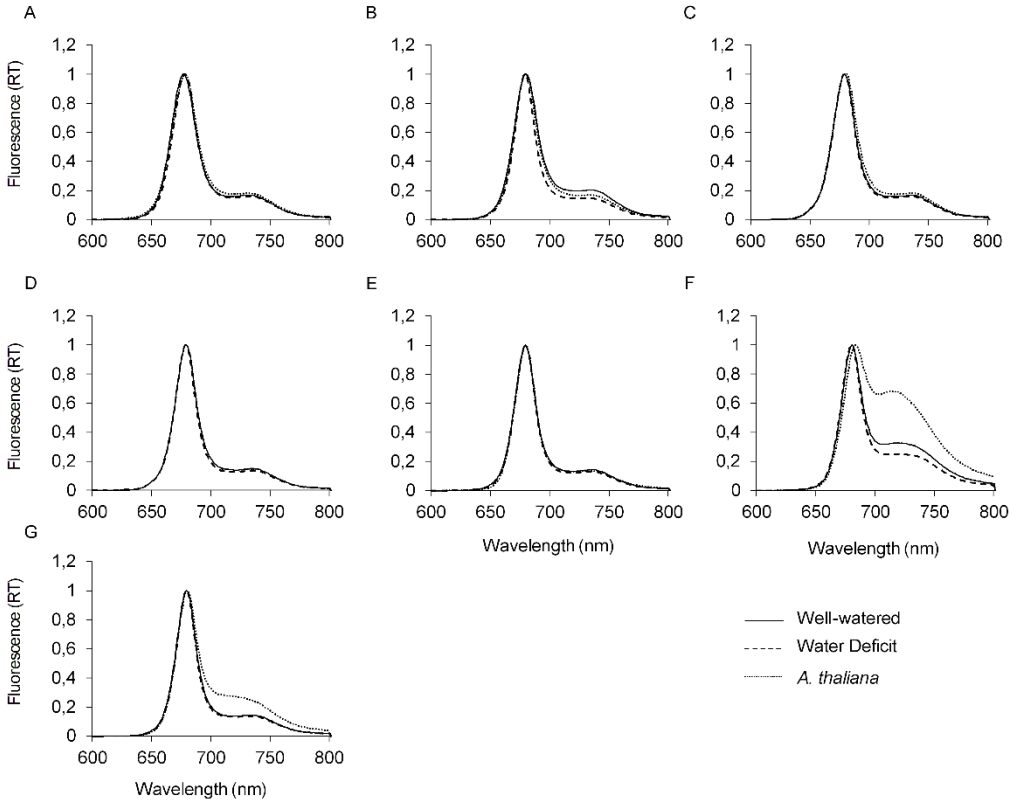

Figure S3. Fluorescence spectrum at room temperature (excitation at 440 nm) of (A) band one, free pigments; (B) band two, lhcb monomer; (C) band three, LHCII trimer; (D) band four, CP29-CP24-LHCII (not present in *A. thaliana*); (E) band five, non-identified; (F) band six, PSI-lhca; and (G) band seven, PSII supercomplexes; separated using a sucrose gradient of *G. monostachia* under two water conditions, well-watered and water deficit for 20 days. *Arabidopsis thaliana* was used as a control.

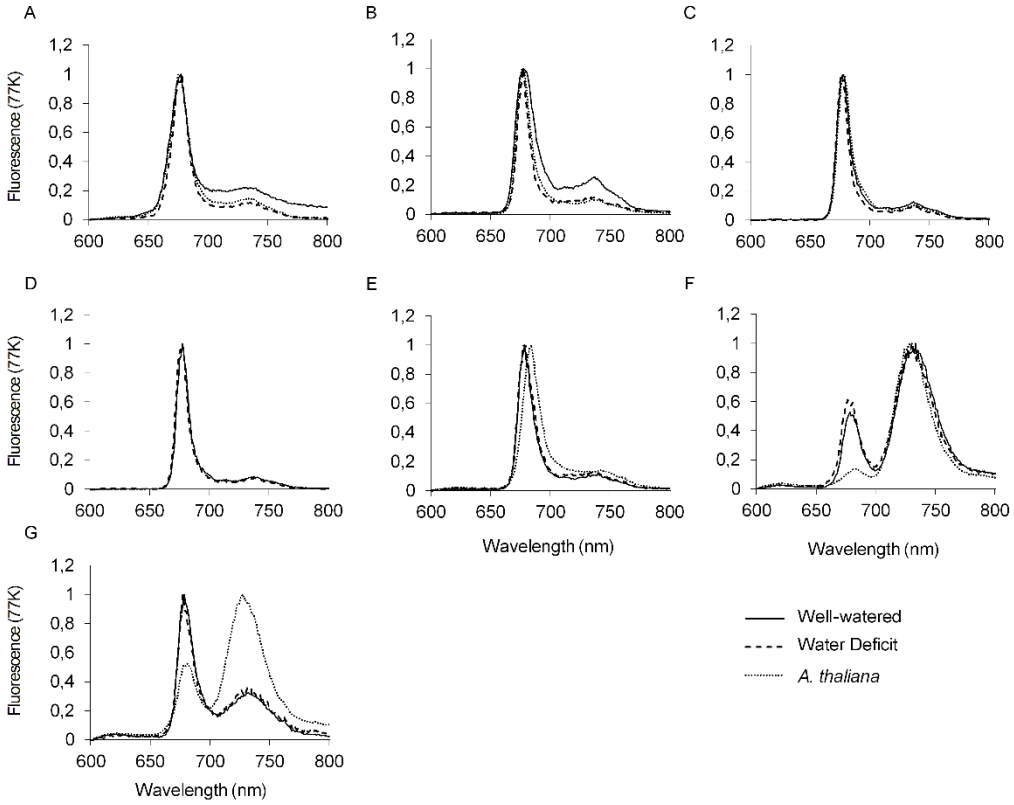

Figure S4. Fluorescence spectrum at 77K (excitation at 440 nm) of (A) band one, free pigments; (B) band two, lhcb monomer; (C) band three, LHCII trimer; (D) band four, CP29-CP24-LHCII (not present in *A. thaliana*); (E) band five, non-identified; (F) band six, PSI-lhca; and (G) band seven, PSII supercomplexes; separated using a sucrose gradient of *G. monostachia* under two water conditions, well-watered and water deficit for 20 days. *Arabidopsis thaliana* was used as a control.

5. Considerações finais

Plantas de *Guzmania monostachia* apresentaram indução ao CAM com 20 dias de déficit hídrico imposto pela suspenção da rega, observado pelo aumento significativo no acúmulo noturno de ácidos, característica marcante desse tipo fotossintético. Após sete dias de reidratação, as plantas que passaram pelo período de seca recuperaram o conteúdo hídrico, a obtenção de CO_2 atmosférico, assim como apresentaram valores equivalentes aos do controle em relação à máxima capacidade fotoquímica. Sugere-se dessa forma que plantas de *G. monostachia* possuem capacidade de sobreviver a períodos de seca de, pelo menos, 20 dias.

A espécie quando submetida à seca apresentou regulação transcricional e pós-traducional da enzima PEPCK, observado pelo aumento nos transcritos da *PEPCK* e na modulação da atividade de descarboxilação, possivelmente devido a um controle por fosforilação. Essa regulação resultou na maior atividade de descarboxilação observada às 12:00h, momento em que também ocorreu uma redução no conteúdo de ácidos, resultando em uma provável elevação da concentração interna de CO_2. Os resultados indicam fortemente uma modulação no uso da energia pelo PSII de plantas CAM, já que foi observada uma flutuação no uso fotoquímico e não-fotoquímico da energia pelo PSII. De forma interessante, ocorreu aumento no uso fotoquímico e redução no não-fotoquímico no mesmo momento em que ocorreu a máxima atividade de descarboxilação. Adicionalmente, sugere-se que o incremento no nível de transcritos das cinases STN7 e STN8, principalmente ao meio dia, acarretou a fosforilação de componentes do centro de reação do PSII e do LHCII. A fosforilação desses componentes pode ter levado a alterações na conformação das membranas do tilacoide, propiciando o transporte linear de elétrons e a produção de NADPH, necessário para a fixação do CO_2.

A condição de seca resultou na regulação positiva da transcrição de *PRIN2* e *CSK*, o que, possivelmente, resultou em acréscimo do aparato que regula a transcrição de genes do PSII e PSI e que é responsivo ao estado redox, principalmente a tiorredoxina reduzida. De fato, foi observado aumento na transcrição de *PsbA*, *PsbC* e *PsaA* às 7:00h e 17:00h, momentos do dia com menor eficiência fotoquímica do PSII e, possivelmente, abundância da

tiorredoxina na sua forma reduzida. Adicionalmente, os resultados indicam que a regulação positiva na transcrição de *PsaA* foi mantida ao meio dia, já que os níveis de seus transcritos e do seu regulador (*STN7*) estavam aumentados. Entretanto, mesmo com o acréscimo na abundância de transcritos de genes *Psb*, foi observado redução nos níveis das proteínas analisadas, sugerindo um controle na tradução/processamento dessas proteínas do PSII. Dessa forma, os resultados indicam uma modulação na maquinaria fotossintética, caracterizada pela redução no conteúdo de proteínas do PSII e aumento de componentes do PSI. Foi observado também incremento na abundância da subunidade gama da ATP sintase, o que, possivelmente, resultou na adequação da capacidade de síntese de ATP, ajustando o metabolismo à demanda energética da fotossíntese CAM.

Dessa forma, os resultados apresentados indicam um ajuste tanto bioquímica quanto molecular, influenciando o uso da energia pelo PSII de plantas induzidas ao CAM como resposta à seca.

6. Perspectivas

Esse projeto propiciou a elaboração/adequação de dois protocolos para a espécie *G. monostachia*, um para investigar a atividade de descarboxilação da enzima PEPCK e o outro para o isolamento de tilacoides. A adaptação do segundo protocolo abriu a possibilidade de estudos com proteínas do tilacoide, no seu estado nativo ou desnaturadas, de outras plantas CAM ou CAM facultativas induzidas pela seca ou até por outros fatores. Dessa forma, será possível averiguar se espécies CAM apresentam de forma geral uma proporção PSII/PSI diferente de espécies C_3 ou se uma alteração é induzida, no caso de facultativas.

Essa pesquisa indica que projetos que visam inserir o CAM em espécies C_3 devem considerar também os processos que ocorrem durante o dia, como a descarboxilação e absorção e uso da energia da luz. Nesse sentido, a necessidade de entender a regulação dos componentes do controle transcricional e pós-traducional da enzima PEPCK parece ser essencial para alcançar o efeito concentrador de carbono proporcionado por esse tipo de fotossíntese. Dessa forma, sugere-se a investigação da AMP-ACTIVATING PROTEIN KINASE, responsável por regular a transcrição da *PEPCK* através do fator de transcrição ARBP (Inoue & Yamauchi, 2006). Estudos com fornecimento de ^{32}Pi podem ser feitos para averiguar a variação diurna na fosforilação da PEPCK e, combinado com a análise da atividade de descarboxilação, confirmar a modulação na fosforilação e controle sobre a atividade de descarboxilação em plantas induzidas ao CAM. Além disso, identificar a cinase que regula pós-traducionalmente essa atividade parece ser essencial para alcançar avanços na identificação de elementos regulados com a indução do CAM. Assim, sugere-se um estudo que identifique possíveis cinases que apresentam afinidade pelo mesmo resíduo que é fosforilado na PEPCK (Thr_{58} em espécies C_3, Shen *et al.*, 2017), seguido de ensaios com ^{32}Pi, confirmando a atividade da cinase sobre a PEPCK. Por fim, sugere-se o uso de uma planta CAM mutante deficiente no processo de acidificação noturna (por exemplo, *Mesembryanthemum crystallinum* linhagem 351, Cushman *et al.*, 2008*a*) para avaliar se a mesma modulação no uso da energia pelo PSII acontece mesmo sem o suprimento do CO_2 provindo da descarboxilação dos ácidos.

Referências bibliográficas

Abreu ME, Carvalho V, Mercier H. 2018. Antioxidant capacity along the leaf blade of the C_3-CAM facultative bromeliad *Guzmania monostachia* under water deficit conditions. Functional Plant Biology 45, 1–10.

Adams WW, Demmig-Adams B. 1992. Operation of the xanthophyll cycle in higher plants in response to diurnal changes in incident sunlight. Planta 186, 390–398.

Allen JF. 2002. Photosynthesis of ATP-electrons, proton pumps, rotors, and poise. Cell 110, 273–276.

Baker NR. 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annual review of plant biology 59, 89–113.

Benzing DH. 2000. Bromeliaceae: profile of an adaptive radiation. Systematic Biology 54, 340–344.

Bilger W, Björkman O. 1990. Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. Photosynthesis Research 25, 173–185.

Borland AM, Hartwell J, Weston DJ, Schlauch KA, Tschaplinski TJ, Tuskan GA, Yang X, Cushman JC. 2014. Engineering Crassulacean acid metabolism to improve water-use efficiency. Trends in Plant Science 19, 327–338.

Busch FA. 2013. Current methods for estimating the rate of photorespiration in leaves. Plant Biology 15, 648–655.

Caffarri S, Tibiletti T, Jennings R, Santabarbara S. 2014. A comparison between plant photosystem I and photosystem II architecture and functioning. Current Protein & Peptide Science 15, 296–331.

Crayn DM, Winter K, Schulte K, Smith JAC. 2015. Photosynthetic pathways in Bromeliaceae: phylogenetic and ecological significance of CAM and C_3 based on carbon isotope ratios for 1893 species. Botanical Journal of the Linnean Society 178, 169–221.

Crayn DM, Winter K, Smith JAC. 2004. Multiple origins of Crassulacean acid metabolism and the epiphytic habit in the neotropical family Bromeliaceae. Proceedings of the National Academy of Sciences of the United States of America 101, 3703–8.

Croce R, van Amerongen H. 2013. Light-harvesting in photosystem I. Photosynthesis Research 116, 153–166.

Cushman JC, Agarie S, Albion RL, Elliot SM, Taybi T, Borland AM. 2008a. Isolation and characterization of mutants of common ice plant deficient in crassulacean acid metabolism. Plant physiology 147, 228–38.

Cushman JC, Meyer G, Michalowski CB, Schmitt JM, Bohnert HJ. 1989. Salt stress leads to differential expression of two isogenes of phosphoenolpyruvate carboxylase during Crassulacean acid metabolism induction in the common ice plant. The Plant cell 1, 715–725.

Cushman JC, Tillett RL, Wood JA, Branco JM, Schlauch KA. 2008b. Large-scale mRNA expression profiling in the common ice plant, *Mesembryanthemum crystallinum*, performing C_3 photosynthesis and Crassulacean acid metabolism (CAM). Journal of Experimental Botany 59, 1875–1894.

Dekker JP, Boekema EJ. 2005. Supramolecular organization of thylakoid membrane proteins in green plants. Biochimica Et Biophysica Acta 1706, 12–39.

Demmig-Adams B, Adams III WW. 1992. Responses of plants to high light stress. Annual Review of Plant Physiology and Plant Molecular Biology 43, 599–626.

Díaz MG, Hernández-Verdeja T, Kremnev D, Crawford T, Dubreuil C, Strand Å. 2018. Redox regulation of PEP activity during seedling establishment in *Arabidopsis thaliana*. Nature Communications 9, 1–12.

Drop B, Webber-Birungi M, Yadav SKN, Filipowicz-Szymanska A, Fusetti F, Boekema EJ, Croce R. 2014. Light-harvesting complex II (LHCII) and its supramolecular organization in *Chlamydomonas reinhardtii*. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1837, 63–72.

Eaton-Rye JE, Tripathy BC, Sharkey TD. 2012. Photosynthesis (JJ Eaton-Rye, BC Tripathy, and TD Sharkey, Eds.). Dordrecht: Springer Netherlands.

Ferreira KN, Iverson TM, Maghlaoui K, Barber J, Iwata S. 2004. Architecture of the photosynthetic oxygen-evolving center. Science 303, 1831–1838.

Freschi L, Rodrigues MA, Domingues DS, Purgatto E, Van Sluys M-A, Magalhaes JR, Kaiser WM, Mercier H. 2010a. Nitric oxide mediates the hormonal control of Crassulacean acid metabolism expression in young pineapple plants. Plant physiology 152, 1971–85.

Freschi L, Takahashi CA, Cambui CA, Semprebom TR, Cruz AB, Mioto PT, Versieux LM, Calvente A, Latansio-Aidar SR, Aidar MPM, Mercier H. 2010b. Specific leaf areas of the tank bromeliad *Guzmania monostachia* perform distinct functions in response to water shortage. Journal of Plant Physiology 167, 526–533.

Gao J, Wang H, Yuan Q, Feng Y. 2018. Structure and function of the photosystem supercomplexes. Frontiers in Plant Science 9, 1–7.

Givnish TJ, Barfuss MHJ, Van Ee B, et al. 2011. Phylogeny, adaptive radiation, and historical biogeography in Bromeliaceae: insights from an eight-locus plastid phylogeny. American journal of botany 98, 872–95.

Gonçalves AZ, Mercier H, Oliveira RS, Romero GQ. 2016. Trade-off between soluble protein production and nutritional storage in Bromeliaceae. Annals of Botany 118, 1199–1208.

Gutteridge S, Jordan DB. 2001. Dynamics of photosynthetic fixation: control, regulation and productivity. In: Regulation of photosynthesis. Kluwer Academic Publishers, 297–312.

Herrera A. 2009. Crassulacean acid metabolism and fitness under water deficit stress: If not for carbon gain, what is facultative CAM good for? Annals of Botany 103, 645–653.

Inoue E, Yamauchi J. 2006. AMP-activated protein kinase regulates PEPCK gene expression by direct phosphorylation of a novel zinc finger transcription factor. Biochemical and Biophysical Research Communications 351, 793–799.

Jansson S. 1994. The light-harvesting chlorophyll a b-binding protein. BBA - Bioenergetics 1184, 1–19.

Junge W. 2019. Oxygenic photosynthesis: history, status and perspective. Quarterly Reviews of Biophysics 52, e1.

Kangasjärvi S, Neukermans J, Li S, Aro EM, Noctor G. 2012. Photosynthesis, photorespiration, and light signaling in defense responses. Journal of Experimental Botany 63, 1619–1636.

Kindgren P, Kremnev D, Blanco NE, De Dios Barajas López J, Fernández AP, Tellgren-Roth C, Small I, Strand Å. 2012. The plastid redox insensitive 2 mutant of *Arabidopsis* is impaired in PEP activity and high light-dependent plastid redox signalling to the nucleus. Plant Journal 70, 279–291.

Kleingesinds CK, Gobara BNK, Mancilha D, Rodrigues MA, Demarco D, Mercier H. 2018. Impact of tank formation on distribution and cellular organization of trichomes within *Guzmania monostachia* rosette. Flora: Morphology, Distribution, Functional Ecology of Plants 243, 11–18.

Krause GH, Weis E. 1991. Chlorophyll fluorescence and photosynthesis: the basics. Annual Review of Plant Physiology and Plant Molecular Biology 42, 313–349.

Males J. 2016. Think tank: water relations of Bromeliaceae in their evolutionary context. Botanical Journal of the Linnean Society 181, 415–440.

Martinelli G, Moraes M. 2013. Livro vermelho da flora do Brasil. Rio de Janeiro: Instituto de Pesquisas Jardim Botânico do Rio de Janeiro.

Martinelli G, Vieira CM, Gonzalez M, Leitman P, Piratininga A, Costa AF da, Forzza RC. 2008. Bromeliaceae da Mata Atlântica brasileira: lista de espécies, distribuição e conservação. Rodriguésia 59, 209–258.

Martorell C, Ezcurra E. 2007. The narrow-leaf syndrome: A functional and evolutionary approach to the form of fog-harvesting rosette plants. Oecologia 151, 561–573.

Matiz A, Tamaso P, Yepes A, Freschi L, Mercier H. 2013. CAM photosynthesis in bromeliads and agaves: What can we learn from these plants? Photosynthesis. InTech, 91–134.

Maxwell C, Griffiths H, Borland A, Young A, Broadmeadow M, Fordham M. 1995. Short-term photosynthetic responses of the C_3-cCAM epiphyte *Guzmania monostachia* var. monostachia to tropical seasonal transitions under field conditions. Functional Plant Biology 22, 771–781.

Maxwell C, Griffiths H, Young AJ. 1994. photosynthetic acclimation to light regime and water stress by the C_3-CAM epiphyte *Guzmania monostachia*: Gas-exchange characteristics, photochemical efficiency and the xanthophyll cycle. Functional Ecology 8, 746–754.

Mechela A, Schwenkert S, Soll J. 2019. A brief history of thylakoid biogenesis. Open Biology 9, 1–12.

Medina E. 1974. Dark CO fixation, habitat preference and evolution within the Bromeliaceae. Evolution 28, 677–686.

Mioto PT, Mercier H. 2013. Abscisic acid and nitric oxide signaling in two different portions of detached leaves of *Guzmania monostachia* with CAM up-

regulated by drought. Journal of Plant Physiology 170, 996–1002.

Mioto PT, Rodrigues MA, Matiz A, Mercier H. 2015. CAM-like traits in C_3 plants: biochemistry and stomatal behavior. In: Progress in Botany. Progress in Botany. Cham: Springer International Publishing, 195–209.

Nicol L, Croce R. 2018. Light harvesting in higher plants and green algae. In: Light Harvesting in Photosynthesis. CRC Press, 59–76.

Niechayev NA, Pereira PN, Cushman JC. 2019. Understanding trait diversity associated with crassulacean acid metabolism (CAM). Current Opinion in Plant Biology 49, 74–85.

Nieder J, Prosperí J, Michaloud G. 2001. Epiphytes and their contribution to canopy diversity. Entomologia Experimentalis et Applicata.51–63.

Nievola CC, Kraus JE, Freschi L, Souza BM, Mercier H. 2005. Temperature determines the occurrence of CAM or C_3 photosynthesis in pineapple plantlets grown in vitro. In Vitro Cellular and Developmental Biology - Plant 41, 832–837.

Pereira PN, Purgatto E, Mercier H. 2013. Spatial division of PHOSPHOENOLPYRUVATE CARBOXYLASE and nitrate reductase activity and its regulation by cytokinins in CAM-induced leaves of *Guzmania monostachia* (Bromeliaceae). Journal of Plant Physiology 170, 1067–1074.

Pikart FC, Marabesi MA, Mioto PT, Gonçalves AZ, Matiz A, Alves FRR, Mercier H, Aidar MPM. 2018. The contribution of weak CAM to the photosynthetic metabolic activities of a bromeliad species under water deficit. Plant Physiology and Biochemistry 123, 297–303.

Pinheiro C, Chaves MM. 2011. Photosynthesis and drought: Can we make metabolic connections from available data? Journal of Experimental Botany 62, 869–882.

Pittendrigh CS. 1948. The bromeliad-anopheles-malaria complex in Trinidad. I-The bromeliad flora. Evolution 2, 58–89.

Puthiyaveetil S, Ibrahim IM, Allen JF. 2012. Oxidation-reduction signaling components in regulatory pathways of state transitions and photosystem stoichiometry adjustment in chloroplasts. Plant, Cell and Environment 35, 347–359.

Ranson SL, Thomas M. 1960. Crassulacean acid metabolism. Annual Review of Plant Physiology 11, 81–110.

Rochaix J. 2011. Regulation of photosynthetic electron transport. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1807, 375–383.

Shen Z, Dong XM, Gao ZF, Chao Q, Wang BC. 2017. Phylogenic and phosphorylation regulation difference of PHOSPHOENOLPYRUVATE CARBOXYKINASE of C_3 and C_4 plants. Journal of Plant Physiology 213, 16–22.

Silva LD, Gomes FP, Oliveira PS, Almeida FR, Pirovani CP, Laviola BG, do Amaral JFT. 2019. Plasticity of photosynthetic metabolism in *Jatropha curcas* genotypes under water deficit. Genetics and Molecular Research 18, 1–17.

Smith JAC, Griffiths H, Luttge U. 1986. Comparative ecophysiology of CAM and C_3 bromeliads. I. The ecology of the Bromeliaceae in Trinidad. Plan 9, 359–376.

Takahashi CA, Ceccantini GCT, Mercier H. 2007. Differential capacity of nitrogen assimilation between apical and basal leaf portions of a tank epiphytic bromeliad. Brazilian Journal of Plant Physiology 19, 119–126.

Winter K. 2019. Ecophysiology of constitutive and facultative CAM photosynthesis. Journal of experimental botany, 1–14.

Winter K, Holtum JAM. 2014. Facultative Crassulacean acid metabolism (CAM) plants: Powerful tools for unravelling the functional elements of CAM photosynthesis. Journal of Experimental Botany 65, 3425–3441.

Winter K, Holtum J a M, Smith JAC. 2015. Crassulacean acid metabolism: a continuous or discrete trait? New Phytologist, 1–6.

Wunder T, Liu Q, Aseeva E, Bonardi V, Leister D, Pribil M. 2013. Control of *STN7* transcript abundance and transient STN7 dimerisation are involved in the regulation of STN7 activity. Planta 237, 541–558.

Yang X, Cushman JC, Borland AM, et al. 2015. A roadmap for research on crassulacean acid metabolism (CAM) to enhance sustainable food and bioenergy production in a hotter, drier world. New Phytologist 207, 491–504.

Zotz G, Hietz P. 2001. The physiological ecology of vascular epiphytes: current knowledge, open questions. Journal of experimental botany 52, 2067–2078.

Zotz G, Wilhelm K, Becker A. 2011. Heteroblasty—A Review. The Botanical Review 77, 109–151.