LUCAS ROANI PONTE

THE ANTAGONISTIC EFFECT OF AtCCR4a UPON DROUGHT OR HEAT STRESS CONDITIONS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para obtenção do título de *Magister Scientiae*.

Orientador: Pedro Augusto Braga dos Reis

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Orientador

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ABSTRACT

PONTE, Lucas Roani, M.Sc., Universidade Federal de Viçosa, August, 2021. **The Antagonistic Effect of AtCCR4a Upon Drought or Heat Stress Conditions**. Advisor: Pedro Augusto Braga dos Reis.

Climate changes are expected to increase the severity of environmental adversities to plants, such as changes in rainfall regimes and the occurrence of more extreme temperatures, imposing a major risk to the productivity of agricultural crops. Throughout evolution, plants have evolved elegant mechanisms to cope with different adverse conditions, through the activation of different signaling pathways. The occurrence of abiotic stresses, such as osmotic stress or endoplasmic reticulum stress, tend to activate specific cellular pathways, such as the death cell signaling pathway mediated by proteins that contain DCD/NRP domains. The AtNRP1 and AtNRP2 proteins, components of the pathway in Arabidopsis, have been characterized as effectors of the programmed cell death process by the breakdown of the vacuolar membrane. Understanding how this pathway is integrated into cellular stability is essential, which justifies checking the predicted and not yet functionally characterized interactions among the components of the pathway with other proteins, such as AtCCR4a, a deadenylase that is both active in mRNA processing and in sucrose/starch metabolism. Given this context, we aimed to investigate the interaction between AtNRP2 and AtCCR4a, and its role during heat and drought stresses. We did not observe its direct involvement in the NRP-signaling pathway, however *AtCCR4a* was induced by osmotic and heat stresses. Furthermore, *AtCCR4a* disruption provides a drastically heat stress-sensitive phenotype, leading to reductions in photosynthetic pigment contents days after exposure to stress and negative changes in carbohydrate metabolism during this condition. Surprisingly, *AtCCR4a* disruption also promotes a more tolerant phenotype to water deficit, contributing to greater maintenance of relative water contents in leaf tissues and plant survival rate. These results indicate that AtCCR4a may have an antagonistic function in stress-responsive pathways, probably by regulating at a post-transcriptional level the expression of certain genes and/or by selecting specific target mRNAs.

Keywords: Abiotic. Arabidopsis. Deadenylase. Heat. Water.

RESUMO

PONTE, Lucas Roani, M.Sc., Universidade Federal de Viçosa, agosto de 2021**. O Efeito Antagônico de AtCCR4a Perante Condições de Seca ou de Estresse por Alta Temperatura**. Orientador: Pedro Augusto Braga dos Reis.

Prevê-se que as mudanças climáticas incrementarão a severidade de adversidades ambientais, como mudanças nos regimes pluviométricos e a ocorrência de temperaturas mais extremas, impondo um grande risco para a produtividade das culturas agrícolas. Ao longo da evolução, as plantas desenvolveram elegantes mecanismos para lidar com distintas condições adversas por meio da ativação de diferentes vias de sinalização. A ocorrência de estresses abióticos, como estresse osmótico ou do retículo endoplasmático, tende a ativar vias celulares específicas, como a via de sinalização de morte celular mediada por proteínas contendo domínios DCD/NRP. As proteínas AtNRP1 e AtNRP2, componentes da via em Arabidopsis, foram caracterizadas como efetores do processo de morte celular programada via a ruptura da membrana vacuolar. Entender como esta via está integrada à estabilidade celular é essencial, justificando verificar as previstas e ainda não caracterizadas interações entre os componentes da via com outras proteínas, como AtCCR4a, uma deadenilase ativa tanto no processamento de RNAm quanto no metabolismo de sacarose/amido. Neste contexto, nós tivemos como objetivo investigar a interação entre AtNRP2 e AtCCR4a, e a sua função na ocorrência de estresses por alta temperatura e seca. Não foi observado o seu envolvimento direto com a via de sinalização mediada por proteínas NRP, mas *AtCCR4a* foi induzido por estresses osmótico e por alta temperatura. Além disso, a supressão de *AtCCR4a* promove um fenótipo drasticamente sensível ao estresse térmico, acarretando em reduções nos teores de pigmentos fotossintéticos dias após a exposição ao estresse e alterações negativas no metabolismo de carboidratos durante esta condição. Surpreendentemente, a supressão de *AtCCR4a* também promove um fenótipo mais tolerante ao estresse hídrico, contribuindo para maiores manutenção dos teores relativos de água em tecidos foliares e taxa de sobrevivência de plantas. Estes resultados indicam que AtCCR4a pode apresentar um papel antagônico em vias de resposta a estresses, provavelmente por regular a expressão de determinados genes a nível pós-transcricional e/ou selecionando RNAms-alvo específicos.

Palavras-chave: Abiótico. Água. Arabidopsis. Calor. Deadenilase.

SUMMARY

1. INTRODUCTION

The estimated world population will be approximately 10 billion individuals by the year 2050 (FAO, 2017). On a global scale, the accelerated population growth may generate considerable gaps between the current rate of food production with the future population demand, making the maintenance of food security uncertain in future scenarios (Godber and Wall, 2014).

In parallel, from the 2030s on, reduced productivity levels of crops may be observed as a result of the effects of climate changes. Such effects, as the occurrence of anomalous temperatures and/or changes in rainfall regimes, could impair the growth and development of crops, especially during their most critical stages, such as seedling establishment and flowering (Iizumi and Ramankutty, 2016; Ray et al., 2019).

Sub-Saharan African and Southeast Asian countries, as well as China and India, represent the most vulnerable group to the effects of climate changes (Knox et al., 2012; Thornton et al., 2014). It is estimated that around 1 billion people suffer from chronic hunger, with China and India containing more than 40% of the world's malnourished population (Sharma et al., 2016). This situation is alarming, since it is predicted that the Asian population will have reached its peak by the mid-2040s and the African population will continue to expand until the end of this century (FAO, 2017).

It is indispensable that actions are taken to boost food production. However, it is known that the increase in food production cannot be achieved only by the expansion of the agricultural frontiers around the world, for the sake of reducing greenhouse gas emissions and preserving several ecosystems (Hertel, 2015; Tyczewska et al., 2018; de Pinto et al., 2020). In this context, it is essential to develop cultivars that are not only more productive, but also more tolerant to pathogen and/or pest attacks, and also to climatic adversities, such as drought and more severe temperatures (Hatfield et al., 2011; Anderson et al., 2020).

In a biotechnological perspective, there are several molecular approaches that provide more tolerant plant materials through induced gene editing (Erpen-Dalla Corte et al., 2019; Bohra et al., 2020). There is the study of cell signaling pathways in plants, in which the objective is the identification, characterization, and selection of effector and/or regulatory genes of these pathways. Such strategy contributes to the elucidation of their functions in plant physiology and their contribution to the occurrence of phenotypes with desirable agronomic traits (Ahmad and Mukhtar, 2017; Georges and Ray, 2017).

Under water deficiency conditions, osmotic and/or endoplasmic reticulum (ER) stresses can be triggered in plants, which in turn can activate specific signaling pathways, such as the NRP-mediated cell death signaling pathway. Although it consists of a defense mechanism, the activation of this pathway can lead to the senescence of plant tissues, which can irreversibly result in the death of the plant (de Camargos et al., 2019). This is due to the activation of genes encoding hydrolytic proteins that break down the vacuolar membrane, leading to cell lysis (Mendes et al., 2013). Investigating the predicted protein-protein interactions among the components of this signaling pathway with other proteins becomes very relevant to unravel molecular mechanisms that can potentially collaborate to the generation of plant materials that are more tolerant to different stress conditions (Fucile et al., 2011)

In this work, we first investigated a predicted interaction of AtNRP2, a component of the NRP-mediated cell death signaling pathway, with AtCCR4a, a deadenylase active in mRNA processing and also involved in sucrose/starch metabolism in Arabidopsis (Suzuki et al., 2015; Reis et al., 2016). We also evaluated the induction of *AtCCR4a* in the occurrence of different abiotic stresses, which led us to investigate the phenotypic and metabolic responses of *ccr4a* knockout mutant plants subjected to heat stress as well as their phenotypic responses to water stress.

2. BIBLIOGRAPHIC REVIEW

2.1. N-rich protein (NRP)-mediated cell death signaling pathway

The NRP-mediated cell death signaling pathway was first characterized in soybean (*Glycine max*) (Irsigler et al., 2007; Costa et al., 2008). In this pathway, the transcription factor GmERD15 (*Glycine max* Early Responsive to Dehydration 15) is known to be induced by osmotic and/or endoplasmic reticulum (ER) stress. Upon activation, this factor binds to the promoter region of the protein-encoding genes that contain DCD/NRP domains (Alves et al., 2011b; Alves et al., 2011a). The DCD domain, involved in the processes of cell development and death, is located in the C-terminal region of these proteins and is apparently highly conserved in plants. In turn, the N-terminal portion contains the NRP domain, which is rich in residues of the amino acid asparagine (Tenhaken et al., 2005).

The NRP proteins described in soybean are NRP-A, NRP-B and NRP-C (Costa et al., 2008; Reis et al., 2016). In addition, the orthologous genes characterized in *Arabidopsis thaliana* are responsible for encoding the AtNRP1 and AtNRP2 proteins (Hoepflinger et al., 2011; Reis et al., 2016). Regarding *AtNRP2*, Reis *et al.* (2016) found differences in its induction kinetics when compared to *AtNRP1*, with the latter showing higher levels of induction after Arabidopsis seedlings were subjected to osmotic and ER stresses. The authors noted that the expression of *AtNRP2* depends on the prior activity of *AtNRP1*. Furthermore, the suppression of *AtNRP1* or *AtNRP2* expression resulted in the maintenance of chlorophyll contents in Arabidopsis seedlings submitted to osmotic and ER stresses. In parallel, more prominent levels of cell death were observed in *Atnrp1* knockout seedlings complemented with the gene itself and seedlings overexpressing *AtNRP2* when submitted to these same treatments. Taken together, these results indicate that *AtNRP2*, as well as *AtNRP1*, are effectors of the programmed cell death process.

Recently, contrary to the previous reports, Yang et al. (2021) reported that the cell death process can be dramatically induced by loss of function of both *AtNRP1* and *AtNRP2*. The authors observed that AtNRP1 and AtNRP2 may be modulators of the cell death process upon occurrence of ER stress. While AtNRP2 is constitutively expressed, AtNRP1 is activated by bZIP60, an unfolded protein response (UPR) key regulator. Both proteins apparently inhibit the transcription of cell death-related genes. Such controversy demonstrates that more detailed studies will be needed to understand how the NRP-mediated cell death signaling pathway is integrated into cellular balance in the face of environmental stresses.

The high level of expression of NRP proteins triggers a signaling cascade that induces genes of the NAC superfamily (NAM, ATAF and CUC), such as GmNAC030, GmNAC081 and GmNAC085 in soybean and ANAC036 in Arabidopsis (Mendes et al., 2013; Pimenta et al., 2016; Melo et al., 2021). The induction of these genes triggers the process of programmed cell death (PCD) through the expression of genes encoding hydrolytic enzymes, most notably the vacuolar processing enzyme (VPE). As its name implies, this enzyme causes the breakdown of the tonoplast in plant cells (Hara-Nishimura et al., 2005; Hatsugai et al., 2015).

It is also known that this pathway is negatively modulated by BiP proteins, classified as molecular chaperones (Reis et al., 2011; Carvalho et al., 2014b). BiP proteins assist other proteins to obtain their proper folding and to keep inactive transcription factors, located in the

ER membrane, that respond to stress events (Pobre et al., 2019). It was observed that its induced overexpression in Arabidopsis, soybean and tobacco (*Nicotiana tabacum*) plants confers a higher level of tolerance to water deficit. These plants showed a higher survival rate and lower degree of leaf wilting compared to the control treatment (Alvim et al., 2001; Valente et al., 2009; Carvalho et al., 2014a; Reis et al., 2016; Coutinho et al., 2019).

2.2. AtCCR4a, AtCCR4b and other CAF1-CCR4-NOT complex proteins

Carbon catabolite repressor 4 (CCR4) proteins are part of the CAF1-CCR4-NOT complex, which is located in cytosolic mRNA processing bodies (P-bodies) and apparently highly conserved among eukaryotic organisms. The process of deadenylation (degradation) of mRNA molecules by this complex is performed by cleavage of the poly(A) tail from a free 3'-OH end (3'-5'-exoribonuclease activity) and it has already been described in yeasts, insects, and humans (Tucker et al., 2001; Temme et al., 2004; Mittal et al., 2011). The structure of this complex consists of 7 subunits: two 3'-5'-exoribonucleases, CAF1 and CCR4; the NOT1 protein, which probably functions as the scaffold protein of the complex; and the NOT2, NOT3, NOT4 and NOT5 proteins, which interact with NOT1, alongside CAF1 (Basquin et al., 2012).

The first studies investigating CAF1 functions in plants indicate the role of these genes in mediating biotic stress responses (Sarowar et al., 2007). Single and double mutants of *AtCAF1a* and *AtCAF1b* showed to be more susceptible to infection of *Pseudomonas syringae* pv *tomato* DC3000 (Pst DC3000), whereas over-expressing *AtCAF1a* plants showed an increased resistance (Liang *et al.*, 2009). The authors proposed that AtCAF1 proteins promote the degradation of specific mRNAs, which repress the transcription of certain genes, like the pathogenesis-related (PR) genes *PR1* and *PR2*. Walley *et al.* (2010) verified that *AtCAF1a* and *AtCAF1b* are also involved in mediating abiotic stress responses. *Atcaf1a* seedlings showed to be more tolerant to salt treatment compared with the wild-type and *Atcaf1b* seedlings, indicating that these proteins are not functionally redundant in all cases.

Arae *et al.* (2019) evaluated the interaction of AtCCR4a or AtCCR4b with the AtCAF1 protein isoforms. Through yeast two-hybrid assays, 4 isoforms of AtCAF1 (h/i/j/k) showed interaction with AtCCR4b, while the tested AtCAF1 isoforms did not show significant interaction with AtCCR4a. AtCAF1a/b showed no interaction with both AtCCR4 isoforms. This allowed the authors to conclude that there is probably a different version of the complex in which AtCAF1 functions without interacting with AtCCR4 isoforms. Similarly in rice (*Oryza sativa*), OsCAF1b also does not interact with OsAtCCR4a/b (Chou *et al.*, 2017).

Arae *et al.* (2019) also reported that AtCCR4a and AtCCR4b interact with the APUM2 and APUM5 proteins, most likely in the P-bodies. The APUM proteins belong to the Pumilio RNA-binding protein family (PUF family), which is characterized by binding specifically to their mRNA targets. Once bound, they interact with other proteins to inhibit translation or activate mRNA deadenylation (Wang et al., 2018; Joshna et al., 2020).

APUM5 is involved in a defense mechanism by binding directly with *Cucumber mosaic virus* (CMV) RNAs, impairing its replication and consequently attenuating the viral infection in the host plant (Huh et al., 2013; Un Huh and Paek, 2013). In addition, Huh and Paek (2014) verified that the expression of *APUM5* was up-regulated in 10-day-old Arabidopsis seedlings exposed to abiotic stressors such as mannitol, salt and abscisic acid (ABA). Interestingly, the authors also observed that *35S-APUM5* plants showed a hypersensitive phenotype to salinity and drought stress in soil, while the *APUM5*-RNAi plants showed to be more tolerant to water losses compared to the wild-type.

AtCCR4a and AtCCR4b are also involved in sucrose/starch metabolism (Suzuki et al., 2015). In this study, double mutant lines were generated, which were subjected to a high sucrose concentration condition (200 mM), which is harmful for seedling establishment. The double mutant showed better growth and development compared to wild-type and single knockout seedlings. In this condition, double mutant seedlings also showed higher sucrose and starch contents, mainly of the amylose fraction. A higher accumulation of GBSS1 (Granule Bound Starch Synthase 1) transcripts, a protein involved in amylose biosynthesis, was observed in double mutant seedlings compared to the wild-type. Furthermore, it was evidenced that the transcripts in these seedlings presented poly(A) tails with greater lengths, suggesting that AtCCR4a and AtCCR4b together may be involved with the modulation of the poly(A) tail of transcripts involved in starch biosynthesis.

3. MATERIAL AND METHODS

3.1. Plasmid Constructs

All recombinant constructs were generated from the Gateway® cloning system (Thermo Fisher Scientific™). Primers used for the amplification of genes, described in this study, are listed at the Supplemental Table 1 (Table S1). The open reading frame (ORF) of *AtCCR4a* (AT3G58560) was amplified from cDNA from leaves of *Arabidopsis thaliana* ecotype Columbia (Col-0) and inserted into the entry vector pDONR207. The resulting clone was named AtCCR4a-pDONR207 (pUFV3150). Subsequently, the inserted DNA sequence was transferred through recombination from the entry vector to the expression vector in plants, pEarleyGate 103, under control of the 2x35S promoter of CaMV, and which also allows the fusion of AtCCR4a to GFP. The resulting construct, AtCCR4a-pEarleyGate103 (pUFV3369) was used for stable plant transformation.

The expression vectors SPYCE-GW and SPYNE-GW, which contain the C-terminal (cYFP) or N-terminal (nYFP) region of YFP, respectively, were used in BiFC assays. AtCCR4a-pDONR207 was transferred to SPYCE-GW and SPYNE-GW, resulting in AtCCR4a-SPYCE (pUFV3372) and AtCCR4a-SPYNE (pUFV3373) clones, respectively.

3.2. Plant Material and Growth Conditions

Arabidopsis thaliana, Columbia (Col-0) ecotype, was used as wild-type (WT). T-DNA insertion mutant seeds *ccr4a* (SALK_144072) were obtained through the Arabidopsis Biological Resource Center. Complemented AtCCR4a plants were obtained by transformation via *Agrobacterium tumefaciens* via the floral dipping method (Bent, 2006). For this, *A. tumefaciens* strain GV3101 was transformed with the DNA construct (AtCCR4a-pEarleyGate103; pUFV3369) by electroporation and the transformed colonies were confirmed by PCR using the primers MC36 and AtCCR4a-RVS (Table S1). T1 seeds were put to germinate in commercial substrate (MEC PLANT®), under photoperiod of 8h/16h light/dark at 22°C (Osram® tube light T8 18W). Transforming seedlings were selected by performing successive sprays of FINALE® (glufosinate-ammonium; 120 μg mL^{-1}) for 7 days. Then, the selected seedlings were transferred to individual pots. The resulting plants were genotyped by PCR using the primers MC36 and AtCCR4a-RVS (Table S1).

3.3. RNA extraction and cDNA synthesis

The extraction procedure of Arabidopsis RNA samples was performed using the Trizol reagent (Invitrogen™), followed by precipitation with isopropanol. The integrity of the extracted samples was verified by 1% agarose gel electrophoresis analysis, with ethidium bromide 0.1 mg mL^{-1}. Subsequently, the quantification of the samples was estimated using a

BioSpec-nano spectrophotometer (Shimadzu™). Approximately 4 µg of RNA were treated with DNase I, RNase-free (Thermo Fisher Scientific™) and then used for cDNA synthesis, using 1U of M-MLV Reverse Transcriptase (Thermo Fisher Scientific™) and Oligo (dT)18 primer.

3.4. RT-qPCR analyses

RT-qPCR were performed in a 7500 Real-Time PCR Systems thermocycler (Thermo Fisher Scientific™). The reaction mix consisted of 20 ng of cDNA, 0,6 µM of each respective primer and SYBER™ Green PCR Master Mix (Thermo Fisher Scientific™), totaling 10 µL. The stages of the reactions were: 10 min at 94°C, followed by 40 cycles (15 s at 94°C and 1 min at 60°C) and finally the dissociation step. The expression of target genes was normalized compared to the expression of the endogenous *AtActin2* gene (At3g18780) and quantified by the method and $2^{-\Delta\Delta CT}$ for relative quantification. Primer sequences are found in Table S1.

3.5. Protein extraction and western blotting

For the characterization of the complemented lines (*ccr4a*/35S:AtCCR4a-GFP), seeds were first surface-sterilized and then put on plates containing half-strength MS medium (Murashige and Skoog, 1962). After 3 days in the dark at 4°C, the plates were placed under a 16h/8h light/dark photoperiod. After 7 days, seedlings were collected and frozen in liquid nitrogen. The pulverized material was added 1 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 1% IGEPAL CA-630, 1 mM PMSF and 2 mM benzamidine) to obtain the protein extract. Subsequently, the following steps were executed: addition of the elution buffer, previously heated to 95°C, to the protein extracts; separation of the proteins by polyacrylamide gel electrophoresis under denaturing conditions, containing 10% sodium dodecyl sulfate (SDS-PAGE); transfer of the proteins onto nitrocellulose membrane, which was blocked with TBS-T plus 5% skimmed milk powder and probed with anti-GFP antibody. The Clarity Western ECL® kit was used to visualize the resulting bands via the ChemiDoc® photodocumentator (Bio-Rad™).

3.6. Biomolecular fluorescence complementation (BiFC)

To verify the interaction between the AtCCR4a and AtNRP2 proteins, the AtNRP2-SPYCE (pUFV3185) and AtNRP2-SPYNE (pUFV3186) clones were also used. Different combinations of *A. tumefaciens* strain GV3101 expressing the indicated NYFP/CYFP-fused proteins were co-infiltrated into the abaxial surface of *Nicotiana benthamiana* leaves at an OD_{600nm} ratio 1:1 for infiltration. Fluorescence was analyzed in epidermal cells 3 days after infiltration using a Zeiss inverted LSM510 META laser scanning microscope equipped with an argon/helium laser as excitation source. YFP was excited at 514 nm using an argon laser, and YFP emission was detected using a 560-615 nm filter. The images were captured and processed with the Zeiss LSM Image Browser 4 software. Also, to determine whether the interaction between the respective proteins is necessarily caused by the presence of some stress inducer, in the first assay, tunicamycin (10 µg mL^{-1}) infiltration was performed on the abaxial surface of the co-infiltrated leaves 8 hours before the fluorescence analysis. In the second assay, the AtMBD2 and AtWWP1 proteins were coexpressed together as a positive control (Calil, 2017).

3.7. Osmotic and Endoplasmic Reticulum Stress Assays

For the osmotic stress assay with mannitol, seeds were first surface-sterilized and then put on plates containing standard half-strength MS medium or containing 200 mM mannitol (Murashige and Skoog, 1962). After 3 days in the dark at 4°C, the plates were placed at an incline of approximately 45° under a 16h/8h light/dark photoperiod. After 7 days, seedlings were collected and the length of their roots was measured using a digital caliper.

For the osmotic stress assay with polyethylene glycol (PEG: MW 8000; Sigma-Aldrich®) and endoplasmic reticulum (ER) stress assay with tunicamycin (TM; Sigma-Aldrich), seeds were first surface-sterilized and then put on plates containing half-strength MS medium (Murashige and Skoog, 1962). After 3 days in the dark at 4°C, the plates were put under a 16h/8h light/dark photoperiod. After 14 days, seedlings were collected and placed in 12-well plates containing distilled water. After one day of acclimation, 10% PEG (w/v) or 2.5 µg mL^{-1} TM treatments were applied. H_2O was used as control for PEG and DMSO for TM. For each treatment, 3 replicates consisting of 10 seedlings were used.

3.8. Drought Tolerance Assays

For the drought tolerance assays, seeds were germinated in substrate under photoperiod of 8h/16h light/dark at 22°C. After 15 days from sowing, the seedlings were transferred to individual pots and grown under photoperiod of 8h/16h light/dark at 22°C. The individual amount of substrate in each pot was previously weighed using a digital balance (Ke Home SF-

400). For 4 weeks, the plants were irrigated for 2 times weekly. Then, the irrigation was stopped for 15 days. During this period, the pots were randomly distributed on the trays. In addition, the water status of the plants was evaluated by collecting fully expanded leaves, adopting the relative turgidity technique (Barrs and Weatherley, 1962). Thus, the following equation was used: RT = [(FW-DW)/(TW-DW)] x 100, where RT = Relative Turgidity (%); FW = Initial Fresh Weight (g); DW = Dry Weight (g); TW = Turgid Fresh Weight (g). Leaf collection was performed on days 0, 7, 10, 13 and 15 of water restriction treatment. On day 15 of water restriction, all plants were rehydrated. After 3 days, the survival rate (%) was estimated by the following equation: (Quantity of surviving plants/Total quantity of plants) x 100.

3.9. Heat Stress Assays

For all heat stress assays performed in this study, the temperature of 38°C was established, due to the fact that the 37-42°C range is extensively used in heat stress studies in Arabidopsis (Wang et al., 2020).

For the first and second heat stress assays, seeds were put to germinate in substrate under photoperiod of 8h/16h light/dark at 22°C. After 15 days from sowing, the seedlings were transferred to individual pots and grown under photoperiod of 8h/16h light/dark at 22°C. In the first assay, 6-week-old plants were transferred to a pre-heated BOD at 38°C for 18 h. Leaf samples were collected at times of 0, 3, 6 and 18 h of heat stress treatment for gene expression analyses. In the second assay, 6-week-old plants were transferred to a pre-heated BOD at 38°C for 24 h. Then, they were transferred to the normal growth conditions and kept for 14 days. Rosette samples were collected for metabolic analyses at times of 0 (one hour after the light period began), 1, 6, 12 and 24 h of heat stress treatment and 24 h after the plants had returned to normal growth conditions.

In the third assay, the plants were grown under the photoperiod of 16h/8h light/dark at 22°C. 6-week-old plants were transferred to a pre-heated BOD at 38°C for 24 h. Then, they were transferred to the normal growth conditions and kept for 14 days. Rosette samples were collected for metabolic analyses on days 0, 3, 7 and 14 after the heat stress treatment, one hour after the light period began.

3.10. Metabolic analyses

The extraction of chlorophylls, glucose, fructose, sucrose, starch and total free amino acids was performed according to according to Lisec *et al.* (2006) with minor changes. Shortly, approximately 10 mg of lyophilized material was subjected to extraction with 700 µL of 100% methanol, being heated at 80°C for 20 min under 750 rpm agitation and then centrifuged at 16840 *g* for 15 min at 4°C. The pellet generated was used for extraction of starch, while the supernatant was used to quantify photosynthetic pigments, soluble sugars, organic acids, total free amino acids and proline. The supernatant was then transferred to another microtube, and pigment quantification (chlorophylls and carotenoids) was then proceeded, and to the remaining supernatant, 375 µL of chloroform and 750 µL of ultrapure water were sequentially added.

The material was then centrifuged for 10 min at 16840 x g at 4°C, resulting in two very characteristic phases: an aqueous upper polar phase and a lower non-polar phase. The aqueous phase, containing the metabolites of interest was then collected and transferred to another microtube for further analysis, and the organic phase was discarded. The pellet was washed with 1 mL of 70% ethanol, treated with 0.1 M NaOH and heated for 60 min at 95°C for protein extraction and then neutralized with 1M acetic acid for starch quantification.

The quantification of these metabolites was performed according to the following references: pigments (Wellburn, 1994); the contents of glucose, fructose, sucrose and starch (Fernie et al., 2001); total free amino acids (Yemm et al., 1955); proline (Sithtisarn et al., 2009); and the contents of malate and fumarate (Nunes-Nesi et al., 2007).

4. RESULTS

4.1. AtCCR4a apparently does not interact with AtNRP2

Many components of the NRP-mediated cell death signaling pathway have not yet been characterized or discovered. Thus, protein-protein interactions among the components of this pathway with other proteins need to be evidenced, allowing us to better understand how this pathway is integrated into cellular metabolism under stress episodes. By checking the network of interactions derived from the interactome of Arabidopsis, a possible interaction between the AtNRP2 protein, a component of the NRP-mediated cell death pathway, and the AtCCR4a protein is assumed (Fucile et al., 2011).

Accordingly, we aimed to confirm the predicted interaction between these two proteins. However, we could not verify it by BiFC assays, through transient expression in tobacco (*N. benthamiana*) leaves (Fig 1 and 2). In the first BIFC assay, tunicamycin (10 µg mL^{-1}) was infiltrated into the previously agroinfiltrated leaves 8 hours before fluorescence analysis was performed. This was done in order to verify whether the interaction between AtCCR4a and AtNRP2 necessarily depends on the presence of some stress inducer to occur (Fig. 1). Although tunicamycin infiltration was not performed, we emphasize that in the second BIFC assay, we used the interaction between AtMDB2 and AtWWP1, previously verified by Calil (2017), as a positive control (Fig. 2).

Though we have not been able to prove that AtCCR4a and AtNRP2 interact in the cytosol of plant cells, we do not discard the possibility that this interaction occurs *in vivo*. We emphasize the need to perform yeast two-hybrid assays and other protein-protein interaction assays or even BIFC assays with shorter time gaps between infiltrating tunicamycin or another stress inducer and performing fluorescence analysis.

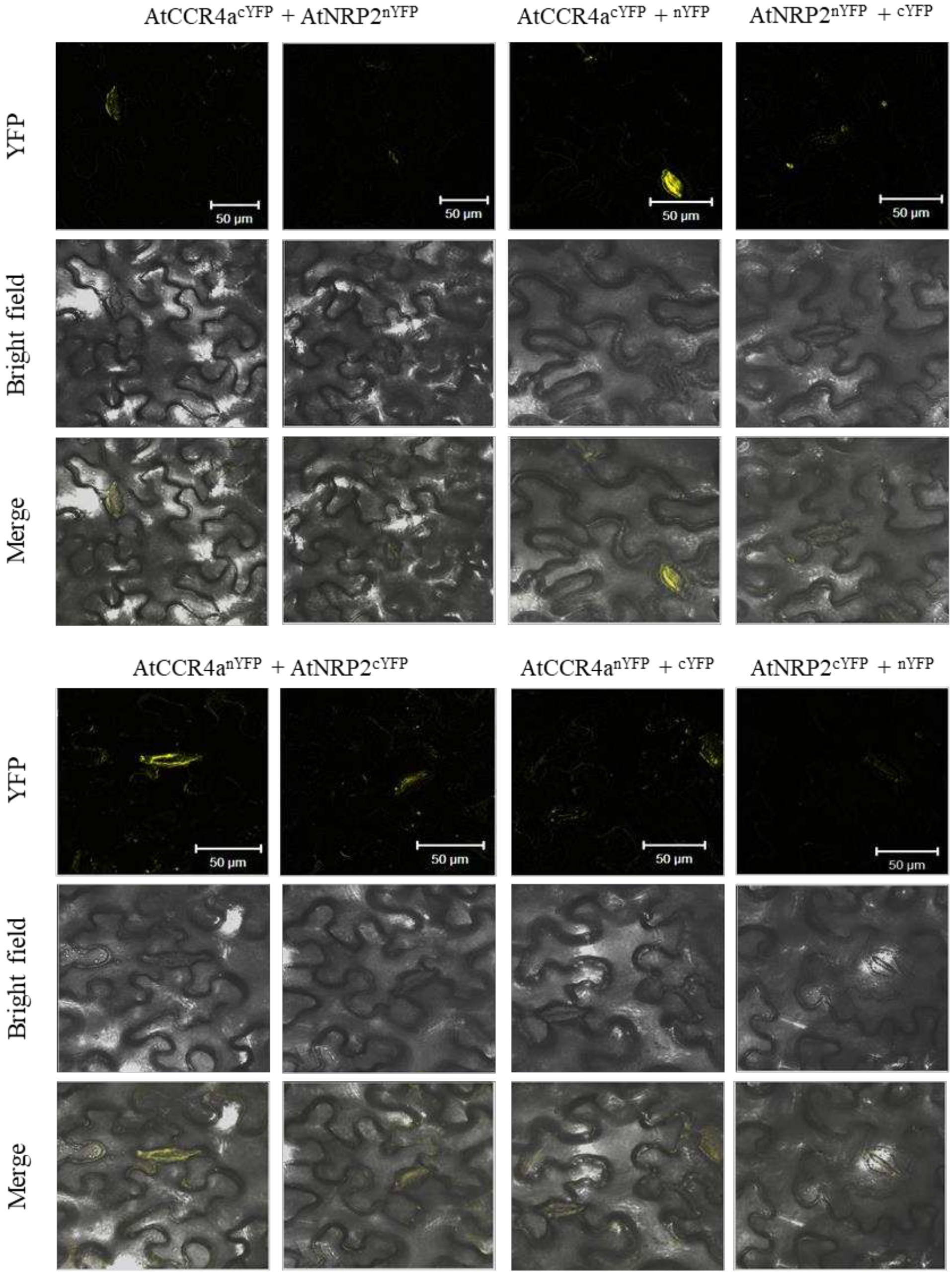

Figure 1. Interaction analysis with BiFC. Similar images were obtained in biological replicates 3 days after agroinfiltration. AtCCR4a tagged with the C-terminal fragment of YFP (cYFP) and AtNRP2 tagged with the N-terminal fragment of YFP (nYFP) and vice versa were coexpressed together in tobacco leaves by agroinfiltration. Tunicamycin (10 µg mL^{-1}) infiltration was performed 8 hours before analysis on the previously agroinfiltrated tobacco leaves.

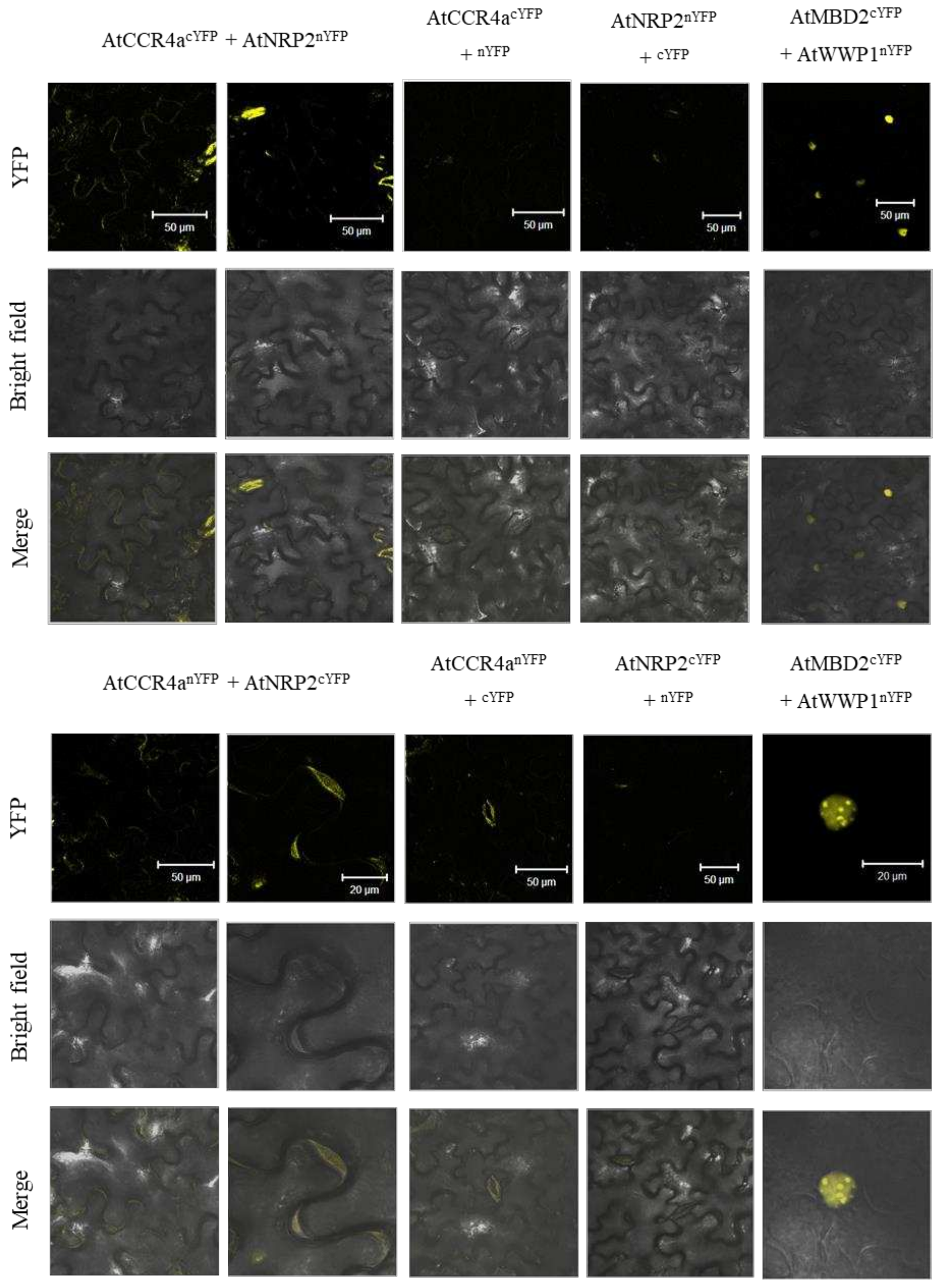

Figure 2. Interaction analysis with BiFC. Similar images were obtained in biological replicates 3 days after agroinfiltration. AtCCR4a tagged with the C-terminal fragment of YFP (cYFP) with AtNRP2 tagged with the N-terminal fragment of YFP (nYFP) and vice versa were coexpressed together in tobacco leaves by agroinfiltration. Coexpression of AtMDB2 tagged with the C-terminal fragment of YFP (cYFP) with AtWWP1 tagged with the N-terminal fragment of YFP (nYFP) was used as a positive control for transfection.

4.2. *AtCCR4a* is induced by osmotic and heat stresses

Due to the fact that other components of the NOT1-CCR4-NOT complex have already been characterized as responsive to certain abiotic stresses, we decided to check the expression of *AtCCR4a* in wild-type seedlings subjected to osmotic or ER stresses, which are also the main types of stress that lead to activation of NRP-mediated cell death signaling pathway (Walley et al., 2010; Huh and Paek, 2014; Reis et al., 2016). An increasing in the relative expression of *AtCCR4a* was observed in 14-day-old seedlings after they had been subjected to osmotic stress for 3 hours (Fig. 3A). In the same period, however, we did not observe changes in *AtCCR4a* expression in seedlings subjected to ER stress (Fig. 3B). For such analyses, we also checked the relative expression level of stress marker genes, such as *AtRAB18* and *AtCNX1* for osmotic and ER stresses, respectively. Both marker genes had their expression increased at the treatment times evaluated (Fig. 3A and B).

Because *AtCCR4a* was induced in the occurrence of osmotic stress and *nrp1* seedlings previously showed a sensitive phenotype under this condition, characterized by the occurrence of shorter root lengths, we decided to evaluate the growth of *ccr4a* seedlings under the same condition (Hoepflinger et al., 2011; Reis et al., 2016). We did not observe any difference in growth between the 7-day-old *ccr4a* seedlings compared to the wild-type when grown on medium containing 200 mM mannitol (Fig. S1). Thus, the induction of *AtCCR4a* by osmotic stress supports the possibility that *AtCCR4a* may at least be induced by some component of the NRP-mediated cell death signaling pathway. We emphasize that although we did not observe *AtCCR4a* being induced in the presence of tunicamycin (TM), we do not rule out the possibility of it being induced at intervals longer than 3 hours in the occurrence of ER stress, which has already been observed with *AtNRP1* and *AtNRP2* genes (Reis et al., 2016).

Relative expression of *AtCCR4a* was also checked in 6-week-old wild-type plants exposed to high temperature (38°C) by the fact that *AtCAF1b* had its expression increased in 7-day-old seedlings subjected to heat stress (Sun et al., 2020). We could observe an increasing at the 3-hour exposure time. The effect of heat stress treatment was validated by checking the expression of *AtHSP70*, another stress marker gene, which was markedly induced at 1 and 3 hours of high temperature exposure (Fig. 3C).

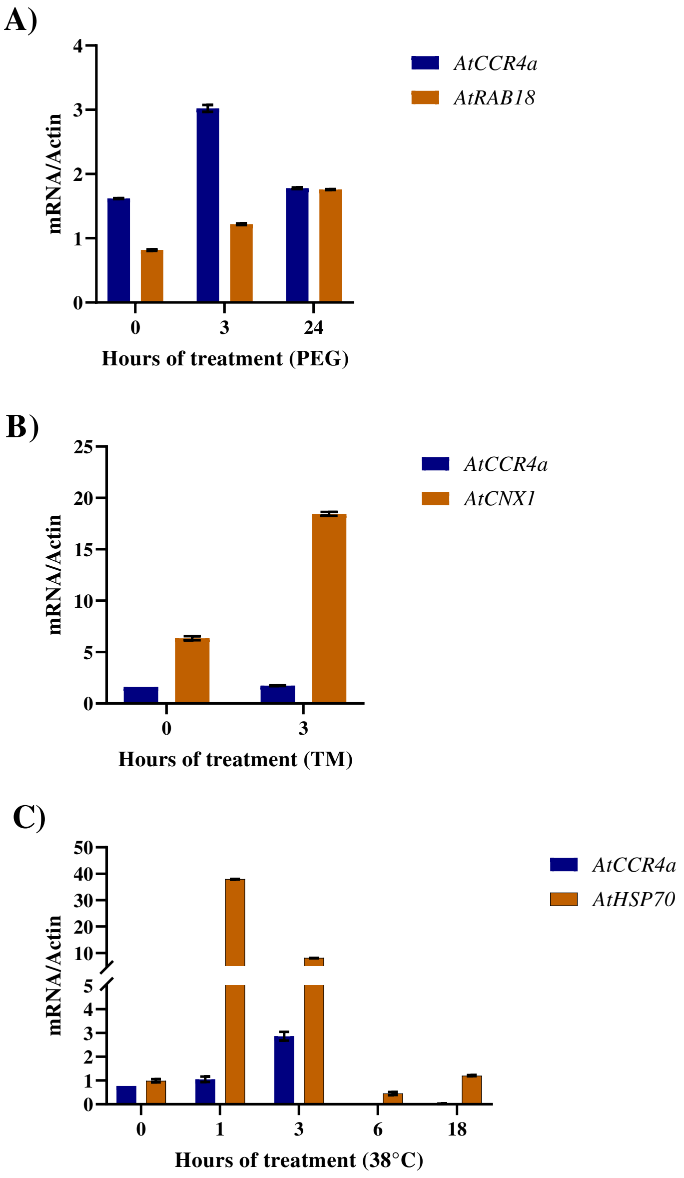

Figure 3. Transcript accumulation of *AtCCR4* and stress marker genes (*AtRAB18*, *AtCNX1* and *AtHSP70*) in 14-day-old seedlings under osmotic (A; 10% PEG) or ER (B; 2.5 µg mL^{-1} TM) stress and in leaves of 6-week-old plants under heat stress (C; 38°C for 18h). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, and *AtActin2* was used as endogenous control. Error bars represent 95% confidence interval based on three biological replicates. PEG (polyethylene glycol 8000). TM (tunicamycin).

4.3. Phenotypic and metabolic evaluations of *ccr4a* plants during and after heat stress

We next decided to evaluate the phenotypic response of *ccr4a* plants to a heat stress event. The metabolic response of *ccr4a* plants during a 24-hour treatment period was also verified, as a result of AtCCR4a and AtCCR4b being involved in sucrose/starch metabolism (Suzuki et al., 2015). After 14 days of the 24-hour heat stress treatment (38°C), the *ccr4a* plants were shown to be drastically sensitive compared to the wild-type (Fig. 4).

At the end of the heat stress treatment, a reduction in chlorophyll *a* and *b* contents, and consequently in total chlorophylls content can be observed in the *ccr4a* plants compared to the wild-type in the control condition (Fig. 5A, B and C). The same was observed for carotenoid contents (Fig. 5D). This indicates that the photosynthetic performance of *ccr4a* plants may have been impaired at the end of the heat stress, due to reduced pigment contents.

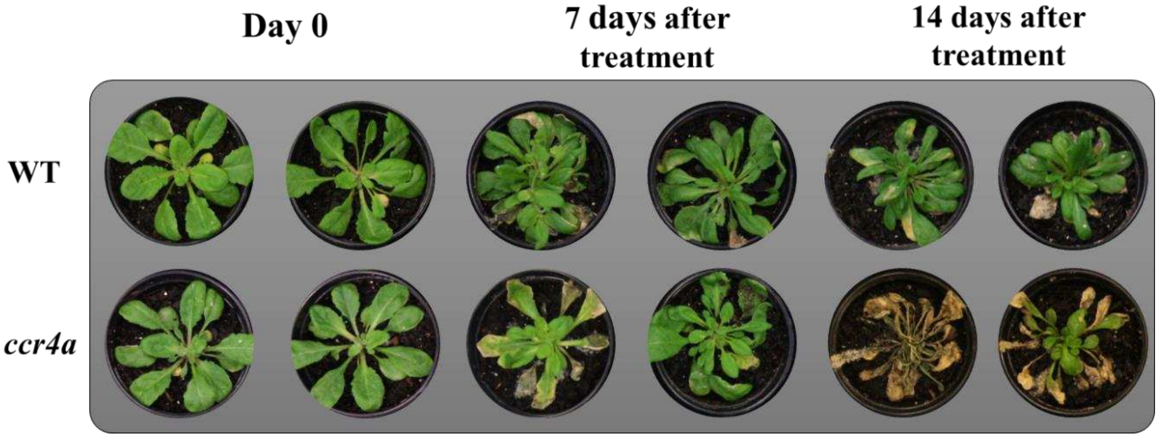

Figure 4. Phenotypic evaluation of 6-week-old wild-type (WT) and *ccr4a* plants 7 and 14 days after 24 h of heat stress treatment (38°C).

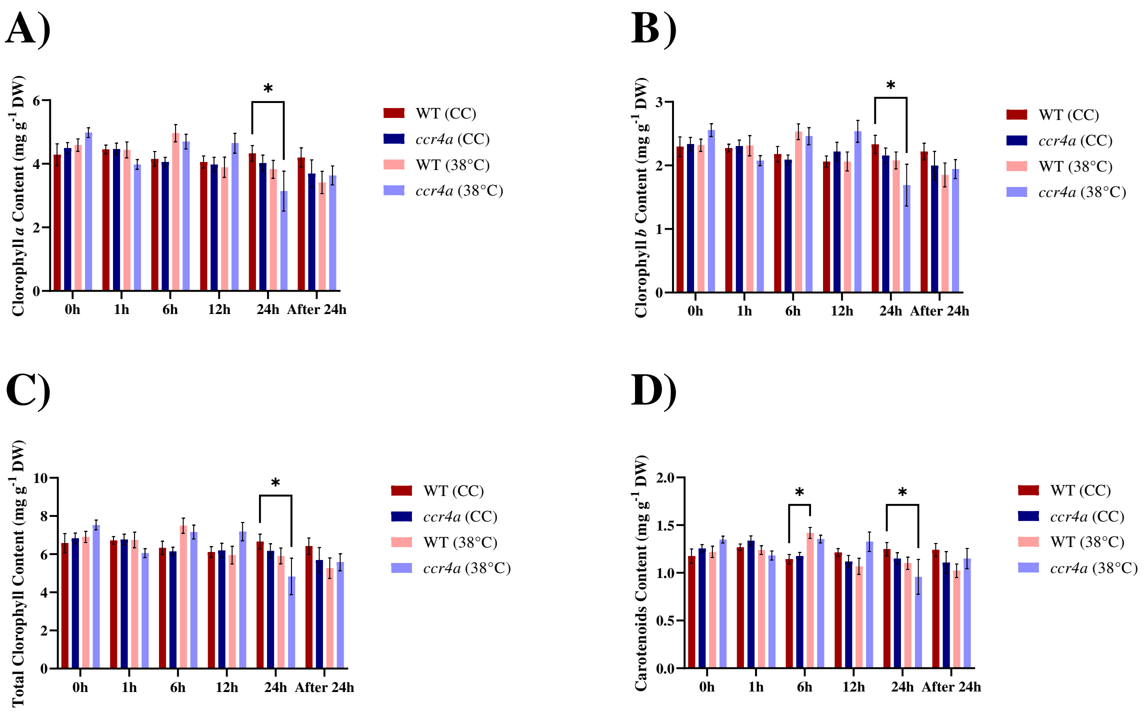

Figure 5. Contents of photosynthetic pigments (n = 5) in rosettes from 6-week-old wild-type (WT) and *ccr4a* plants during and after 24 h of the 24-hour heat stress treatment (38°C) or control condition (CC). A, chlorophyll *a* content. B, chlorophyll *b* content. C, total chlorophyll (*a* + *b*) content. D, carotenoids content. Error bars indicate SE. Significant differences were determined by Tukey's test (* $P < 0.05$). DW, dry weight.

Regarding carbohydrate metabolism, sucrose contents were drastically reduced in both wild-type and *ccr4a* plants during the treatment period (Fig. 6C). Under control conditions, an increase in starch contents was observed at 6 and 12 hours of treatment, due to the circadian rhythm of the plants, that is, by the biosynthesis and accumulation of starch throughout the day by the plants (Streb and Zeeman, 2012). However, this pattern was not observed in plants exposed to high temperature, which demonstrates that heat stress affected their main metabolism (Fig. 6D). Additionally, *ccr4a* plants exposed to high temperature showed lower starch contents compared to *ccr4a* plants in the control condition at the one-hour time point. At the end of the heat stress treatment, the same was observed (Fig. 6D).

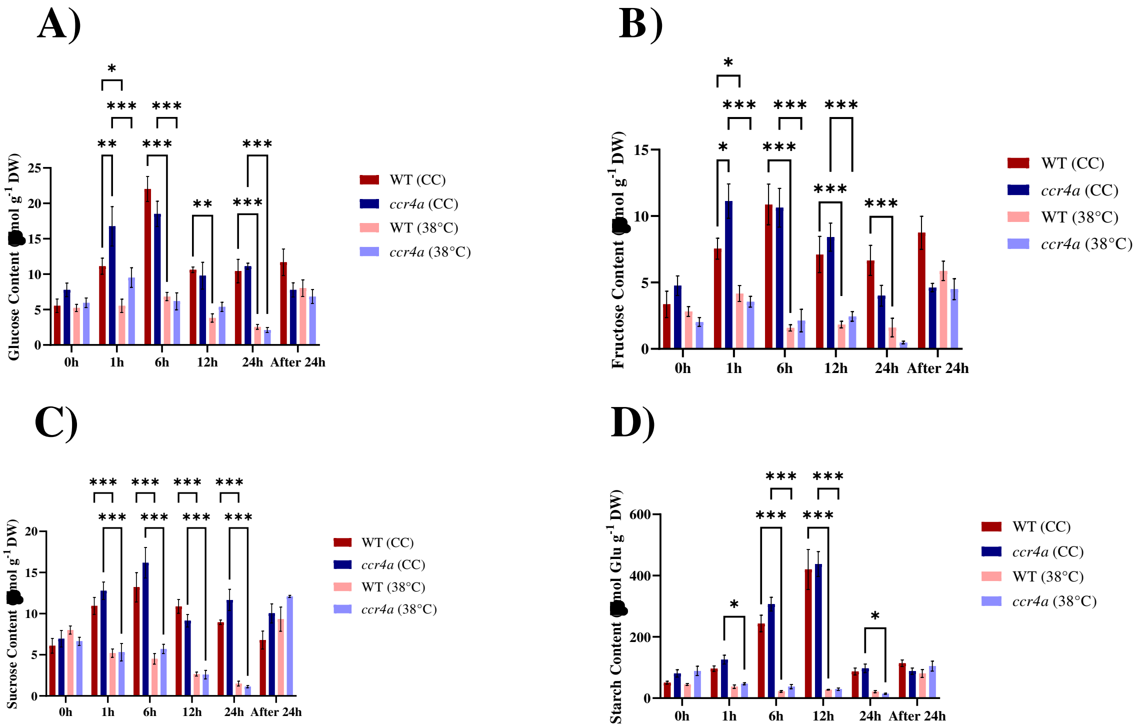

Figure 6. Contents of soluble sugars, sucrose and starch (n = 5) in rosettes from 6-week-old wild-type (WT) and *ccr4a* plants during and after 24 h of the 24-hour heat stress treatment (38°C) or control condition (CC). A, glucose content. B, fructose content. C, sucrose content. D, starch content. Error bars indicate SE. Significant differences were determined by Tukey's test (* P < 0.05; ** P < 0.01; ***P < 0.001). DW, dry weight.

Intriguingly, *ccr4a* plants showed higher glucose and fructose contents compared to the wild-type in the control condition at the one-hour time point. This difference was not observed at later analysis times (Fig. 6A and B). In addition, during the treatment period, reduced glucose and fructose contents were observed in both wild-type and *ccr4a* plants (Fig. 6A and B).

Wild-type and *ccr4a* plants from 12 hours exposed to heat treatment and during their recovery time showed higher total free amino acid contents (Fig. 7A). Notably, both genotypes also showed higher proline contents at 12 hours of treatment (Fig. 7B). These results indicate that plant metabolism has been redirected to cope with this stressful condition. Increases in the levels of amino acids, especially proline, have been observed in plants exposed to abiotic stresses, as these compounds act as osmoprotectants and are involved in the regulation of crucial processes, such as gene expression and redox-homeostasis (Rai, 2002; Batista-Silva et al., 2019).

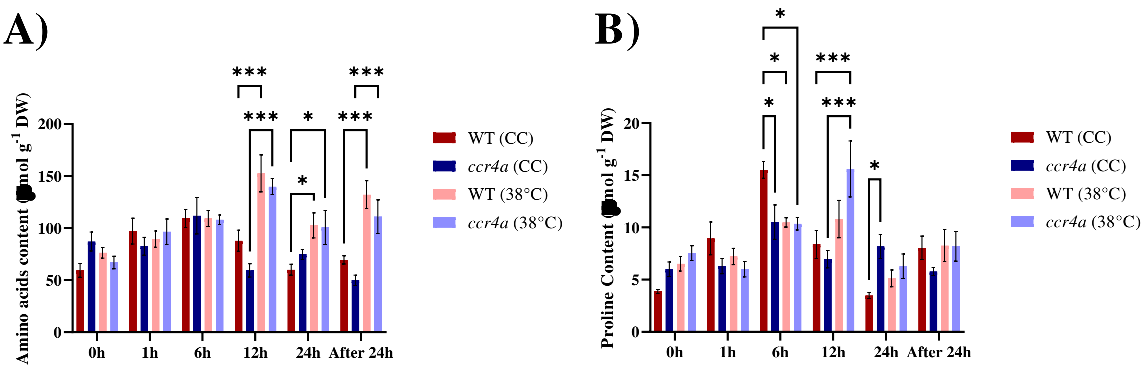

Figure 7. Contents of total free amino acids and proline (n = 5) in rosettes from 6-week-old wild-type (WT) and *ccr4a* plants during and after 24h of the 24-hour heat stress treatment (38°C) or control condition (CC). A, amino acids content. B, proline content. Error bars indicate SE. Significant differences were determined by Tukey's test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). DW, dry weight.

In the second heat stress experiment, we took advantage of the introduction of the complemented *ccr4a*-AtCCR4a lines (Fig. 8) to investigate whether the expression of AtCCR4a could rescue the wild-type phenotype when faced with a heat stress episode. In addition, we decided to metabolically evaluate the plants days after submission to the treatment to investigate whether significative changes in carbohydrates, soluble sugars and especially photosynthetic pigments contents would occur among the tested genotypes.

Similar to the first experiment, the *ccr4a* plants showed a more sensitive phenotype to heat stress compared to the wild-type and also to the complemented lines. The latter showed similar phenotypes to the wild-type, demonstrating that *AtCCR4a* complementation restores the wild-type phenotype to heat stress (Fig. 9).

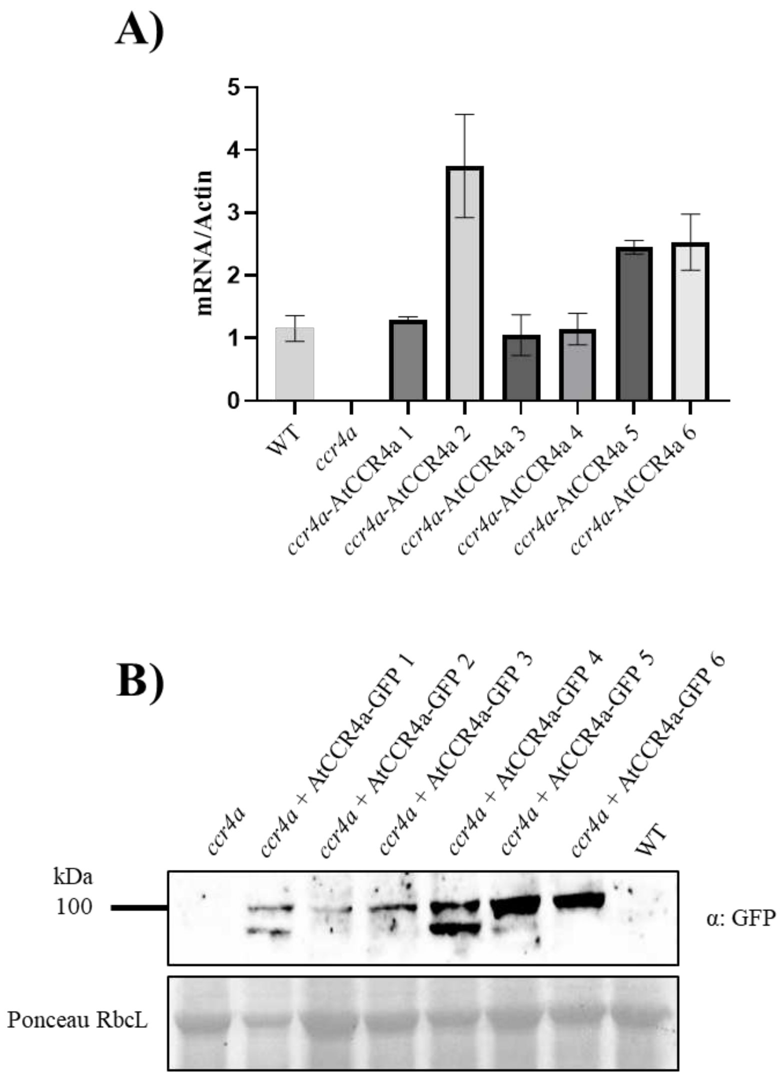

Figure 8. Characterization of independently complemented lines (*ccr4a*/35S:AtCCR4a-GFP). A) Transcript accumulation of *AtCCR4a* in complemented lines. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, and *AtActin2* was used as endogenous control. Error bars represent 95% confidence interval based on three biological replicates. B) AtCCR4a-GFP protein levels of complemented lines. Total protein extracts from complemented lines were separated by SDS-PAGE and immunodetected using anti-GFP antibody. WT, wild-type.

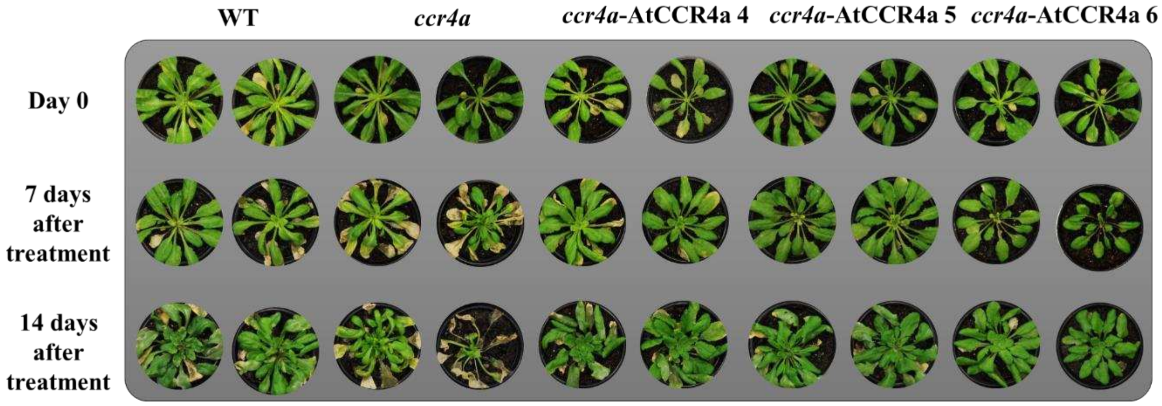

Figure 9. Phenotypic evaluation of 6-week-old wild-type (WT), *ccr4a* and *ccr4a*-AtCCR4a plants 7 and 14 days after 24 h of heat stress treatment (38°C).

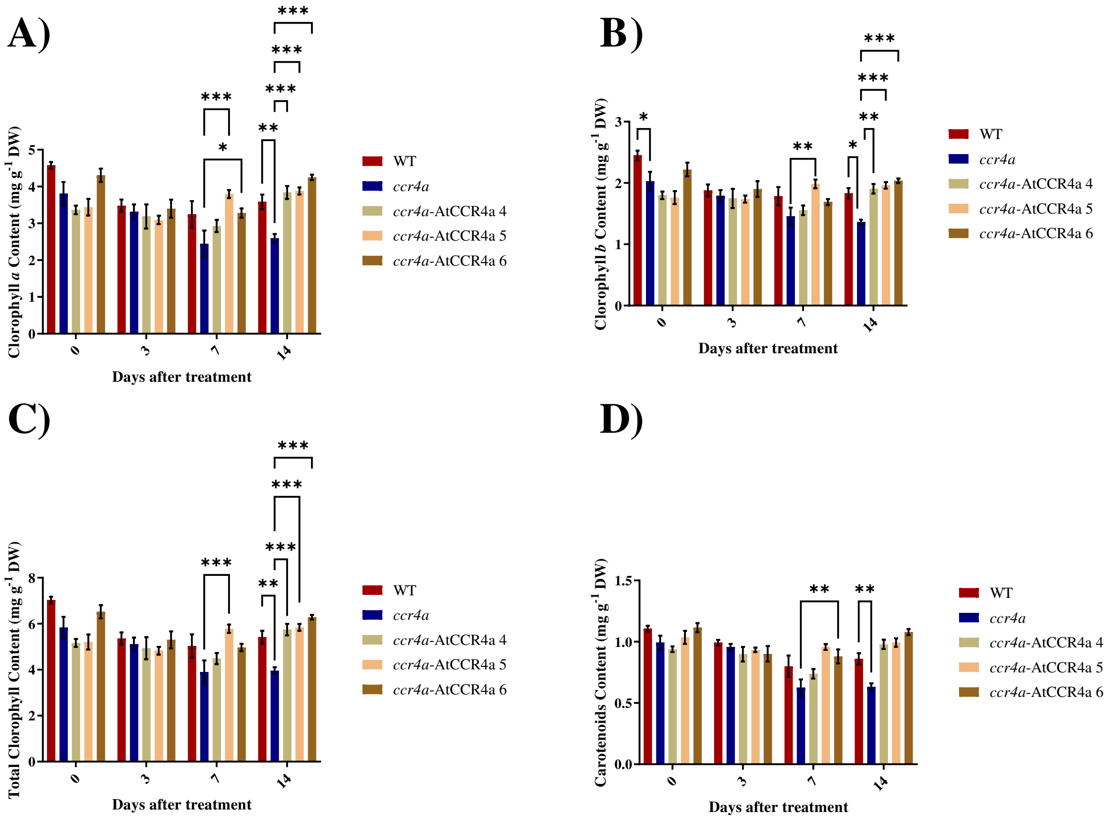

Figure 10. Contents of photosynthetic pigments (n = 5) in rosettes from 6-week-old wild-type (WT), *ccr4a* and *ccr4a*-AtCCR4a plants at 0, 3, 7 and 14 days after the 24-hour heat stress treatment (38°C). A, chlorophyll *a* content. B, chlorophyll *b* content. C, total chlorophyll (*a* + *b*) content. D, carotenoids content. Error bars indicate SE. Significant differences were determined by Tukey's test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). DW, dry weight.

Concerning photosynthetic pigments, the *ccr4a* plants showed lower chlorophyll *a* and *b* contents to at least one and to all complemented lines on day 7 after treatment, respectively. Subsequently, 14 days after the treatment, the *ccr4a* plants showed lower chlorophylls contents to all other genotypes (Fig. 10 A, B and C). In addition, carotenoid contents in *ccr4a* plants were significantly lower compared to *ccr4a*-AtCCR4a 6 line and to the wild-type at 7 and 14 days after treatment, respectively (Fig. 9D). No significant differences in photosynthetic pigment contents were observed among the tested genotypes under normal growing conditions (Fig. S2). This demonstrates that the photosynthetic activity of *ccr4a* plants is most likely impaired after they have been exposed to a heat stress episode.

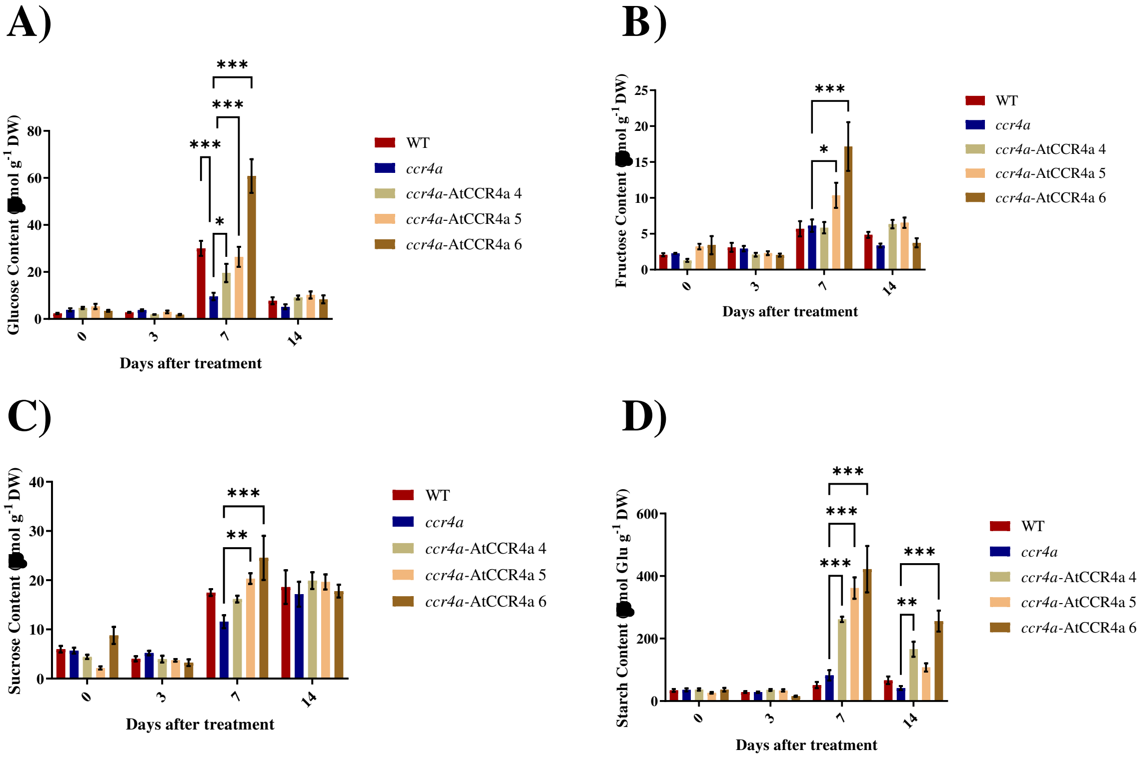

Figure 11. Contents of soluble sugars, sucrose and starch (n = 5) in rosettes from 6-week-old wild-type (WT), *ccr4a* and *ccr4a*-AtCCR4a plants at 0, 3, 7 and 14 days after the 24-hour heat stress treatment (38°C). A, glucose content. B, fructose content. C, sucrose content. D, starch content. Error bars indicate SE. Significant differences were determined by Tukey's test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). DW, dry weight.

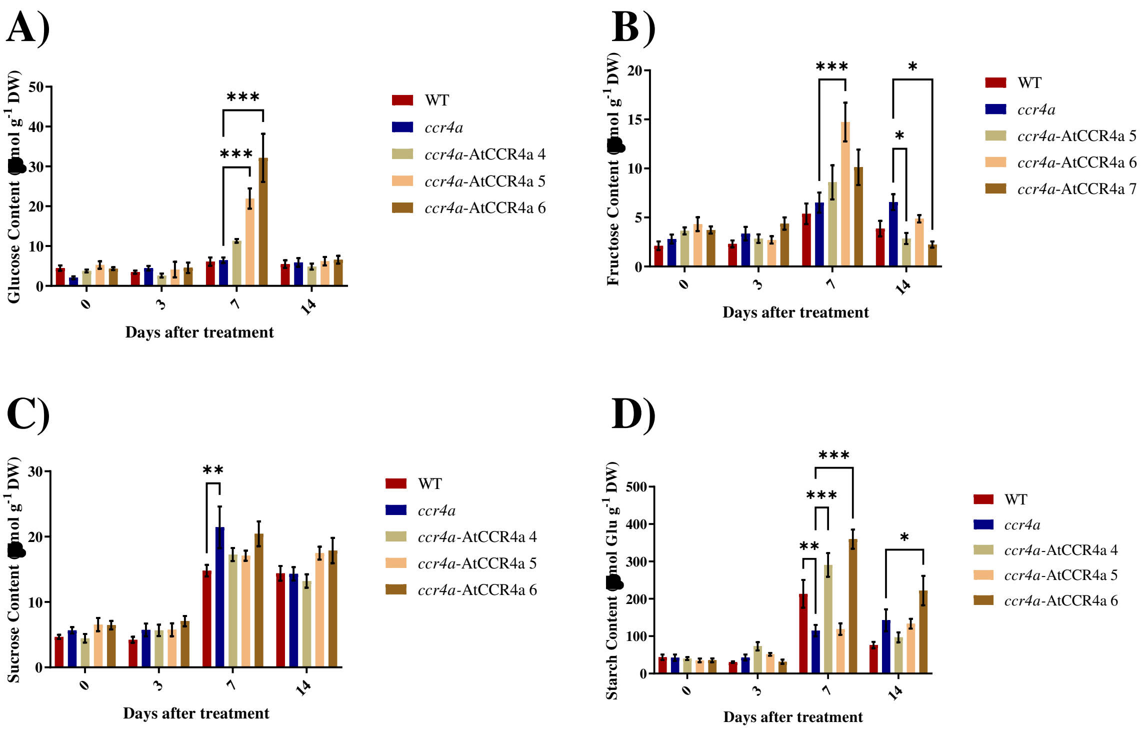

Figure 12. Contents of soluble sugars, sucrose and starch (n = 5) in rosettes from 6-week-old wild-type (WT), *ccr4a* and *ccr4a*-AtCCR4a plants grown on control condition (22°C). A, glucose content. B, fructose content. C, sucrose content. D, starch content. Error bars indicate SE. Significant differences were determined by Tukey's test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). DW, dry weight.

Regarding carbohydrate metabolism, 7 days after exposure to heat stress treatment, *ccr4a* plants showed lower sucrose contents compared to *ccr4a*-AtCCR4a 5 and 6 lines, respectively (Fig. 11C). Interestingly, the wild-type showed lower sucrose contents compared to *ccr4a* plants in this same period when grown under control condition (Fig. 12C).

In addition, *ccr4a* plants showed lower contents of starch compared to all complemented lines and to two complemented lines 7 and 14 days after treatment, respectively (Fig. 11D). In the control condition, *ccr4a* showed lower starch contents compared to the wild-type and to two complemented lines on day 7 of analysis. On day 14, the *ccr4a*-AtCCR4a 6 line showed higher starch contents compared to the *ccr4a* plants (Fig. 12D).

Regarding soluble sugars, 7 days after treatment, the wild-type and all complemented lines showed higher glucose contents than *ccr4a* plants (Fig. 11A). Meanwhile, two complemented lines showed higher fructose contents (Fig. 11B). In the control condition, higher glucose contents were observed in two complemented lines compared to *ccr4a* plants on day 7. Also, *ccr4a*-AtCCR4a 5 line showed higher fructose contents. Curiously, *ccr4a* plants showed higher fructose contents in comparison with two complemented lines on day 14 (Fig. 12A and B).

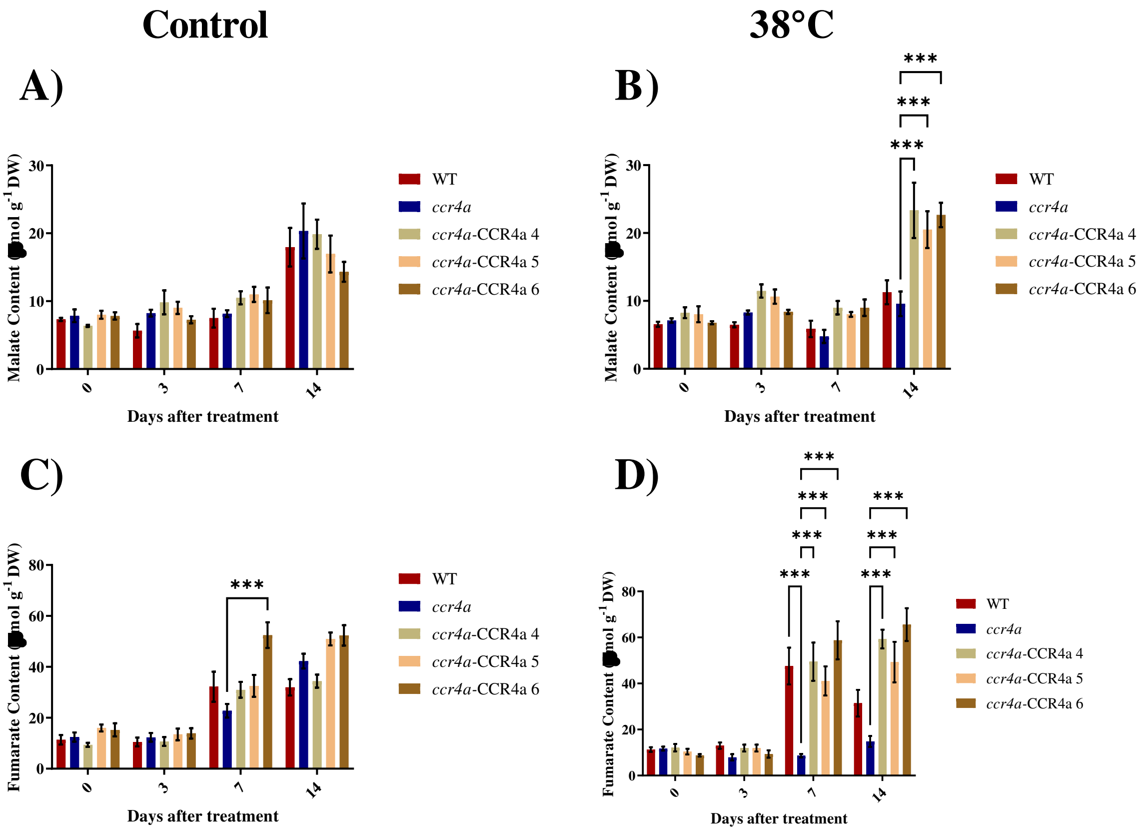

Figure 13. Contents of malate and fumarate (n = 5) in rosettes from 6-week-old wild-type (WT), *ccr4a* and *ccr4a*-AtCCR4a plants at 0, 3, 7 and 14 days after the 24-hour heat stress treatment (38°C) or grown on control condition (22°C). A and B, malate content. C and D, fumarate content. Error bars indicate SE. Significant differences were determined by Tukey's test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). DW, dry weight.

Interestingly, all complemented lines showed significant increments in malate and fumarate contents compared to *ccr4a* plants 7 and 14 days after heat stress treatment (Fig. 13 B and D). In Arabidopsis, malate and fumarate, one of the possible compounds derived from the former, are key organic acids for metabolism, as they are involved in the regulation of stomatal opening and represent an additional carbon source for various molecular processes, such as amino acid biosynthesis (Zell et al., 2010; Araújo et al., 2011). Recently, the accumulation of fumarate in the cytosol of plant cells has been characterized as a crucial process for the acclimation of the photosynthetic process upon heat stress. Compared to its precursor, fumarate is a low-risk compound, that is, it imposes a low risk for the occurrence of metabolic disturbances (Herrmann et al., 2021). This indicates that the complemented lines could be undergoing an acclimation process days after they were subjected to the heat stress treatment, enabling them to better tolerate a possible next episode of heat stress.

These results indicate that the *AtCCR4a* disruption promotes a severe sensitivity in *ccr4a* plants to heat stress (Fig. 4 and 9). This can be observed by the reduction in photosynthetic pigment levels at the end of the treatment and even days after exposure to it (Fig. 5 and 10). Furthermore, although the complemented lines showed some differences in the contents of soluble sugars and carbohydrates in relation to the wild-type, a higher tolerance to heat stress was observed in these lines, indicating that the *AtCCR4a* complementation indeed contributes to the recovery of this phenotype (Fig. 10, 11 and 12).

4.4. Phenotypic evaluation of *ccr4a* plants under drought stress

We also investigated the response of *ccr4a* plants to water stress, since *AtAPUM5*-RNAi plants showed a more water deficit tolerant phenotype (Huh and Paek, 2014). In a preliminary experiment, we could observe that the *ccr4a* plants showed a phenotype of higher tolerance to water deficit compared to the wild-type (Fig. 14A). This was supported by the higher survival rate of the *ccr4a* plants after they were rehydrated (Fig. 14C), although no major difference was observed between the relative water contents of the two genotypes at the end of the water shortage period (Fig. 14B).

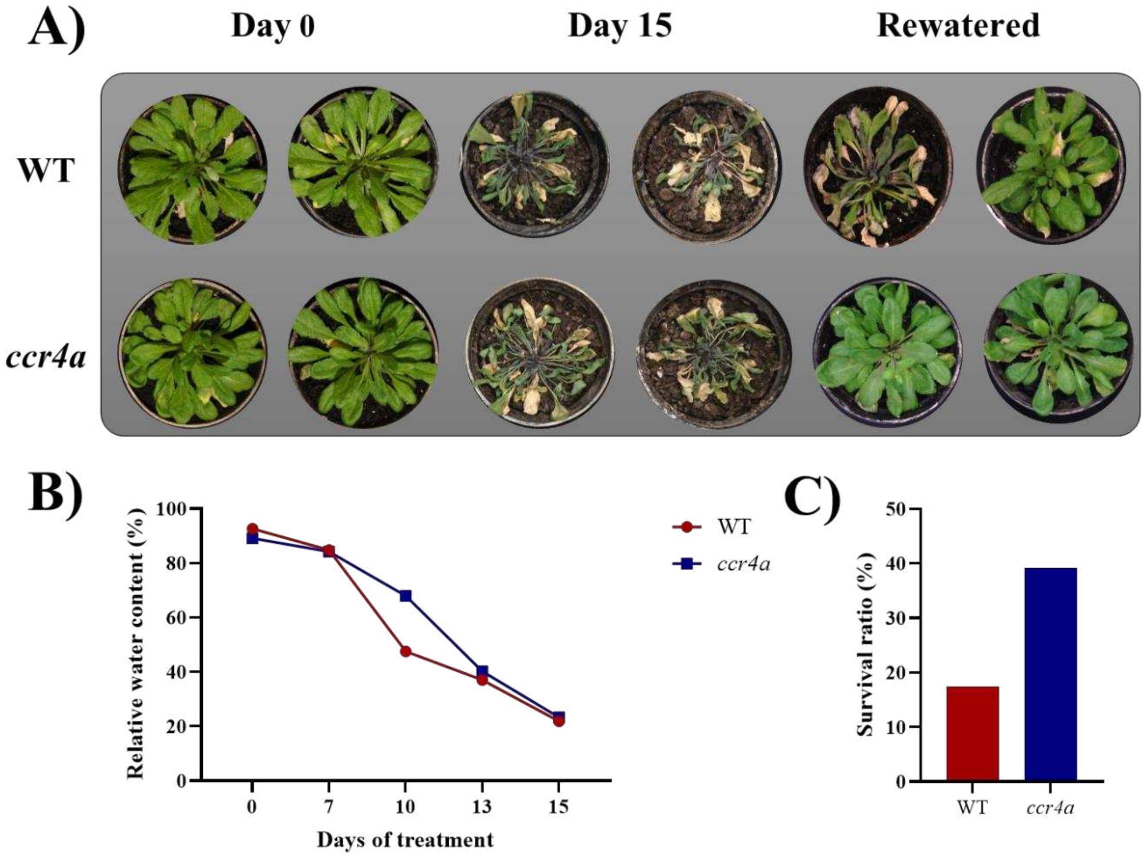

Figure 14. Phenotypic evaluation of 6-week-old wild-type (WT) and *ccr4a* plants under water deficit treatment. A) Representative photographs of plants during the stages of the experiment. B) Estimation of relative water content (%) of wild-type and *ccr4a* plants (n = 5) during 15 days of water shortage. C) Estimation of survival ratio (%) of wild-type and *ccr4a* plants (n = 23) after 3 days of being rehydrated.

With the generation of the *ccr4a*-AtCCR4a complemented lines (Fig. 8), it was possible to conduct a second experiment in which similar results to the first were observed. During the water shortage period, the *ccr4a* plants maintained higher relative water contents compared to the wild-type and the complemented lines (Fig 15B). In addition, they showed a phenotype of higher tolerance to water deficit, in comparison to the other genotypes, along with a higher survival rate (Fig. 15A and C). Similar to the wild-type, the complemented lines showed low survival rates and drastic reductions in relative water contents during the treatment period (Fig. 15A and C). These results indicate that *AtCCR4a* disruption contributes to plants better tolerating a water deficiency condition.

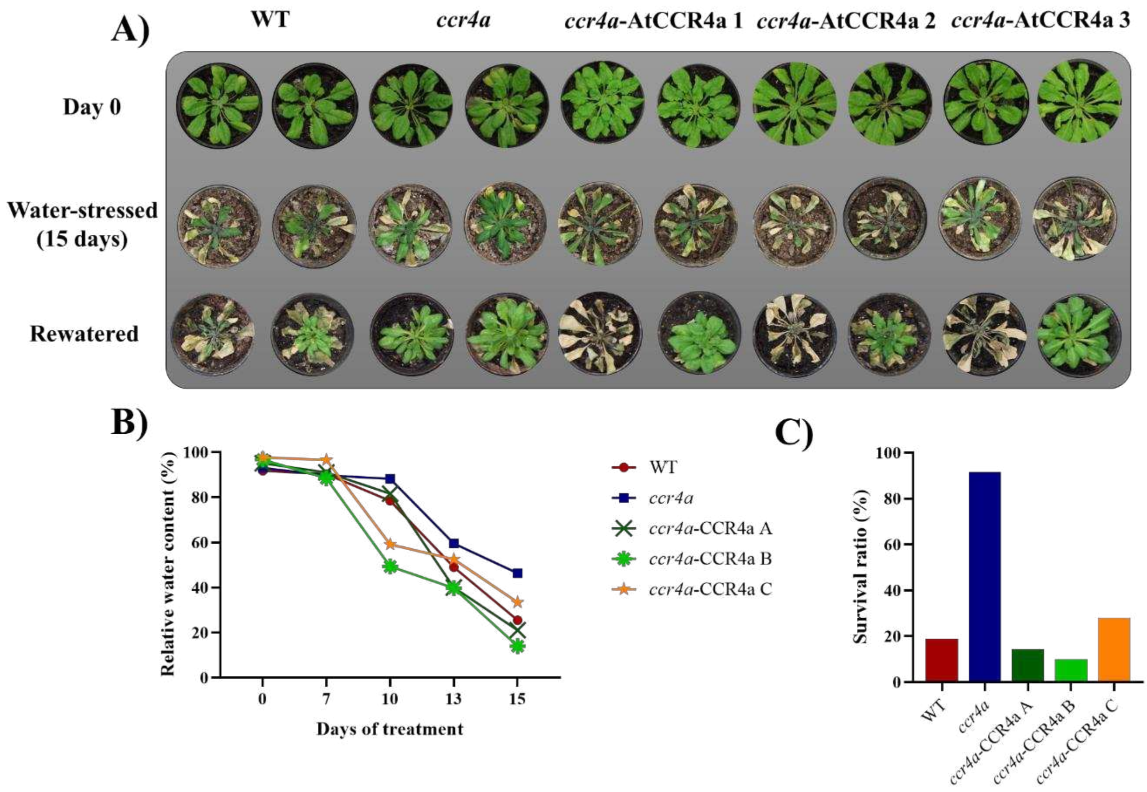

Figure 15. Phenotypic evaluation of 6-week-old wild-type (WT), *ccr4a* and *ccr4a*-AtCCR4a plants under water deficit treatment. A) Representative photographs of plants during the stages of the experiment. B) Estimation of relative water content (%) of the tested genotypes (n = 5) during 15 days of water shortage. C) Estimation of survival ratio (%) of the tested genotypes (n = 22) after 3 days of being rehydrated.

5. DISCUSSION

5.1. The possible link between AtCCR4a and the NRP-mediated cell death signaling pathway

Prolonged exposure of plants to adverse environmental conditions, such as water deficit, leads to the occurrence of cell dehydration, which triggers osmotic and/or endoplasmic reticulum (ER) stresses. Such condition tends to activate the cell death signaling pathway mediated by proteins containing DCD/NRP domains, which although a defense mechanism, leads to senescence of plant tissues and consequently contributes to plant death (de Camargos et al., 2019). In Arabidopsis, the AtNRP1 and AtNRP2 proteins induce genes of the NAC superfamily. These in turn induce genes encoding enzymes with caspase-1-like activity, such as the vacuolar processing enzyme (VPE), which ruptures the vacuolar membrane, inevitably triggering cell lysis (Hatsugai et al., 2015; Reis et al., 2016)

Unraveling how this pathway is incorporated into the metabolism of plant cells presents a great potential to make possible the generation of plant materials more tolerant to the occurrence of environmental adversities, such as drought. However, for this it is critical that the numerous protein-protein interactions between the components of the pathway with other proteins active in cell stability be investigated in detail.

In this approach, we aimed to verify the interaction between AtNRP2 with AtCCR4a, which is predicted in the Arabidopsis interactome (Fucile et al., 2011). AtCCR4a is a deadenylase, that is, an enzyme active in shortening the 3' poly(A) tail of mRNA, performing its function in cytosolic bodies (P-bodies) as a subunit of the CAF1-NOT-CCR4 complex, the major enzyme complex in plants that catalyzes mRNA deadenylation (Walley et al., 2010; Arae et al., 2019). Together with AtCCR4b, it is also involved in sucrose/starch metabolism by determining the poly(A) tail length of transcripts of AtGBSS1, an enzyme active in amylose biosynthesis (Suzuki et al., 2015).

We were unable to visualize the interaction between AtCCR4a and AtNRP2 *in vivo* by their transient coexpression in tobacco leaves (Fig. 1 and 2). We even performed tunicamycin (TM) infiltration into the previously agroinfiltrated leaves to verify that this interaction necessarily occurs in the presence of a stress inducer (Fig. 1). However, we emphasize that we do not rule out the possibility that this interaction occurs mostly in the cytoplasm of plant cells, as is predicted in the Arabidopsis interactome (Fucile et al., 2011). Future assays may consider shorter time intervals (~2 h) between infiltration of some stress inducer, such as TM or polyethylene glycol (PEG), and fluorescence analysis. In addition, performing yeast two hybrid assays should be considered.

RNA metabolism is a critical mechanism to control gene expression in several physiological and metabolic processes, especially in response to environmental adversities (Kawa and Testerink, 2017; Matsui et al., 2019). Since some components of both NRP-mediated death cell signaling pathway and the CAF1-NOT-CCR4 complex have already been characterized as responsive to certain types of stress, such as osmotic stress, we evaluated the expression of *AtCCR4a* upon the occurrence of osmotic and ER stress (Walley et al., 2010; Reis et al., 2016). The expression of *AtCCR4a* was increased upon osmotic stress in 14-day-old seedlings embedded in PEG-containing solution. However, no apparent increasing in *AtCCR4a* expression was observed at the occurrence of ER stress at the 3-hour time point. We emphasize that *AtCCR4a* may indeed be induced in the latter condition at longer evaluation times, as has been observed with *AtNRP1* and *AtNRP2* (Reis et al., 2016). Such results indicate that *AtCCR4a* is induced by some stress conditions as those of the NRP-mediated death cell signaling pathway components. While not necessarily a component of the pathway, AtCCR4a can be activated by them.

5.2. *AtCCR4a* disruption leads to sensitivity to heat stress

Temperature is among the main environmental factors that influence various biochemical and physiological processes that shape plant growth and development. Temperature fluctuations occur naturally during the life cycle of plants. However, extreme variations can negatively alter metabolism and cause irreversible damage to plants, impairing their growth and productivity during their reproductive period (Bita and Gerats, 2013). The exposure of plants to temperatures far above their optimum threshold, even within several minutes to a few hours, can be extremely detrimental to plants, especially depending on their developmental stage (Yeh et al., 2012).

Sun et al. (2020) identified differentially expressed genes in 7-day-old Arabidopsis seedlings that underwent heat stress treatment. Interestingly, *AtCAF1b* is among the upregulated genes. Being subunits of the CAF1-NOT-CCR4 complex, Walley et al. (2010) reported that AtCAF1a and AtCAF1b do not act redundantly neither are required for all types of abiotic stresses. *Atcaf1a* seedlings showed a higher germination rate compared to wild-type and *Atcaf1b* seedlings when grown on saline medium (200 mM NaCl). At the same time, *Atcaf1a* and *Atcaf1b* did not show different tolerance levels than the wild-type under waterlogged condition. *Atcaf1a* and *Atcaf1b* also showed lower survival rates when subjected to oxidative stress (5 μM paraquat dichloride).

In addition, Arae et al. (2019) verified that four of 11 tested AtCAF1 isoforms interacted with AtCCR4b but not with AtCCR4a, suggesting that there may be a version of the CAF1-NOT-CCR4 complex functionally independent of the interaction between AtCAF1 and AtCCR4. However, we first assessed the expression of *AtCCR4a* within an 18-hour heat stress treatment (38°C). *AtCCR4a* was induced within 3 hours of high temperature exposure (Fig.

1C). This led us to check whether there would be any noticeable phenotype in *ccr4a* plants subjected to this condition.

We could observe that the *ccr4a* plants presented a higher sensitivity to heat stress in comparison with the wild-type (Fig. 4 and 9). The *AtCCR4a* complementation led to the recovery of the wild-type phenotype to heat stress in the complemented plants (Fig. 9). This shows us that indeed *AtCCR4a* disruption results in a higher sensitivity of the plants to heat stress.

In the first heat stress experiment, we did not observe major changes in photosynthetic pigment contents, except at the end of the treatment, in which the *ccr4a* plants presented lower total chlorophylls and carotenoids contents (Fig. 5C and D). This may have been caused by a reduction in the biosynthesis of chlorophylls as a result of the deactivation of numerous enzymes by exposure to high temperature and/or the degradation of chlorophylls by the production of reactive oxygen species (ROS) (Dutta et al., 2009).

Furthermore, we could observe that during the treatment period, the carbohydrate metabolism of both wild-type and *ccr4a* plants was altered, as we did not observe an increasing in starch contents at 6 and 12 hours of treatment. Such increasing was observed in both genotypes in the control condition (Fig. 6D), a metabolic consequence promoted by the plant's circadian clock (Streb and Zeeman, 2012).

Daytime starch breakdown has already been observed as a strategy to promote sugar accumulation, which in turn may end up acting as compatible solutes, or even as signaling molecules (Streb and Zeeman, 2012; Smith and Zeeman, 2020). This is probably due to the activation of hydrolytic enzymes that are not required for classical nocturnal starch breakdown, such as AtBAM1 (AtBMY7) (Thalmann and Santelia, 2017). Furthermore, a severe heat increase tends to limit photosynthetic activity, decreasing carbon assimilation and consequently decreasing the starch contents produced (Vasseur et al., 2011; Smith and Zeeman, 2020; Wang et al., 2020).

In both wild-type and *ccr4a* plants, total free amino acids and proline contents increased upon occurrence of the heat stress treatment (Fig. 7). This demonstrates that both genotypes responded to this stress, as the accumulation of amino acids has already been described as a strategy to combat abiotic stresses in plants (Bhaskara et al., 2015; Batista-Silva et al., 2019).

In turn, in the second heat stress experiment, we decided to evaluate the plants days after exposure to stress to assess whether there would be notable changes in photosynthetic pigment and carbohydrate contents. Again, we observed reductions in photosynthetic pigment contents in *ccr4a* plants at 7 and 14 days after being exposed to heat stress treatment (Fig. 10). Besides, at 14 days after the treatment, the *ccr4a* plants presented lower starch contents in relation to one of the complemented lines (Fig. 11D). We highlight the fact that we did not observe notable reductions in starch contents in *ccr4a* plants compared to wild-type and complemented lines in the control condition, probably as a result of *AtCCR4b* acting on sucrose/starch metabolism in the mutant plants, attenuating the effect of *AtCCR4a* disruption.

Last but not least, the complemented lines showed an accumulation of malate and fumarate in comparison with the *ccr4a* plants 7 and 14 days after being subjected to heat stress treatment, which may indicate the occurrence of an acclimation strategy (Fig. 13).

Malate and fumarate are organic acids derived from the tricarboxylic acid (TCA) cycle in the mitochondria. In the cytoplasm of plant cells, malate can be converted to several other compounds. In C_3 plants, like Arabidopsis, malate can be converted into fumarate in mitochondria or cytoplasm through fumarase isoforms activity. Both malate and fumarate probably function as important transient carbon source for different biosynthetic processes, since they show similar diurnal changes as starch (Zell et al., 2010; Araújo et al., 2011). In plant cells, malate is also active in regulating the redox-homeostasis among mitochondria, chloroplasts and peroxisomes (Selinski and Scheibe, 2019).

In turn, pyruvate accumulation showed to be important to photosynthetic acclimation to low temperatures in Arabidopsis. However, the function of pyruvate accumulation in this process is yet unknow (Scott et al., 2014; Dyson et al., 2016). Recently, Herrmann et al. (2021) reported that pyruvate accumulation is also critical for high temperature responses. The authors conclude that AtFUM2, a cytosolic isoform of fumarase, contributes to the maintenance of cellular stability, probably by regulating the accumulation of malate in the occurrence of adverse environmental conditions.

5.3. *AtCCR4a* disruption leads to tolerance to drought stress

Among all the stresses of abiotic nature, water deficiency is singularly the most limiting for the life cycle of plants, since water is an essential resource. Water deficiency, besides limiting plant growth and development, generates secondary stresses, such as osmotic stress, triggering damages at tissue level, and ER stress, impairing the cellular machinery (Fahad et al., 2017; Seleiman et al., 2021).

Given this context, Huh and Paek (2014b) verified that *APUM5*-RNAi plants were shown to be more tolerant to water deficiency than the wild-type. At the same time, 35S-*AtAPUM5* plants showed a hypersensitive phenotype to this condition. Interestingly AtAPUM5, along with AtAPUM2, has been described as a protein that directly interacts with AtCCR4a and AtCCR4b (Arae et al., 2019). The APUM proteins, belonging to the PUF family, are well-conserved RNA-binding proteins among eukaryotic organisms, which regulate molecular events that lead to rRNA processing (Joshna et al., 2020).

Therefore, we decided to verify the response of *ccr4a* plants under a water deficiency condition. Surprisingly, the *ccr4a* plants showed a higher tolerance to water deficit than the wild-type. Furthermore, the *AtCCR4a* complementation restored the wild-type phenotype in the complemented plants, which demonstrates that the *AtCCR4a* disruption somehow collaborates to the plants better tolerating a water shortage situation (Fig. 14 and 15).

Huh and Paek (2014) noted that abiotic stress-responsive genes were downregulated in 35S-*AtAPUM5* plants, which suggests that *AtAPUM5* may be post-transcriptional regulator of the stress-responsive genes by direct binding of target genes 3′ UTRs. Thus, we conclude that *AtCCR4a* may act in a similar way to *AtAPUM5*, which may justify the higher tolerance to water deficit observed in *ccr4a* plants. However, further studies with *AtCCR4a* overexpressing plants must be conducted to verify if they will exhibit a more vulnerable phenotype to water scarcity.

6. CONCLUSIONS

AtCCR4a is a deadenylase involved in both mRNA processing and sucrose/starch metabolism, being localized in cytosolic bodies (P-bodies). In this work, *AtCCR4a* has been shown to be induced by osmotic and heat stresses, with the possibility of it being induced by long-term endoplasmic reticulum stress. Apparently, AtCCR4a may be not a component of the NRP-mediated cell death pathway, as its interaction with AtNRP2 was not observed through BIFC assays. However, we do not exclude the possibility of this interaction occurs in the cytoplasm of plant cells, raising the need for future studies to be performed to confirm this hypothesis.

Moreover, *AtCCR4a* disruption promotes better plant tolerance to water scarcity, contributing to the maintenance of relative tissue water contents and consequently to a higher plant survival. It also promotes a drastic sensitivity to heat stress, contributing to plant death. Indeed, AtCCR4a is critical for cellular stability as it is probably a post-transcriptional regulator of stress-responsive genes. AtCCR4a may interact with different proteins in the CAF1-NOT-CCR4 complex and/or select specific target mRNAs for different stress conditions, which may explain the phenotypic antagonistic responses observed in *ccr4a* plants subjected to heat or water stress.

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SUPPLEMENTAL DATA

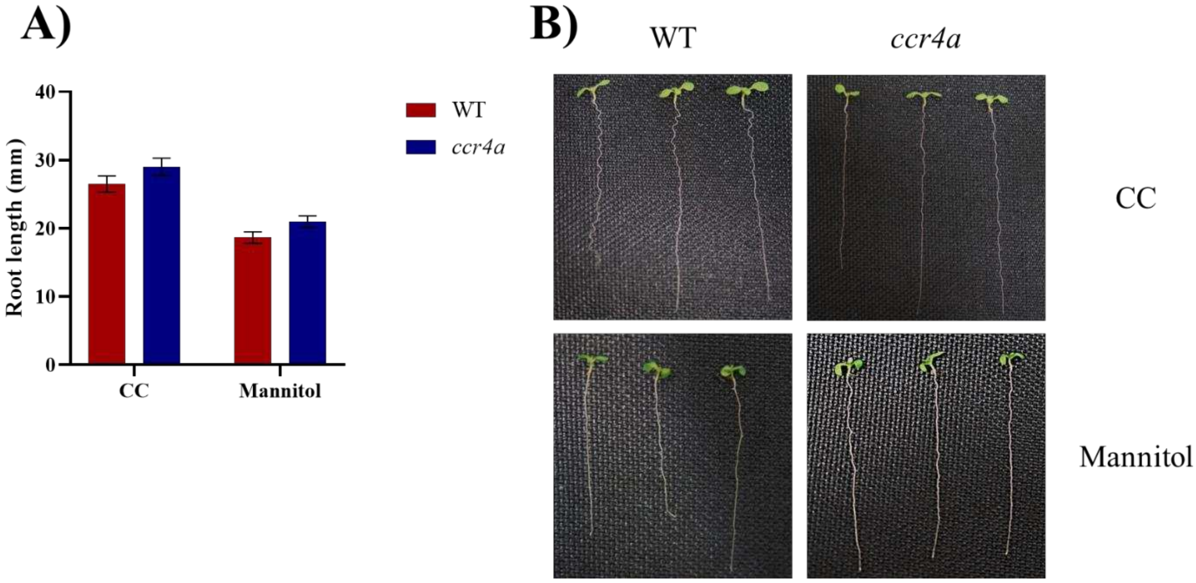

Figure S1. Phenotypic evaluation of 7-day-old wild-type (WT) and *ccr4a* seedlings under osmotic stress. A) Estimation of root length (mm) of the tested genotypes (n = 15) grown on control medium (CC) or medium containing 200 mM mannitol at 7 days after germination. B) Representative photographs of seedlings at 7 days after germination. Error bars indicate SE. CC, control condition.

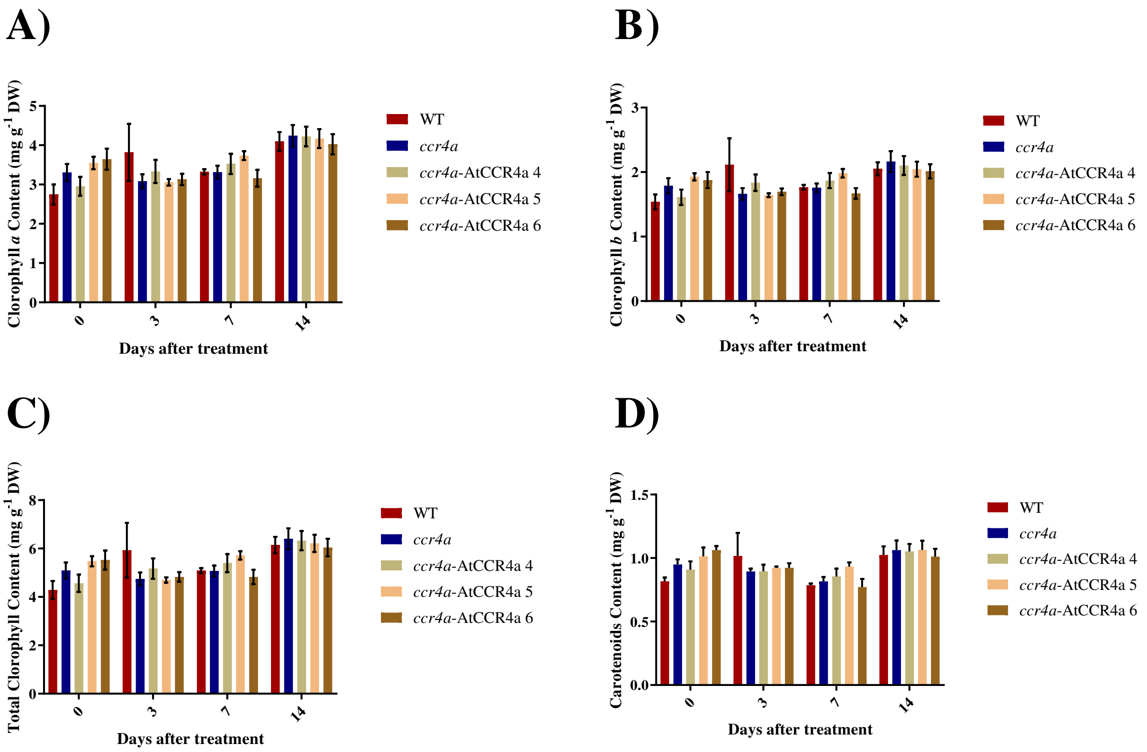

Figure S2. Contents of photosynthetic pigments (n = 5) in rosettes from 6-week-old wild-type (WT), *ccr4a* and *ccr4a*-AtCCR4a plants grown on control condition (22°C). A, chlorophyll *a* content. B, chlorophyll *b* content. C, total chlorophyll (*a* + *b*) content. D, carotenoids content. Error bars indicate SE. DW, dry weight.

Supplemental Table 1 – List of used primers

Primer	**Gene/Flanking sequence**	**Sequence (5´-3´)**
AtCCR4a-FWD	*AtCCR4a*	CAATGCTTAGCGTTATA
AtCCR4a-RVS	*AtCCR4a*	TCTATAAAATATTGTTTCG
MC36	35S Promoter	TCCTTCGCAAGACCCTTCCTC
NRP2-FWD	*AtNRP2*	AGCTTCTGGCAATTA
NRP2-RVS	*AtNRP2*	TTATGCAGAACCAGCTTG
qRT Actin-FWD	*AtActin2*	ATGTCGTGAGCCATCCTGTC
qRT Actin-RVS	*AtActin2*	ACACCGGATTCGTGCGGCAT
qRT AtCCR4a-FWD	*AtCCR4a*	GGGTGCGCTACTTTCTTCAG
qRT AtCCR4a-RVS	*AtCCR4a*	TTCAACAAGGCTGCACAGTC
qRT CNX1-FWD	*AtCNX1*	ATGAGACAACGGCAACTATTTTCC
qRT CNX1-RVS	*AtCNX1*	CCATAATCCTCATGTCCTTCACT
qRT HSP70-FWD	*AtHSP70*	GGCTGAGGCAGATGAGTTC
qRT HSP70-RVS	*AtHSP70*	GTGTCGTCATCCATTCCTC
qRT RAB18-FWD	*AtRAB18*	CGATCCAGCAGCAGTATAG
qRT RAB18-RVS	*AtRAB18*	TTCGAAGCTTAACGGCCACC