FEDERAL UNIVERSITY OF CEARÁ
CENTER OF SCIENCES
DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
POSTGRADUATE PROGRAM IN BIOCHEMISTRY

VICENTE THIAGO CANDIDO BARROS ALENCAR

KNOCKOUT IN PIF14 INDUCES INTERRUPTIONS IN STARCH SYNTHESIS, AFFECTING SEED FORMATION AND DEVELOPMENT OF RICE PLANTS

FORTALEZA
2023

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Research Project presented to the Coordination of the Postgraduate Course in Biochemistry at the Federal University of Ceará as a partial requirement for obtaining a PhD in Biochemistry. Area of concentration: Plant Biochemistry.

Advisor: PhD. Joaquim Albenisio Gomes da Silveira.

FORTALEZA

2023

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Sistema de Bibliotecas
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

A355k Alencar, Vicente Thiago Candido Barros.
Knockout in pif14 induces interruptions in starch synthesis, affecting seed formation and development of rice plants / Vicente Thiago Candido Barros Alencar. – 2023.
108 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica , Fortaleza, 2023.
Orientação: Prof. Dr. Joaquim Albenisio Gomes da Silveira.

1. Transcription factor.. . 2. Oryza sativa. 3. Signalling. 4. Physiological response. 5. Protein-protein interaction. I. Título.

CDD 572

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Approved in 30 / 01 / 2023.

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To

My parents, Fatima, and Alencar

My grandparents, Geraldo and Marilaque

My family

ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

To the Federal University of Ceará and the Biochemistry and Molecular Biology Course.

To BRIDGES, for the financial support with the maintenance of the scholarship in the last period of the doctorate.

To Prof. doctor Joaquim Albenísio Gomes da Silveira, for his guidance and especially for encouraging and passing on excellent scientific knowledge. For being a great example of a professor, researcher, and human being. Thank you.

To Dr. Ana Karla Moreira Lobo, for teaching, scientific collaboration throughout my stay at LABPLANT. In addition, an excellent friendship. Thank you.

To Dr. Fabrício Eulálio Leite Carvalho for contributing to my scientific development, friendship, and valuable collaborations during my long stay at LABPLANT. Thank you.

To Dr. Ana Luiza Sobral Paiva, for contributing at the very end of my academic scientific trajectory, but already contributing with great weight in this cycle, mainly as an example of a strong and determined person. For the friendship linked in the first contact. Thank you.

To Dr. Nelson José Madeira Saibo for providing the plant material used in this work. For scientific collaboration and valuable contributions. Thank you.

To all my family at LABPLANT, especially Ayrton Markos, Rikaely, Yugo, Victor, Adilton, Moaciaria, Dani Alessandro, Paulo Vinicius, Valeria, Eliezer, for always being accessible, for collaboration and moments of relaxation. Thank you.

To all my foreign friends, especially my Portuguese friend Elsa, my Pakistani friends Humaira, Maqsood Allan, Sheheryar, and my great Venezuelan friend Dani Alessandro for the moments of relaxation, laughter and learning and always accessible, mainly to practice English and Spanish. Thank you.

To my brothers Flaviana, Flavio, Ana and Enn.

To my nephews, Iara Raquel, Leobides, Natanael, Ester, Izaque, Marco Antonio.

To my beloved parents, Maria de Fatima and Antonio Alencar for the great support and collaboration in my life.

"Nature is a huge game of chess played by gods, and which we are privileged to observe. How the rules make game are what fundamental physics breathes, and understanding those rules is our goal."

Richard Feynman

ABSTRACT

Light is crucial for the growth and development of plants through their photoreceptors (Cryptochrome - CRY, phototropin - PHOT and phytochrome - PHY) responsible for its uptake and signalling in various mechanisms of biological routes. Therefore, phytochromes play a crucial role in response to light (Photomorphogenesis) and dark (Skotomorphogenesis). Phytochrome is a chromoprotein synthesized in the cytosol as an insoluble protein in its inactive form (Phy-r) when absorbing light in the far-red spectrum (Fr) undergoes a conformation and changes to its active form (Phy-Fr). Once in the active form, Phy migrates to the nucleus where it interacts with PHYTOCHROME TRANSCRIPTION FACTORS (PIFs) triggering various physiological responses in plants. In *Arabidopsis Thaliana*, 8 isoforms of PIFs (1 – 8) are known and characterized and in rice plant (*Oryza Sativa* L.) 6 from the alignment of base pairs of PIF3-Like from Arabidopsis, therefore, they are called PILs (11 – 16). We have previously observed that PIF14-knocked rice plants exhibit several physiological disturbances such as reduced germination and grain filling. With this, we hypothesize that PIF14 deficiency can reduce seed quality by reducing starch synthesis, making these seeds less viable. Furthermore, in the next generations, these seeds will induce disturbances in some stages of development. To test this hypothesis, rice plants were knocked out in PIF14 (CRISPR/Cas9 technique) and grown throughout their development cycle. Transformed plants showed a reduction in tillering and panicle growth rates throughout the growing season. Seeds showed lower growth rates associated with reductions in number, size, and mass. Starch accumulation rates were similar showing strong reductions in content at the end of maturation while sucrose and total soluble sugars showed an inverse trend, as did proteins. Seeds showed lower viability indicated by the electrolyte leakage rate and reduced germination rate. In addition, these seeds showed lower seedling establishment, root and shoot elongation rates, associated with delayed vegetative and reproductive development. These results show that knockout of the PIF14 gene affects rice development, probably starting with seed formation via starch metabolism.

Keywords: transcription factor; *Oryza* sativa L; signalling; physiological response; protein-protein interaction.

RESUMO

A luz é crucial para o crescimento e desenvolvimento das plantas através de seus fotorreceptores (Criptocromo - CRY, fototropina - PHOT e fitocromo - PHY) responsáveis por sua captação e sinalizar em vários mecanismos de rotas biológicas. Por tanto, fitocromos desempenham um papel crucial em resposta ao claro (Fotomorfogênese) e escuro (Escotomorfogêneses). Fitocromos são cromoproteínas sintetizada no citosol como proteína insolúvel na sua forma inativa (Phy-r) ao absorver a luz no espectro do vermelho distante (Fr) sofre uma conformação e muda para sua forma ativa (Phy-Fr). Uma vez na forma ativa o Phy migra para o núcleo onde interage com os FATORES DE TRANSCRIÇÃO DE FITOCROMOS (PIFs) desencadeando várias respostas fisiológicas nas plantas. Em *Arabidopsis Thaliana*, são conhecidas e caracterizadas 8 isoformas de PIFs (1 – 8) e em planta de arroz (*Oryza Sativa* L.) 6 a partir do alinhamento de pares de bases do PIF3-Like de Arabidopsis, por isso, são chamados de PILs (11 – 16). Nós observamos previamente que plantas de arroz com PIF14 nocauteado apresentam diversos distúrbios fisiológicos tais como redução na germinação e no enchimento dos grãos. Com isso hipostenizamos que a deficiência de PIF14 é capaz de reduzir a qualidade das sementes por meio da redução na síntese de amido, tornando essas sementes com menor viabilidade. Além disso, nas próximas gerações, essas sementes irão induzir distúrbios em algumas fases do desenvolvimento. Para testar essa hipótese, plantas de arroz foram nocauteadas em PIF14 (técnica CRISPR/Cas9) e cultivadas durante todo seu ciclo de desenvolvimento. Plantas transformadas apresentaram redução no perfilhamento e nas taxas de crescimento das panículas ao longo do período vegetativo. As sementes apresentaram menores taxas de crescimento associadas a reduções no número, tamanho e massa. As taxas de acumulação de amido foram similares mostrando fortes reduções no conteúdo no final da maturação enquanto sacarose e açucares solúveis totais mostraram uma tendência inversa, assim como as proteínas. As sementes apresentaram menor viabilidade indicada pelo índice de vazamento de eletrólitos e redução na taxa de germinação. Além disso, essas sementes apresentaram menor estabelecimento da plântula, taxas de elongamento de raízes e parte aérea, associadas com atraso no desenvolvimento vegetativo e reprodutivo. Esses resultados evidenciam que o nocaute no gene PIF14 afeta o desenvolvimento de arroz iniciando provavelmente na formação da semente pela via de metabolismo do amido.

Palavras-chave: fator de transcrição; *Oryza sativa* L; sinalização; resposta fisiológica; interação proteína - proteína.

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LIST OF ABBREVIATIONS AND ACRONYMS

AGPase	ADP- glucose pyrophosphorylase
AGPL2	ADP-glucose pyrophosphorylase2
AtPIF	Arabdopsis Phytochrome-Interacting Factor 14
bHLH	Basic Helix-Loop-Helix
CRISP/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR associated protein 9
D14	DWARF14
DNA	Deoxyribonucleic Acid
EMBL	European Molecular Biology Laboratory
FT	Factor transcription
GBSSI	Granule-Bound Starch Synthase
HCl	Hydrochloric acid
$HClO_4$	Perchloric Acid
IPA1	IDEAL PLANTARCHITECTURE1
KO1	Transformed plant line 1
KO2	Transformed plant line 2
KO3	Transformed plant line 3
KOH	Potassium hydroxide
KO-OsPIF14	knockout rice plants
MCW	Methanol:Chloroform:Water
NT	Non-transformed plants
OsASCO1	Rice 1-aminocyclopropane-1-carboxylate synthase1
OsCCA1	Rice CIRCADIAN CLOCK ASSOCIATED1
OsDREB1	Rice Dehydration-Sensitive Element-Binding 1
Osphy	Rice phytochrome
OsphyA	Rice phytochrome A
OsphyB	Rice phytochrome B
OsphyC	Rice phytochrome C
OsPIF14	Rice Phytochrome-Interacting Factor 14
OsPIF4	Rice Phytochrome-Interacting Factor 14 gene
OsPIL	Rice Phytochrome-Interacting Factor 3-Like

OsPPR1	Rice PSEUDO-RESPONSE REGULATOR 1
OsSERF1	Rice SALT-RESPONSIVE-ERF1
OsTB1	Rice TEOSINTE BRANCHED1
PCR	Polymerase Chain Reaction
Pfr	Active phytochrome
pGBSSI	Granule-bound starch synthase I
PGL	POSITIVE GRAIN LENGTH REGULATOR
Phy	Phytochrome
PIF	Phytochrome-Interacting Factor
PIF14	Phytochrome-Interacting Factor 14
PIL	Phytochrome-Interacting Factor 3-Like
PPFD	Photosynthetic photon flux density
Pr	Inactive phytochrome
PRR	PSEUDO-RESPONSE REGULATOR
RNA	Ribonucleic Acid
RPBF	Prolamin box binding factor
RPM	Rotations per minute
SIB	Swiss Institute of Bioinfomatics
SLR1	Slender Rice 1
SS	Starch synthase
SST	Total soluble sugars
StSase	Starch synthase
Suc	Sucrose
SUS	Sucrose synthase
TOC1	TIMING OF CAB EXPRESSION 1
UGPase	UDP - glucose pyrophosphorylase

LIST OF SYMBOLS

ºC	Degree Celsius
η	Etá
h	Hour
μ	Mi
%	Percentage
ϕ	Phi
®	Trademark

SUMMARY

1. CHAPTER I – PHYTOCHROMES AND PHYTOCHROME INTERACTING FACTORS: CENTRAL PLAYERS IN LIGHT-MEDIATED SIGNALING FOR PLANT GROWTH

2. INTRODUCTION

Plant growth and development are directly determined by environmental factors, such as temperature, light quality and intensity, availability of water and nutritional resources (FRANKLIN; QUAIL, 2009; CORDEIRO et al 2016). In addition to using light to convert solar energy into chemistry through photosynthesis to produce its metabolites, plants use light to reach their maximum genetic potential through sensors that capture light-induced stimuli determining cell growth and differentiation. For this, they have sensors such as phototropin (phot) and cryptochromes (cry) that absorb light from ultraviolet-A (UV-A) to regions of the blue spectrum (B) (390 ηm) and phytochromes (PHY) that absorb mainly in the red spectrum (r) and far red (fr) (VOITSEKHOVSKAJA., 2019).

Of these photosensors, phytochrome is of great importance as it participates in then regulates virtually all aspects of plant growth and developmental processes throughout the whole life cycle, covering from seed germination to flowering, among which seedling growth and leaf development are most sensitive to light (PARK et al., 2003). Phytochromes are present in all terrestrial plants and most lineages of green algae, except for chlorophytes (LI et al., 2015). In *Arabidopsis Thaliana*, a model plant for dicots, there are five phytochromes (PhyA, PhyB, PhyC, PhyD and PhyE). In a rice plant (*Oryza Sativa* L.), a model monocot, there are three types of phytochromes (PhyA, PhyB and PhyC) (WANG et al., 2022).

Phytochromes and Phytochrome Interaction Factors (PIFs), together interact and are responsible for capturing light stimuli and translating them into physiological responses. Phytochromes, when absorbing light in the red spectrum (660 ηm), assume the active form (Phy-Pfr), which moves to the cell nucleus and interacts with PIFs degrading them. On the other hand, when far-red light (730 ηm) falls on phytochrome, it assumes the inactive form (Phy-Pr) (PHAM et al., 2018; LEGRIS et al., 2016). This interaction occurs through the protein-protein interaction, where phytochrome interacts with PIFs, ubiquitinating them. Once ubiquitinated, PIF is transported to the 26S proteasome where it is degraded (PHAM et al., 2018).

PIFs are basic transcription factors and form a small subset of basic helix-loop-helix (bHLH) transcription factors that generally have the function of repressing seed germination, acting on seedling skotomorphogenesis, promoting shadow avoidance, thus regulating thousands of genes (LEIVAR; QUAIL, 2011). It is currently known that in Arabidopsis there

are eight types of PIFs (PIF1, PIF3 - PIF8 and PHYTOCHROME INTERACTING FACTOR3-LIKE1 [PIL1] renamed PIF2). All Arabidopsis PIFs have an APB motif; only PIF1 and PIF3 have an APB and an APA motif (LEE; CHOI, 2017). In rice plants, there are six possible PHYTOCHROME-INTERACTING FACTOR-LIKE proteins (OsPIL11 – OsPIL16) with the conserved APB motif; however, only OsPIL15 contains an APA motif (NAKAMURA et al., 2007). Among all rice PILs, only OsPIL14 was shown to interact with OsphyA, OsphyB and OsphyC, preferentially interacting with OsphyB in vitro (CORDEIRO et al., 2016).

In general, it is first known that FIPs have the function of negatively controlling photomorphogenesis (LEIVAR; QUAIL, 2011; LEIVAR; MONTE, 2014). Furthermore, these TFs are also known to regulate many other metabolic pathways, including the circadian cycle (SHOR et al., 2017), hormonal signalling as well as biotic and abiotic responses (PAIK et al., 2017). This occurs through several other cells signalling molecules. In Arabidopsis plants, many mechanisms of regulation of their PIFs are already known (OH et al., 2004; MARUYAMA et al., 2012; TODAKA et al., 2012; STEPHENSON; FANHAUSER; TERRY, 2009; GRAMEGNA et al., 2019; PAIK et al., 2017). However, in rice plants, despite some advances, little is known about this subject.

Nakamura et al., 2007 were the first to characterize a group of the bHLH factor subfamily in rice. Based on sequences like Arabidopsis PIFs, they were then considered homologous (PHITOCROME INTERACTION FACTOR-LIKES-PIL) (OsPIL11 - OsPIL16) which contributes to the knowledge of the studies of PIFs in rice plants (NAKAMURA et al., 2007). Since then, some studies with rice plants have been carried out and characterized to elucidate some of the roles of these factors of interaction with phytochrome in rice plants (ZHOU et al., 2014; CORDEIRO et al., 2016; SAKURABA et al., 2017; JI; DU, 2019; LI et al., 2012).

3. PHYTOCHROME: CHEMICAL, PHYSICAL AND MOLECULAR CHARACTERISTICS

Phytochromes are chromoproteins that assume two reversible forms, one active (Pfr) that absorbs red light spectra (660 ηm) and other inactive (Pr) that absorbs far-red light (730 ηm) spectra (LARNE et al., 2018; INOUE et al., 2017; ROCKWELL et al., 2006). In cells, it is a soluble protein synthesized in an inactive form in the cytosol (LAGARIAS; RAPOPORT., 1980). While in the Pr form, Phy remains in the cytosol, but when the light in the red spectra falls on the phytochrome changes its conformation into the active form and is then translocated

to the nucleus (KLOSE et al., 2015). Thus, depending on the amount of light incident on the plant cell, part of the active form of phytochrome goes to the nucleus and part stays in the cytosol. The Pfr forms of phytochromes in the cytosol regulate the translation of mRNA (PAIK et al., 2012). In the nucleus, phytochromes interact with phytochrome interacting factors (PIFs) (XU et al., 2015).

Molecularly, phytochromes are classified into two categories based on their responses to specific light signals type I and type II. Type I phytochromes (PhyA) are light labile and trigger seed germination and de-etiolation when light is scarce (very low R: FR). Such conditions are found under thin layers of soil and in deep shade. Type II phytochromes (PhyB – PhyE) are light stable but need a significant fraction of the Pfr form to promote signalling. Thus, they are active in more diverse environments over a wide range of irradiance where the R: FR is relatively high (FRANKLIN; QUAIL, 2010).

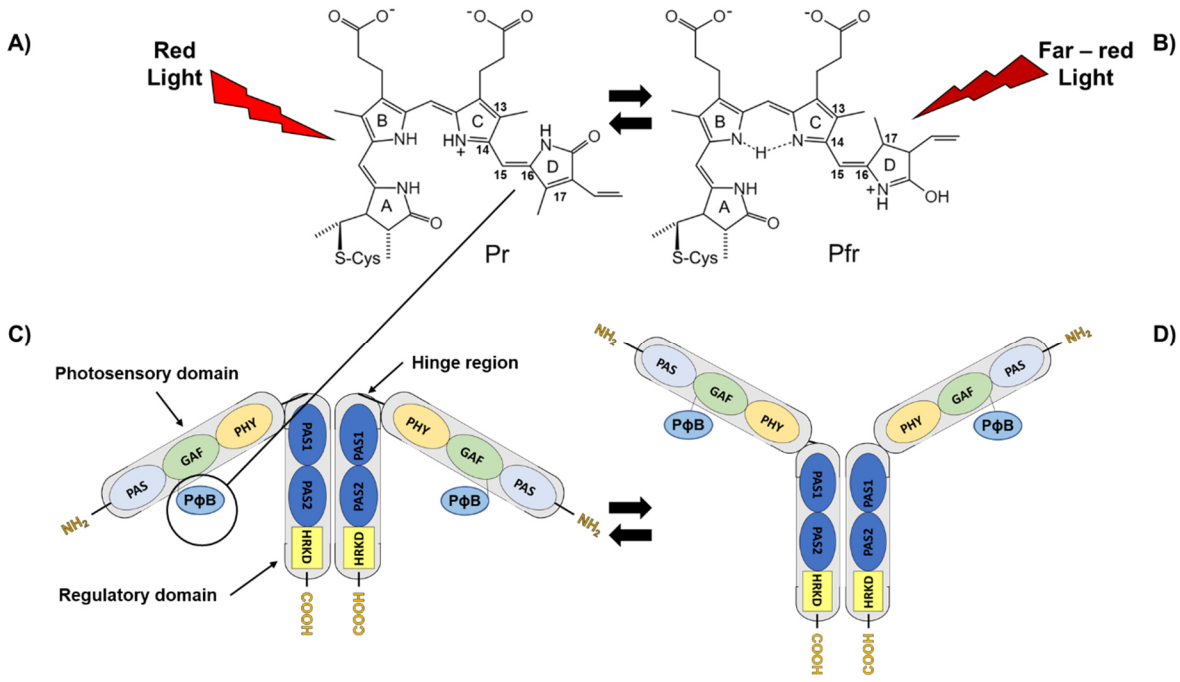
Structurally, Phys consists of an apoprotein that attaches itself to a chromophore through a covalent bond, forming a holoprotein, therefore found as a homodimer (MONTGOMERY; LAGARIAS, 2002; ROCKWELL et al., 2006). Each apoprotein monomer is about 120 kDa in size and comprises two modular structures, the N-terminal chromophore-binding photosensory domain and the C-terminal regulatory domain connected by a flexible hinge region denominated as a region of articulation that permission conformation changes of the phytochrome made in the form Pfr or Pr (PARK; SONG, 2003).

The first modular structure consists of three subdomains, PAS, GAF and PHY. The PHY subdomain is responsible for the stabilization of phytochrome in Pfr form. The GAF subdomain is responsible for making a covalent thioester bond with the chromophore, also known as phytochromobilin (PΦB). The PAS subdomain is in the N-terminal portion of the protein and interacts with the GAF subdomain. Thus, these three subdomains make the portion photosensory function of phytochrome (HALL et al., 2005). The second modular structure, the regulatory domain, is also made up of three subdomains (PAS1, PAS2, HRKD) the last one being a histidine-containing kinase. This subdomain is found in the C-terminal portion of the protein and, as it has kinase activity, it is responsible for interacting with Phytochrome Interacting Factor (PIF), thus being the regulatory portion of the protein (KIM et al., 2003; HALL et al., 2005).

Therefore, phytochromobilin is an open chain tetrapyrrole compound. This way, each pyrrole ring is determined by a letter (A, B, C, and D). In the D ring, there is a double bond between carbons 15 and 16, in which a photoconversion occurs through E - Z isomerization. Functionally, when light shines on PΦB this double bond between C15 and C16

of the D ring, undergoes a rotation causing the D ring to rotate, and then there is a conformational change in the Phy (BRASLAVSKY et al., 2003). Thus, when the red light falls on phytochrome (Pr), the pyrrole ring D rotates resulting in a conformational change from Pr to Pfr. During this conformational change, a nuclear localization site is exposed, so that much of the Pfr is directed to the nucleus (CHEN et al., 2012; MONTGOMERY et al., 2002). Once in the Prf form, phytochrome enters the nucleus where there is an interaction with phytochrome interacting factors (PIFs). This interaction of protein-protein occurs found in the C-terminal portion (HRKD domain) of the phytochrome that for having kinase activity and ubiquitin PIF then is translocated to 26s proteasome when is degraded (PHAM et al., 2018).

Figure 1. Conformational change of phytochrome structure dependent on the light spectrum. **A)** Phytochromobilin before harvesting red light, **B)** Phytochromobilin before harvesting far-red light, **C)** Inactive form of phytochrome (Pr), **D)** Active form of phytochrome (Pfr).



Source: the author himself

4. PHYTOCHROME INTERACTING FACTORS: CHEMICAL AND PHYSICAL PROPERTIES AND MECHANISM OF ACTION

Phytochrome Interacting Factors (PIFs) are basic proteins that belong to a small subset of the basic helix–loop–helix (bHLH) transcription factors family. Therefore, are important commitments responsible for the expression of genes involved in plant development processes, such as photomorphogenesis and skotomorphogenesis, two phenomena that occur in the presence of light or darkness, respectively (WANG et al., 2022).

The basic/helix-loop-helix (bHLH) proteins are a superfamily of transcription factors defined by the bHLH signature domain, which consists of 60 amino acids with two functionally distinct regions. The basic region, located at the N-terminal end of the domain, is involved in DNA binding, and consists of 15 amino acids with a high number of basic residues. The HLH region, at the C-terminal end, functions as a dimerization domain and is constituted mainly of hydrophobic residues that form two amphipathic -helices separated by a loop region of variable sequence and length (TOLEDO-ORTIZ et al., 2003).

In Arabidopsis plants, there are 167 members of the bHLH protein family 8 subgroups of PIFs known and up to 211 in rice plants and 6 subgroups of PIFs (Toledo-ortiz et al., 2003). In addition, the main to have the characteristics of the bHLH family, all the PIF proteins contain a conserved N-terminal sequence, named the active phytochrome B-binding (APB) motif, which is necessary and sufficient for binding to the active form of phyB (KHANNA et al., 2004; LEIVAR; QUAIL, 2011).

Known that FTs directly regulate the expression of downstream genes by binding to either G-box (CACGTG) and/or the E-box (CANNTG) motifs present in their promoters (MARTINEZ-GARCIA et al., 2000; HUQ; QUAIL, 2002; HUQ et al., 2004; SHIN et al., 2007; HORNITSCHEK et al., 2009; ZHANG et al., 2013). When binds to the promoter region of DNA, the PIFs regulate the expression of genes, both repressor and activator (CORDEIRO et al., 2022). Such, in light conditions, function this FT is controlled by Phy interaction that is responsible to control the level of PIFs cells. But, in dark conditions, when the interaction between this FT and phytochrome is non-existent, PIFs abundance in the cellular nucleus is regulated to several factor transcriptions (helix-loop-helix (HLH) proteins, kinases, and E3 ubiquitin ligases). Therefore, in the darks PIFs are stabilised by interacting with De-etiolated (DET1), HECATE2 [HEC2], and the COP1/SPA complex responsible to promote skotomorphogenesis, while that interaction with BIN2, DELLA proteins, HFR1, and COP1, promote the degradation of PIFs in darkness providing the photomorphogenesis (PHAM et al.,

2018). Thereby, both skotomorphogenesis and photomorphogenesis introduce themselves through the transcriptional regulation of thousands of genes. Therefore, to occur transition from skotomorphogenesis to photomorphogenic development is necessary an optimum level of PIFs.

Nonetheless, dark-grown plants display an etiolated phenotype characterized by longer hypocotyl length and smaller yellowish cotyledons, characteristics denominate as skotomorphogenesis (WANG et al., 2020). On the other hand, in the light presence, photomorphogenesis is manifested by short hypocotyl lengths and open and expanded green cotyledons, thereby promoting greening to allow the seedling to adjust for optimal light-harvesting capacity and autotrophic growth (WANG et al., 2022).

5. WHAT IS ALREADY KNOWN ABOUT PIFS AND PHYSIOLOGICAL RESPONSES IN RICE PLANTS

The first study carried out with phytochrome transcription factor–like PIF from Nakamura et al., 2007) was conducted through a characterization of the six OsPIL of the rice plant. These authors identified that the sequence of these transcription factors is like the PIFs of Arabidopsis, and so they were considered homologous (PHITOCROME INTERACTION FACTOR 3-LIKES - PIL, OsPIL11 – OsPIL16). Therefore, to validate whether these rice PILs were indeed homologous to those of Arabidopsis, it was suggested to have functional physiological knowledge (NAKAMURA et al 2007). Since then, some studies with rice plants have been carried out and characterized to elucidate some of the roles of these factors of interaction with phytochrome in rice plants (ZHOU et al., 2014; CORDEIRO et al., 2016; SAKURABA et al., 2017; JI et al., 2019).

In an initial characterization Nakamura et al., 2007 showed that OsPIF11, OsPIF12, and OsPIF13 interact with PSEUDORESPONSE REGULATOR1 (OsPPR1) (NAKAMURA et al., 2007). OsPPR1 is a crucial protein that helps to better understand the molecular links between circadian rhythms, control of flowering timing through photoperiodic, and photosensory pathways signal transduction in plants (MURAKAMI et al., 2003).

Other findings report that OsPIL11, OsPIL13, and OsPIL16 regulate grain size (TODAKA et al., 2012; YANG et al., 2018). These OsPILs, OsPIL15 has more characterized research proving that this transcription factor represses etiolate grain-filling growth (ZHOU et al., 2014), regulation of grain size (JI et al., 2019), in the regulation of tiller angle through the regulation of AUXIN, a phytohormone known to promote cell expansion (XIE et al., 2019). Zhou et al (2014) showed that rice plants overexpressing OsPIL15 exhibited smaller roots and

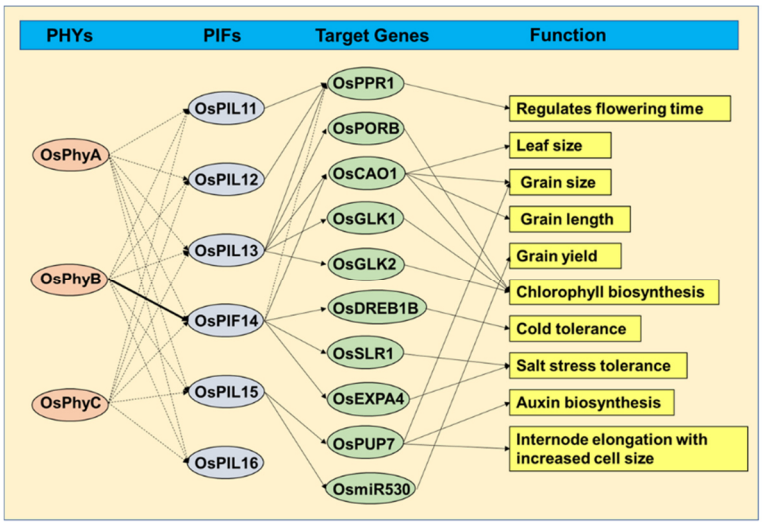
shoots under dark conditions, indicating that this TF may be involved in the growth of etiolated seedlings and that it may be acting as a repressor of genes that are involved with skotomorphogenesis (ZHOU et al., 2014). Besides, this TF has been reported to control grain size and production (JI et al., 2019: SUN et al., 2020).

Todaka et al., 2012 showed by microarray analysis that OsPIL1/OsPIL13 (hereinafter called OsPIL1) is an abiotic stress-responsive gene. Overexpression of OsPIL1 has also been reported to promote internode elongation by increasing cell size, especially under water stress conditions (TODAKA et al., 2012). Regarding growth and development, plants overexpressing OsPIL1 are significantly higher than wild-type ones, but transgenic rice plants expressing OsPIL1-RD (fused to a transcriptional repression domain) are shorter (TODAKA et al., 2012). In the same study, Todaka et al., 2012 observed that rice plants overexpressing OsPIL1 promoted internode elongation. Sakuraba et al., 2017, showed that OsPIL1 is involved with chlorophyll biosynthesis. In the same study, it was also found that the deletion (knockdown) of ospil1 expression caused a pale green phenotype when these plants were grown in a natural rice field (SAKURABA et al., 2017).

Cordeiro et al., 2016, characterized the *OsPIL14* gene and in this work, it was demonstrated that the expression of the *OsPIL14* gene is responsive to circadian rhythms and abiotic stress stimuli (salinity, temperature, water deficit). In addition, OsPIF14 has been shown to act as a repressor. This was confirmed through the repression of the OsBREB1B gene repressor, a TF known as a plant stress regulator, mainly by temperature (CORDEIRO et al., 2016). Recent studies have shown that OsPIL14 interacts with SLR1 by directly regulating downstream gene expression and with light and GA signals, which precisely controls grain-filling growth in response to salt stress (MO et al., 2020).

OsPIL14 is unique to the six OsPIL that bind active phytochrome B forms and, hence was recognized as OsPIF14 (CORDEIRO et al., 2016). Huang et al, 2022 showed that this interaction, PhyB - OsPIF14 is crucial to control the ethylene level in the rice plant. Phys interacts with OsPIF14 promoting its degradation, in turn, OsPIF14 which is linked to the promoter of 1-aminocyclopropane-1-carboxylic acid (OsACO1) releases this protein so that there are transcripts and thus increases the level of ethylene. However, despite reports of regulation of these TFs in rice plants, more research is needed to elucidate their role upstream of OsPILs.

Figure 2. The phytochrome signalling module - PIL - target-function genes in rice plants. The schematic illustration shows the interactions between phytochromes (PhyA - C), PILs (OSPIL11 - 16) and rice plant target genes. The scheme was based on published data on interaction and downstream regulation of phytochromes with PILs and interaction and upstream regulation with target genes in rice plants. The connection line with bold black color indicates interaction confirmed by experiments with phyB. The connecting lines with black protein-protein interaction (Phy - PIL - Target Genes - Function) illustrate the interaction of each phytochrome with different PIFs.



Role of PIFs in crop improvement.

Crop	PIFs	Target Genes	Function	Reference
Rice	*OsPIL11*	*OsPRR1*[2]	Hypocotyl length[1], leaf size[1], Grain size[3], Grain length[4,5]	1. (Li et al., 2012a) 2. (Nakamura et al., 2007) 3. (Yang et al., 2018) 4. (Heang and Sassa, 2012a) 5. (Heang and Sassa, 2012b)
	OsPIL12	*OsPRR1*[1]	Hypocotyl elongation[1]	1. (Nakamura et al., 2007)
	OsPIL1 *OsPIL13*	*OsPRR1*[2], *OsPORB*[3], *OsCAO1*[3], *OsGLK1*[3], *OsGLK2*[3]	Internode elongation with increased cell size[1], drought stress tolerance[1], Chlorophyll biosynthesis[2], Grain size[1]	1. (Todaka et al., 2012) 2. (Nakamura et al., 2007) 3. (Sakuraba et al., 2017)
	OsPIL14 *OsPIF14*	*OsDREB1B*[1], SLR1[2], *OsEXPA4*[2]	Cold tolerance[1], salt stress tolerance[2]	1. (Cordeiro et al., 2016) 2. (Mo et al., 2020)
	OsPIL15	*OsPUP7*[2], *OsmiR530*[3]	Hypocotyl length[1], Auxin biosynthesis[1] Grain Size[2], Grain yield[3]	1. (Zhou et al., 2014) 2. (Ji et al., 2019) 3. (Sun et al., 2020)

Source: Wang et al., 2022

Therefore, after a little more than a decade of the first characterization study of the family of rice plant PIFs, detailed molecular work to structurally understand these transcription factors has been carried out, but little functional work has been carried out with OsPILs. In this way, a range of responses of the role of these transcription factors needs to be elucidated.

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6. CAPITULO II - OSPIF14 KNOCKOUT TRIGGERS DISTURBANCES IN STARCH SYNTHESIS AFFECTING SEED FORMATION AND GENERATING A VICIOUS CYCLE IN RICE DEVELOPMENT

OsPIF14 knockout triggers disturbances in starch synthesis affecting seed formation and generating a vicious cycle in rice development

Abstract - PHYTOCHROME TRANSCRIPTION FACTORS (PIFs), in addition to interacting with phytochromes inside the nucleus, are also involved, direct or indirectly, with several physiological processes which are still poorly understood. We have previously observed that OsPIF14 knockout (CRISPR/Cas9 transformation) affects seed germination and grain filling in rice. In this work, we hypothesized that this gene could be associated with grain filling and seed formation via sugar metabolism, affecting the starch accumulation and seed viability. Consequently, the generated plants should present a lower development and a progressive vicious cycle until the reproductive phase. OsPIF14 knockout induced decreases in tiller number, what was positively associated with the number of panicle formation and grain filling intensity. The seed and panicle growth rates throughout the reproductive phase were significantly reduced, resulting seeds smallest in size and amount. This growth model was paralleled by the starch accumulation rates and transformed plants seeds presented lower starch content and higher contents of sucrose and total sugars at end of the maturation phase (R9) associated with lower contents of proteins and soluble amino acids. In addition, these seeds displayed lower viability, as indicated by low germination rates, and increased electrolyte leakage. Further, OsPIF14 knockout seeds presented lower seedling establishment, shoot and root elongation and delayed development in all vegetative stages. Thus, the proposed hypothesis must be accepted, evidencing that OsPIF14 knockout is capable to induce several physiological disturbances in rice development performing a vicious cycle which could be initiated during seed formation, probably involving starch synthesis and sugar metabolism.

Key words: germination; grain filling; *Oryza sativa*; plant development; sugar metabolism; starch synthesis.

7. INTRODUCTION

Rice (*Oryza Sativa* L.) is a cereal consumed worldwide, thus being the main food source of the population. However, the production and development of rice are complexes processes that involve abiotic factors, especially light that is sensed by the plant photoreceptors (VOITSEKHOVSKAJA., 2019). Among these receptors responsible for sensing light, phytochrome (Phy) takes two forms: the active (Phy-fr) after absorbing far-red light (730 nm wavelength) and the inactive (Phy-r) related to the the red spectrum (660 nm wavelength). When in the active form, phy-fr moves to the nucleus and interacts with specific PHYTOCHROME TRANSCRIPTION FACTORS (PIFs). This interaction results in a series of upstream responses, regulating the expression of genes that are involved in plant development and growth (WANG et al., 2022).

PIFs belong to the bHLH family. In Arabidopsis thaliana there are eight PIFs isoforms (AtPIF1 - AtPIF8) and many PIF-like candidates (LEE; CHOI, 2017). In rice plants, there are six PHYTOCHROME-INTERACTING FACTOR 3-LIKE (OsPIL11 - OsPIL16) - (NAKAMURA et al., 2007). All OsPILs have a binding site to interact with the three phytochrome types of rice (OsphyA, OsphyB and OsphyC). However, OsPIL14, OsPIL15 and OsPIL16 interact with OsphyB (CORDEIRO et al., 2022), but OsPIL14 is the unique that binds active phytochrome B form, hence was called OsPIF14 (CORDEIRO et al., 2016). In general, it is known that this interaction results in several physiological responses, such as changes in seedling etiolation, plant architecture, development, flowering, and reproductive phase (PAIK et al., 2017).

Several studies on upstream responses are widely reported in Arabidopsis and although various studies involving PIFs have been carried out in rice plants, little is known about the physiological responses regulated by these transcription factors in this plant species (WANG et al., 2022). Recently, molecular assays showed some possible upstream responses related to OsPIL interactions. Nakamura et al., (2007) showed that OsPIL11, OsPIL12 and OsPIL13 interact with PSEUDO-RESPONSE REGULATOR 1 (OsPPR1), an important protein that regulates the circadian cycle of plants. Cordeiro et al, 2016, characterized the rice OsPIF14 gene and, in this work, it was demonstrated that its expression is responsive to circadian rhythms and abiotic stress stimuli (salinity, temperature, water deficit). Furthermore, OsPIF14 act as a repressor through repression of the OsBREB1B repressor gene, a known TF regulator of cold stress (CORDEIRO et al., 2016).

However, to validate the physiological responses of PIFs interactions and the signalling routes associated, in vivo studies are still needed. Zhou et al., 2014 showed that OsPIL15 acts as a repressor of etiolated plantlet growth through the regulation of auxin- related genes (ZHOU et al., 2014), a phytohormone known to promote cell expansion (XIE et al., 2019). This regulation modifies the tiller angle and grain size (JI et al., 2019). In addition to OsPIL15, OsPIL11, OsPIL13 and OsPIL16 have been reported to regulate grain size (TODAKA et al., 2012; YANG et al., 2018). Mo et al., 2020 showed an OsPIF14-SLR1 interaction, which directly regulates genes associated with gibberellin metabolism (MO et al., 2020), a phytohormone that regulates several vital plant processes including seed germination and dormancy. Furthermore, OsPIF14 reactivates 1-Aminocyclopropane-1-Carboxylate Synthase1 (OsASCO1), a precursor of ethylene (JIE et al., 2022).

OsPIF14 is antagonistically regulated by gibberellic and jasmonic acid, supported by its interaction with Slender Rice 1 (SLR1) (UM et al., 2018), and related to chlorophyll biosynthesis by controlling the activation of the OsFLU1 protein (LI et al., 2019). Recently, Mo et al., 2020 showed that OsPIF14 overexpression promotes mesocotyl and root growth, specifically in the dark and under salt stress. Moreover, it was found that OsPIF14 is negatively regulated by salt and that it physically interacts with SLR1, which negatively regulates its expression. Cordeiro et al., 2016 characterized the OsPIL14 gene and during in vivo analysis OsPIF14 gene expression was modulated by different treatments, such as cold, drought, salt, and abscisic acid.

Previously, we have empirically observed that OsPIF14 knockout induces reduction in seed germination and growth in rice. As some PIFs are involved with several physiological processes and metabolically associated with plant hormones, we postulated that the lack of these genes could be capable to trigger generalized physiological changes able to affect plant development in specific stages. What mechanisms could induce reduction in seed germination and plant growth caused by PIF14 knockout? Could the absence of PIF-phytochrome interaction triggers the expression of other genes and inducing crosstalk involving different pathways associated with hormonal balance capable to affect grain filling and seed formation?

Interestingly, Wei et al., 2017 studying the involving of starch and sugar metabolism in rice seed formation, characterized the physiological role of the GIF2 (GRAIN INCOMPLETE FILLING 2) gene demonstrating that it encodes to an ADP-glucose pyrophosphorylase large subunit. Grains of gif2 showed a slower filling rate and reduction in grain weight and the starch content was noticeably decreased. Moreover, gif2 endosperm cells showed defects in compound granule formation. The authors concluded that GIF2 genes are

involved in various sugar metabolism, seed formation and grain filling steps. Despite the PIFs are involved in several physiological processes in rice via hormonal balance, the evidence for their contribution to grain filling and yield are lacking (CORDEIRO et al., 2022).

Here, we hypothesized that PIF14 knockout could affect the rice seed germination due to impairment its formation. This problem could alter plant development in different stages, including starch accumulation, grain filling and seed formation. To test this hypothesis, we performed experiments from seed to seed, using two consecutive generations. Seeds generated from a previous generation of KO-OsPIF14 rice plants presented alterations in the morphology associated with low starch accumulation. In addition, these seeds displayed high contents of sucrose and total soluble sugars and in the next generation, rice transformed plants exhibited low rates of grain filling in parallel to starch accumulation. We postulate that this physiological condition can have generated a vicious cycle in rice development, starting provably by a decrease in starch accumulation and synthesis in seeds. These finds are discussed in an integrative way involving gene expression, crosstalk among metabolic pathways and plant plasticity.

8. HYPOTHESIS

PIF14 knockout could affect the rice seed germination due to impairment its formation. This problem could alter plant development in different stages, including starch accumulation, grain filling and seed formation.

9. OBJECTIVES

9.1. General objectives

Understand how the Phytochrome Interaction Factor 14 (PIF14) acts on the physiology of rice plants throughout development.

9.2. Specific objectives

- **a.** To evaluate the sugars content of knockout PIF14 quiescent rice seeds;
- **b.** To analyse seed germination of knockout PIF14 rice lines;
- **c.** Evaluate PIF14 absence responses in rice plants in physiological processes such as germination, seedling establishment, vegetative and reproductive stage.

10. MATERIAL AND METHODS

10.1. Plant material and growth conditions

Seeds of rice plants (Oryza sativa ssp. japonica cv. Nipponbare) including no-transformed plant (NT) and the three OsPIF14 knockout lines (KO1, KO2 and KO3) used in all experiments were supplied by Genomics of Plants Stress Unit, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa and Instituto de Biologia Experimental e Tecnológica (Portugal). Seeds were germinated in Germitest® paper under controlled conditions on growth chambers (25 ºC, 70 % relative humidity, 100 µmol photons m^{-2} s^{-1} and 12 h photoperiod). The former was used for germination and grain-filling development assessment for 16 days. From the latter, ten-day-old grain-filling were transplanted to 3.5 L plastic pots filled with ¼ diluted nutrient solution (Hoagland and Arnon., 1950) in greenhouse conditions (day/night mean temperature of 30/25 °C, mean relative humidity of 65%, maximum photosynthetic photon flux density (PPFD) of 1000 µmol m−2 s−1 at noon, and a photoperiod of 12 h). Nutrient solution pH was adjusted to 6.0 ± 0.5 with 1 M KOH or 1 M HCl and fully changed weekly. Three weeks after transplantation, plants were transferred to nutrient solution in total force.

10.2. Germination and phenotype analysis

The germination rate was obtained using six replicates of each genotype (NT or KO-PIF14), using 1 mm coleoptile length as a marker for germination. The shoot and root length of seedling were measured daily with a calliper and shoot, and root length of the knockout and no-transformed plants were performed daily under controlled conditions using a ruler. The grain length, width and thickness of the transgenic and WT rice grain-filling were performed using 25 grains of each genotype under controlled conditions with a calliper. Plant yield and grain viability analyses were adapted from Prasad et al. (2006). Ten plants of each genotype were randomly collected at physiological maturity and the reproductive parts (panicles) were dried in an oven at 30 ºC for 5 days. After drying, the panicles were threshed manually and the dry mass of 100 grains was recorded. Ten plants of each genotype were randomly collected at physiological maturity and the reproductive parts (panicles) were dried in an oven at 30 ºC for 5 days. After drying, the panicles were threshed manually and the dry mass of 100 grains was recorded. For time course analyzes of the grain filling period, the plants

were collected from the reproductive stage - R4 (after anthesis) of each genotype (KO-OSPIF14 and NT) every 7 days until the grains reach their fully mature stage - R8 (28 days after anthesis). The number of panicles and grains per were manually estimated from each collection. Fresh mass of panicles was estimated from the pots and spikelet and the fresh mass of the grains were performed by manually highlighting the panicle grains of each genotype and recording the weights.

10.3. Grains integrity analysis and weight of 100 seeds

Grain vigor was adapted from Mckersie; Tomes; Yamamoto (1981). Five replicates containing 150 mg of grains immersed in 5 mL of deionized water were homogenized at room temperature (25º C). The conductivity (L1) was measured 24h after homogenization. Grain vigor was calculated by dividing the electrical conductivity (L1) for the sample weight (g). And for membrane damage replicates were boiled at 95 ºC for 1h. The samples were cooled to room temperature and the electric conductivity (L2) was measured as described by Lima Neto et al. (2014) and calculated using the following equation MD = (L1/L2) x 100. The weight of 100 grains was performed by weighing quiescent grains by randomly selecting 100 dry grains of each genotype and recording the weights.

10.4. Carbohydrate and nitrogenous compounds content quantification

Soluble sugars were extracted from 20 mg of lyophilized grain material with 1,5 mL of MCW solution (Methanol: Chloroform: Water 12:5:3 v/v/v) and incubated in thermomixer for 30 min at 30 ºC and 400 rpm, twice. The homogenate was centrifuged at 10,000 g for 10 min at room temperature and the supernatants were collected and reunited. The starch content in the MCW pellet was determined by hydrolysing it with 1,5 mL of HClO4 (30% v/v) following the same specifications for soluble sugars. Soluble sugars from MCW and acid extraction were measured by the phenol-sulphuric acid method (Dubois et al., 1956) and sucrose was quantified through direct micro determination (Van Handel, 1968).

10.5. OsPIF14 interaction network

To build the protein interaction network, the STRING protein-protein interaction database (Version 11.5) was used, enriched by means of genomic prediction data, high-throughput laboratory experiments, co-expression analyses, automated text mining and information from other databases, currently covering a total of 5,090 organisms and 24,584,628 proteins. The software is formed by a consortium between SIB (Swiss Institute of Bioinfomatics), CPR (Novo Nodisk Protein Foundation Center) and EMBL (European Molecular Biology Laboratory). For the construction of the interactome, OsPIL/PIF14 with entry code A0A0P0X2F6, identification Os07g0143200 protein, containing 398 amino acids for the organism Oryza sativa Japonica Group, was used as input protein. For the enrichment of the interactions network, only interactions with a score ≥ 0.700 (high confidence), obtained only from experimental origin, that are part of a physical interaction complex were selected. At the end, the network was transferred to the Cytoscape 3.9.1 software, where the protein-protein interaction network was assembled.

10.6. Cloning of CRISPR/Cas9 transformation cassettes

Cas9-mediated genomic insertion and/or deletion events at PIF14 loci were generated using pMiao vector (Miao., et al 2013), a binary vector containing a hygromycin resistance gene driven by the cauliflower mosaic virus 35S promoter. The target guide RNA 5' – ACCGCCATCCGCGAACCACGCGG – 3' was selected for PIF14 locus and cloned into pMiao. The final vector was introduced into Agrobacterium tumefaciens EHA105 for rice transformation.

10.7. Rice calli transformation and identification of pif14 mutants

Stable transformations were generated using wild-type rice cv. Nipponbare following established methods (Hiei and Komari 2008). Plant lines obtained from tissue culture harboring the hygromycin cassette were analyzed for the presence of insertions or deletions (InDels) in the PIF14 locus. The target region was amplified by PCR in WT and transformed plants. PCR products were Sanger sequenced and the resulting chromatograms were compared

using the decomposition tool TIDE (Brinkman., et al 2014). Identified lines with InDels in the target locus are compiled in Supplemental table 1. Primers used for genotyping are available in Supplemental table 2.

10.8. Statistical analysis

The experiments were arranged in a completely randomized design. For seed germination measurements, seeds of each genotype (NT, KO1, KO2, KO3) were divided into five groups (each consisting of 10 seeds distributed in individual paper roll which each one represented a statistical replicate) and measurement were performed daily. For experiments during vegetative and reproductive phases, was employed four replicates being each one represented by 3L pots containing three plants. Data were submitted to analysis of variance (ANOVA) and Tukey's test at 5% of probability. All statistical analyzes were performed using SigmaPlot 12.0 program (Systat Software, San Jose, USA).

11. RESULTS

11.1. Molecular characterization of rice lines transformed for OsPIF14 knockout

We used three independently transformed lines of PIF14 knockout and non-transformed rice plants (NT, KO1, KO2, KO3) for this study, as previously described. After the successful in editing and transformation, DNA changes were confirmed by gene sequencing. All lines obtained in this work were confirmed through CRISPR/Cas9_OsPIF14 _Transcripts. The results were observed by base pair deletion from the initiation codon. In line #1 knockout, called KO1, we had a deletion of 4 base pairs (-4pb), while in row #2, 37 base pairs (-37pb) were deleted, which we now call KO2. For line #3, we observed that there was a deletion of only one guanine (-1bp) after the initiation codon and here we call this line of KO3 (Table S1).

11.2. OsPIF14 knockout induced alterations in seed morphology and vigour

In this first experiment, we utilized seeds obtained from the T2 generation of three lines of OsPIF14 knockout (KO1, KO2, KO3) and non-transformed (NT) rice plants after previous phenotyping and genotyping. To verify the effects of the lack of the OsPFI14 gene

on seed integrity, we analysed electrolyte leakage and germination rate (24h) in quiescent seeds of transformed and non-transformed plants. Seeds from transformed rice plants showed higher electrolyte leakage (a membrane integrity indicator) than non-transformed ones, showing an average among the three KO lines corresponding 59% higher of membrane damage compared to NT, indicating that seed integrity was drastically affected after PIF14 knockout (Fig. 1A). In addition, the germination rate, other seed integrity indicator, was drastically reduced after 24h of imbibition in KO-OsPIF14 lines, reaching a maximum decrease of 60% compared to NT (Fig. 1B), corroborating the previous results of electrolyte leakage.

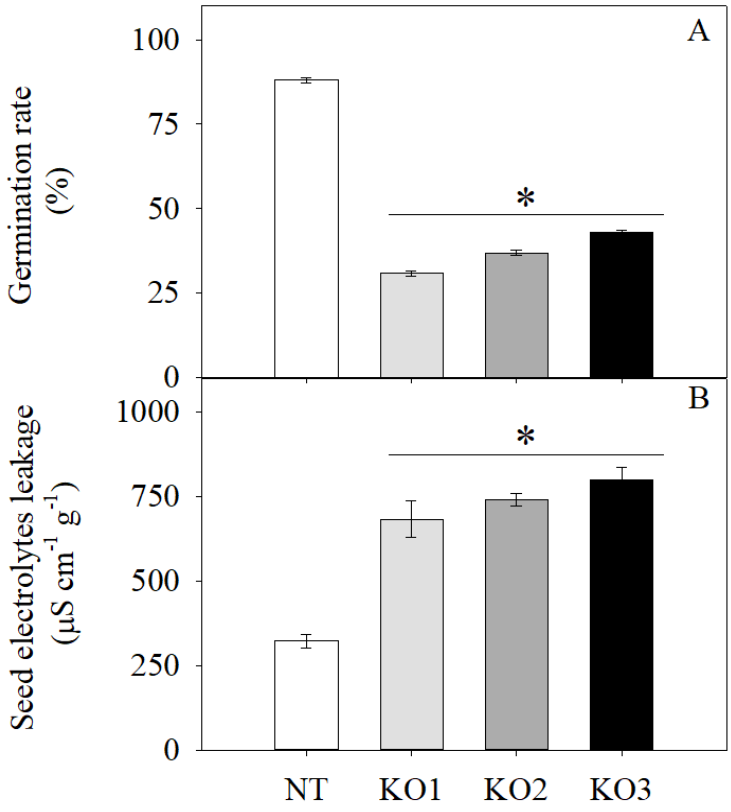

Figure 3. Integrity and vigour of the KO-OsPIF14 rice seeds. Changes in (A) germination rate (%) after 48h of sowing and (B) electrolyte leakage of quiescent seeds after 24h in three OsPIF14 knockout lines (KO1, KO2 and KO3) and in non-transformed plants (NT). The values are average of five independent replicates (±SE), where * represents significant difference between NT and transformed plants ($P < 0.05$) according to the Tukey's test.

The productivity parameters, such as grain size, length, width, and thickness, were reduced by approximately 12%, 14%, 23% and 13%, respectively, in comparison with NT (Fig. 2). This was reflected in the weight of 100 seeds which was 6% lower in KO-PIF14 plants compared to NT. In parallel to these alterations, KO-PIF14 seeds also exhibited great changes

in the contents of starch (which was intensely decreased), sucrose and total soluble sugars, that were largely increased (Table S3). These results indicate that the OsPIF14 knockout plants induces negative effects on grain filling, generating seeds with lower growth and low vigour or viability associated with alterations in the levels of starch and sucrose, suggesting a regulatory role for OsPIF14 during the rice grain filling via starch metabolism.

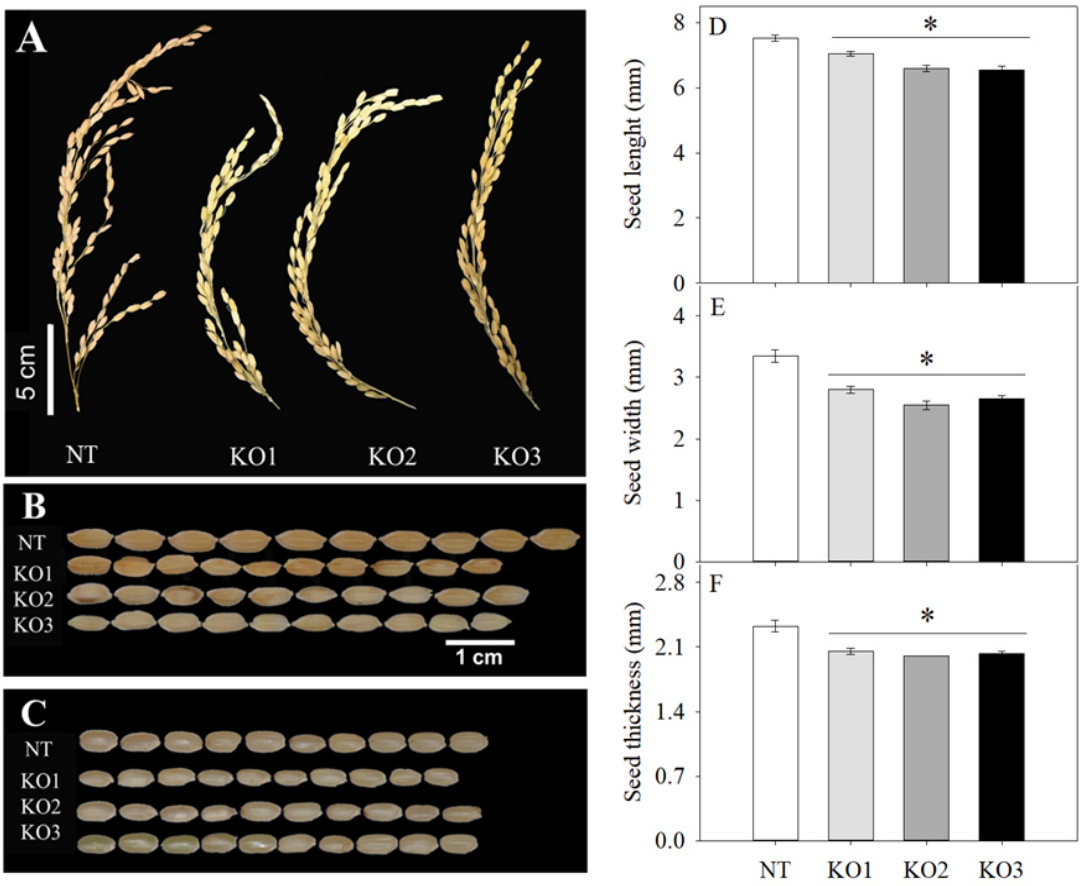

Figure 4. Morphological characterization of KO-OsPIF14 rice seeds. Morphological characteristics in (A) panicles of non-transformed (NT) plants and three knockout OsPIF14 lines plants (KO1, KO2, and KO3) at maturation phase (R9), (B) seeds with the hull, and (C) seeds without hull. Scales in cm are shown inside pictures. The right figures show mature grain size parameters from without hulls quiescent seeds: (D), total seed length, (E) width, and (F) thickness). Values indicate the averages of five independent replicates (±SE).

11.3. OsPIF14 knockout affected various phases of vegetative development: seedling establishment, root and shoot elongation, tillering and dry matter accumulation

Since T2 seeds of KO-PIF14 lines displayed formation problems, as indicated low viability associated with disturbance in starch accumulation and sugar metabolism, we performed a detailed experiment involving all phases of vegetative development of rice plants (seed germination index, seedling establishment, root and shoot growth and tillering intensity). This study was performed to verify if those localized problems in seeds by effect of OsPIF14 knockout could be transferred to plant development and in which phenological stage. Transformed plants showed a significant reduction in germination rates after 48h of sowing (88%, as average of the three lines), in comparison to NT (Fig. 1A). After germination, these seedlings showed strong delay in establishment and afterwards they presented strong reduction in the rates of root and shoot elongation (cm/plant) throughout all vegetative phases (Fig. 3). For instance, after 16 days of germination KO-PIF14 plants exhibited 65% and 67% reduction in shoot and root length, respectively, compared to NT plants (Fig. 4). When the growth reduction was expressed in terms of dry matter, the knockout lines exhibited a decrease of 30% and 35% in shoot and roots, respectively, compared to NT plants, whereas the shoot/root ratios were decreased by 21% (Fig. S3).

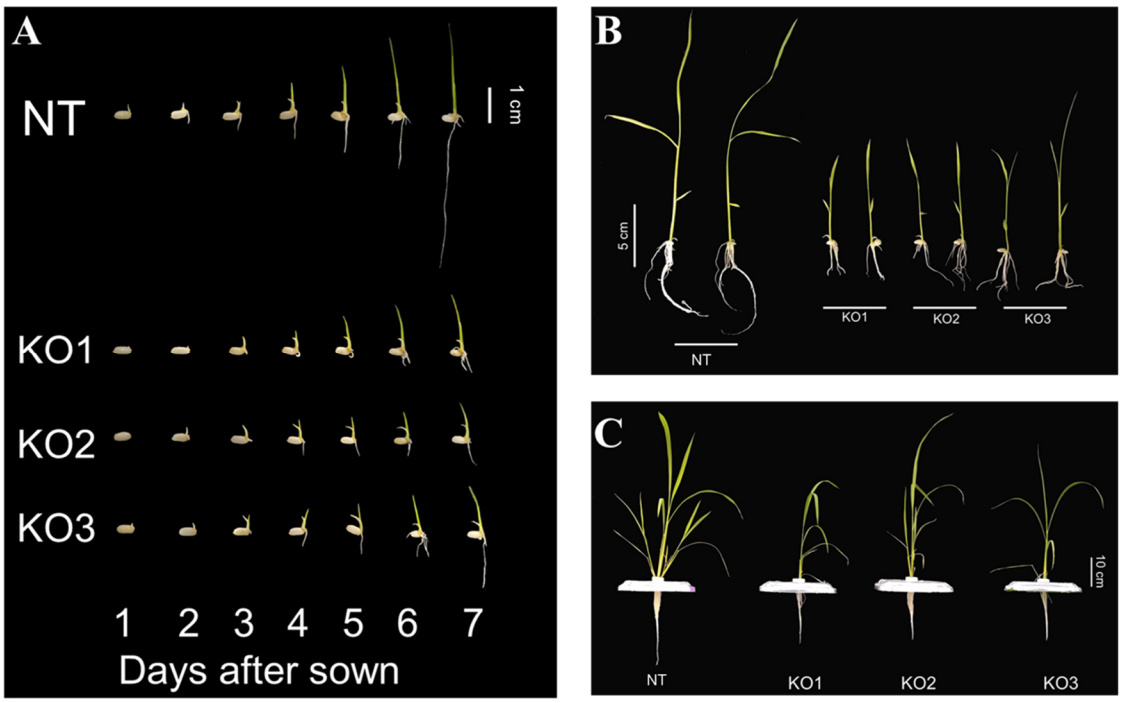

Figure 5. Morphological characterization of KO-OsPIF14 rice seedlings during post-germination and initial vegetative development stages. Pictures show phenotypic differences

between non-transformed (NT) and three knockout OsPIF14 lines seedlings (KO1, KO2, and KO3) in different days after sown: (A) throughout 7 days, (B) at 16-day-old, and (C) at 20-day-old. Scales are in cm and seedlings and plants are representative from five replicates.

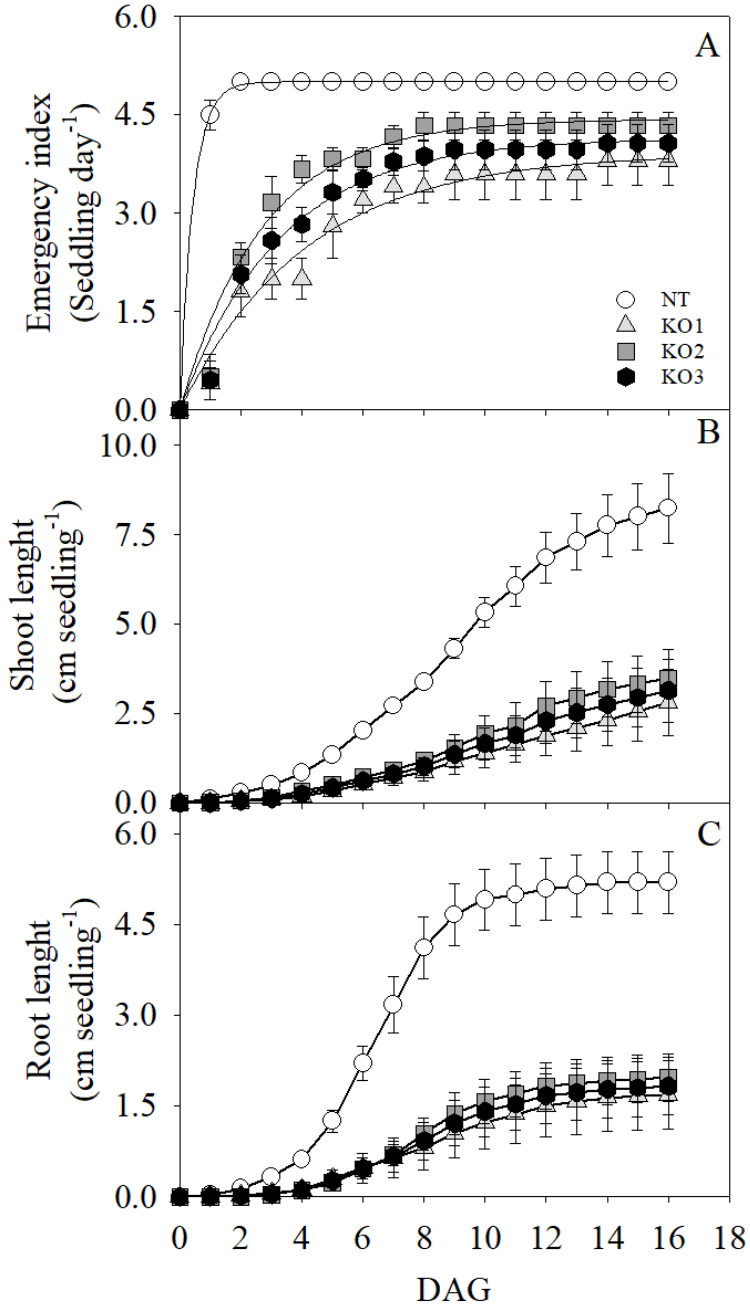

Figure 6. Alterations in rates of emergency and root and shoot elongation of KO-OsPIF14 seedlings. KO-OsPIF14 (KO1, KO2, KO3) seedlings presented delay in (A) seedling emergency index, (B) shoot elongation, and (C) root elongation throughout 16 days of growth in comparation with non-transformed plants (NT). Points are averages (± SE) of three replicates

Nevertheless, after this period, when plants reached the mature phase (from V8 to V13 stages), the negative effects induced by OsPIF14 knockout on root and shoot elongation were minimized, especially in shoot, in comparison to NT plants (Fig. 5A – B, Fig. S2). For instance, after 55 days after germination, KO-PIF14 lines showed decrease in root and shoot length by 83% and 14%, respectively. In contrast, the effects caused by OsPIF14 knockout on shoot dry mass accumulation were much more accentuated in comparison to shoot elongation, exhibiting decreases of 30% and 35%, in shoot and roots, respectively (Fig. 6A - B). At the same time, the shoot/root ratios decreased by 21% (Fig. S3), compared to NT plants, indicating that in vegetative phase the root growth was more affected by OsPIF14 knockout than shoot. In contrast, OsPIF14 knockout affected drastically the tillering, as expressed as number of tillers per plant (Fig. 5C). This important physiological process was initiated approximately 20 days after germination at V4 stage and after 55 days the tilling in transformed plants was 47% lower than that presented by NT plants. These drastic effects of OsPIF14 knockout on tilling directly affected the plant growth capacity as expressed in dry matter accumulated in roots, shoot and whole plant. These results evidence that shoot and root elongation rates (cm/part) are not good parameters to indicate plant growth, as is widely known.

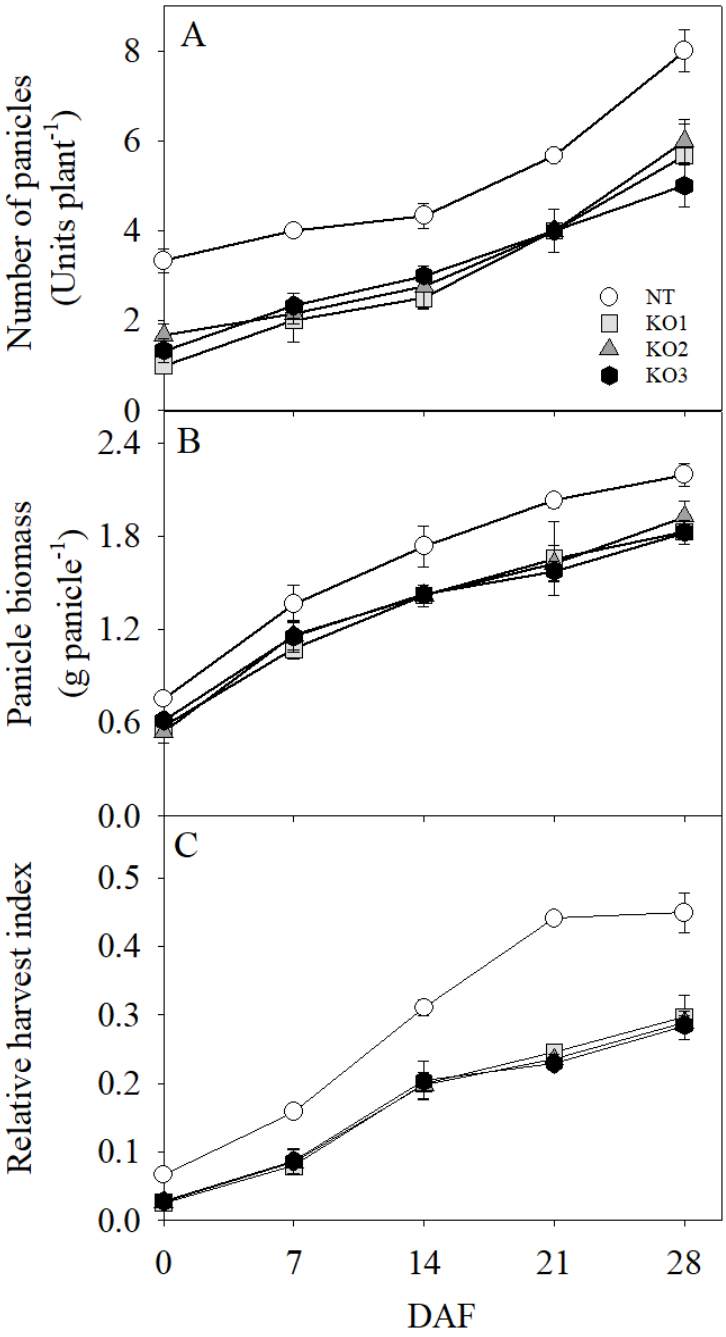

Figure 7. Panicle development and grain filling in KO-OSPIF14 plants. Changes in panicle development during grain filling in different reproductive stages measured in days after anthesis (DAA) in non-transformed (NT) and three knockout OsPIF14 lines plants (KO1, KO2, and KO3). (A) The number of panicles per plant, (B) panicle biomass, (C) relative harvest index. Points are averages of three replicates and standard deviation (±SE).

11.4. OsPIF14 regulates starch accumulation in parallel to grain filling and seed growth

Previously, we showed that OsPIF14 knockout regulates significantly various physiological processes throughout entire vegetative phase, especially seed germination, initial growth, and tillering. To understand better the effects of KO-PIF14, we performed a detailed study involving entire reproductive phase since anthesis from day 0 of anthesis until almost complete grain maturation (R8 stage). Five samples were harvested during this period after anthesis: 0, 7, 14, 21 and 28 days. The time-course data clearly indicate that KO-PIF14 lines suffered strong reduction in the rates of panicle formation associated with lower growth (fresh biomass), intense decrease in seed growth and slight decrease in number of seeds per panicle (Fig. 6 - 7).

The negative effects induced by OsPIF14 knockout on the panicle development were more intense on the panicle number in rice shoots (Fig. 6A) – a reduction of approximately 2-fold since the begin of the period of panicle formation compared to NT plants and this effect persisted during all reproductive phases. The adverse effects on the panicle growth (biomass accumulation) occurred in minor extension but the reduction was highly significant in all reproductive stages (Fig. 6B). These harmful effects caused by KO-PIF14 on panicle development reflected directedly on the harvest index (total seed mass/whole plant mass), which was decreased by approx. 50% after 28 days of anthesis in comparison to NT plants (Fig. 6C).

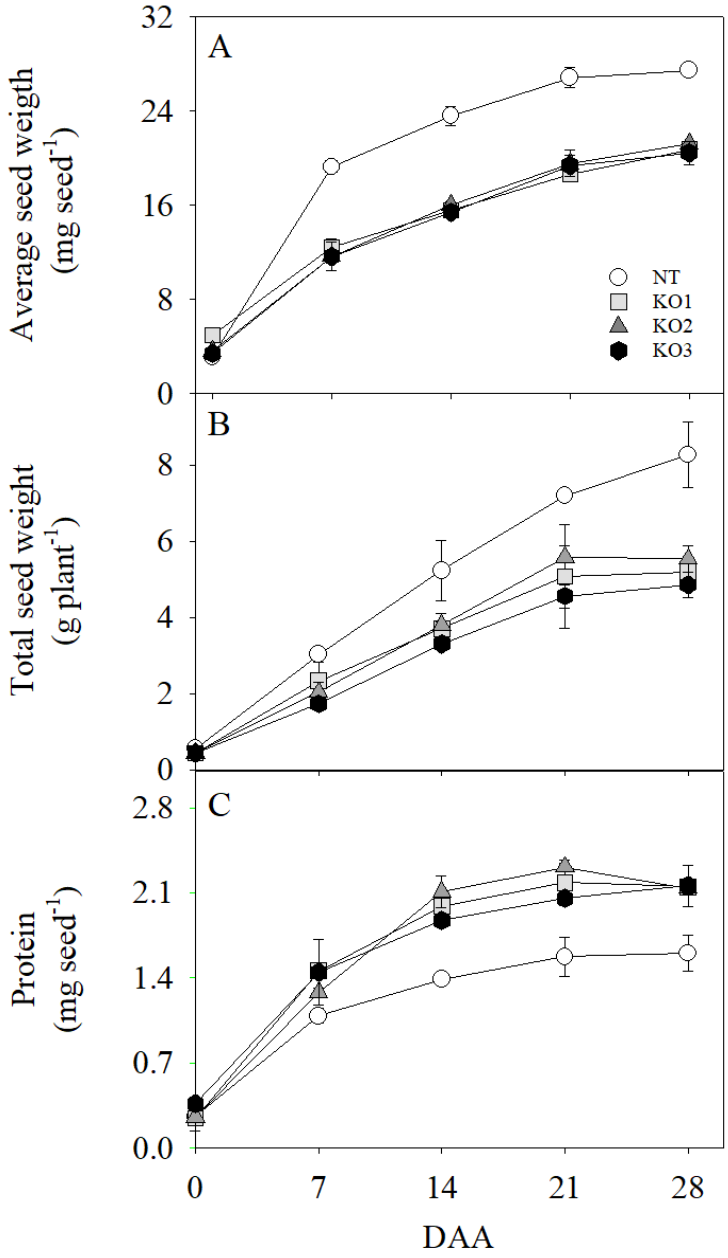

Figure 8. Seed growth and protein accumulation in KO-OSPIF14 plants. Changes in seed growth during grain filling evaluated at different days after anthesis (DAA) in non-transformed (NT) and three OsPIF14 knockout lines plants (KO1, KO2 and KO3). (A) Average seed weight, (B) total seed weight, and (C) seed protein accumulation. Points are averages of three replicates and standard deviation (±SE).

As expected, the deleterious effects induced by PIF14 knockout on panicle development also reflected directly on the seed development as expressed as average of individual seed weight (Fig. 7A) which was reduced by approx. 40% at maturation phase compared to NT plants. A similar reduction was observed in the rice yield (total seed weight

per plant) – (Fig. 7B), which was directly proportional to that decrease previously observed in the harvest index. Interestingly, the KO-PIF14 lines presented higher total protein content in seeds (Fig. 7C) which was inversely proportional to starch content as will further shown. Indeed, the starch accumulation (μmol seed^{-1}) was drastically reduced in OsPIF14 knockout lines compared to NT plants. After 7 days after anthesis the values reached in transformed plants were approximately 3-fold lower than NT genotype and this response was an additive effect of lower starch content (μmol g-1) and lower size growth (mg seed^{-1}). Thus, it is interestingly to observe that the reduction in the starch synthesis/accumulation rates induced by KO-PIF14 occurred in the first periods of seed formation (7 days after anthesis) and after this time the rates were maintained nearly constant, as indicated by the slopes of the curves. In quiescent seeds, the KO-PIF14 seeds showed a reduction of approximately 40% in starch content in comparison to NT seeds (Table S3). Inversely to observed for starch accumulation in seeds, OsPIF14 knockout rice seeds higher sucrose accumulation (μmol seed^{-1}) – a increase by 40% compared to NT plants (Fig. 7B), at begin of seed formation (at R4 stage). In all studied phenotypes the sucrose accumulation decreased abruptly throughout the reproductive phases reaching low values at seed maturation. Besides sucrose, the KO-PIF14 seeds showed also higher accumulation of total soluble sugars (TSS), reaching an increase of approx. 50% at seed maturation phase (Fig. 7C). However, in contrast to sucrose the KO-PIF14 seeds showed a progressive TSS accumulation during seed formation and maturation.

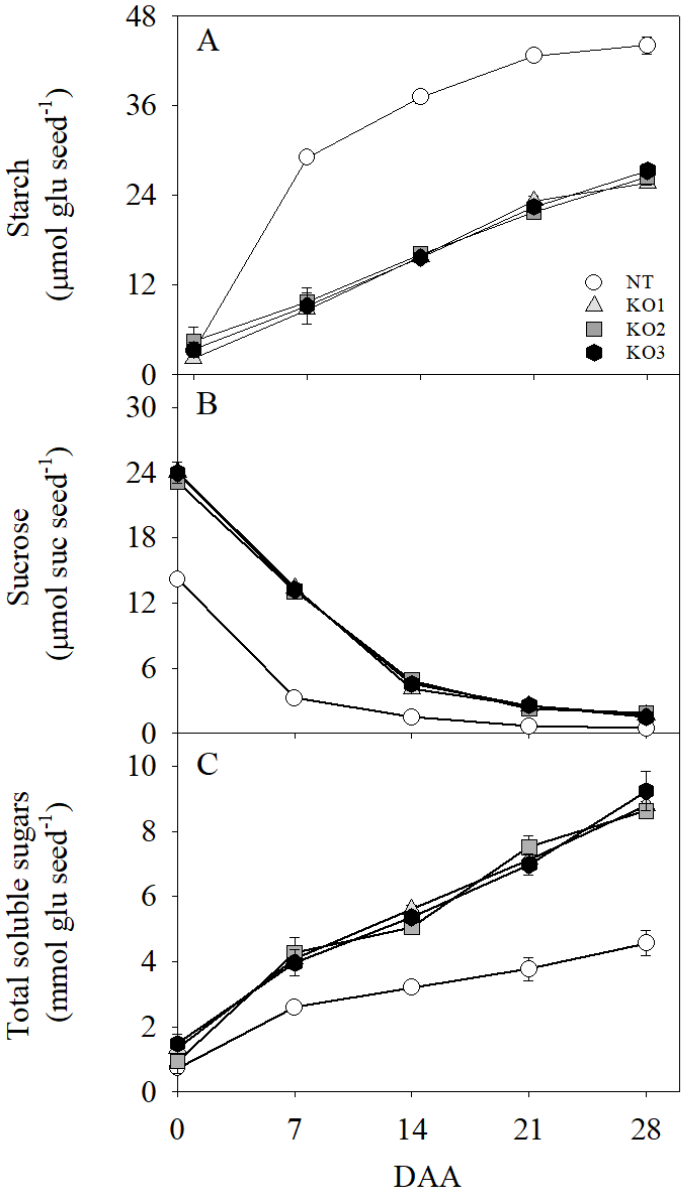

Figure 9. Changes in the accumulation of starch, sucrose, and total soluble sugars in seeds of KO-OSPIF14 plants. Changes in sugar accumulation during grain filling: (A) Starch, (B) sucrose and (C) total soluble sugars, evaluated in different days after anthesis (DAA) in seeds of non-transformed plants (NT) and three lines KO-OsPIF14 (KO1, KO2 and KO3). Points are averages of three replicates and standard deviation (± SE).

12. DISCUSSION

In a previous work, we characterized PHYTOCHROME INTERACTING FACTOR 3-LIKE 14 (OsPIL14; Os07g0143200) henceforth called OsPIF14 due to the evidence of the interaction of this transcription factor with the active form of phytochrome B (Cordeiro et al., 2016). In that work it was observed that OsPIF14 has 85% APB homology with Phytochrome Interacting Factor 4 (PIF4) and Phytochrome Interacting Factor 5 (PIF5) of Arabidopsis thaliana. PIF4 is known to target high temperature-responsive genes (Nusinow et al., 2011; Wigge, 2013), as well as regulate flowering (Nusinow et al., 2011), and growth (Wigge., 2013). Thus, PIF4 and PIF5 are known in Arabidopsis to play key regulatory roles in plant growth and development. In the present study we provide, for the first time, evidence that the OsPIF14 knockout affects negatively various physiological processes related to rice development.

Initially, we empirically observed that KO-PIF14 seeds from a T2 generation exhibit reduced size associated with low starch content. As has been previously demonstrated that reduction in starch synthesis in rice seed induced by knockout of GIF2 (GRAIN INCOMPLETE FILLING 2) gene is associated with the grain filling process (Wei et al. 2017), we hypothesized that OsPIF14 could be involved with a suitable seed formation mechanism. Overall, our results corroborate that hypothesis and add new insights for a comprehensive role displayed by that gene in other physiological processes still not reported in literature. Besides these finds, this current study adds light to an integrate view about OsPIF14 roles which start provably during seed formation involving essentially starch synthesis in seeds. By unknown mechanisms yet, knockout of that gene or it lack can affect the seed integrity during its formation and, in the next generation, these seeds can generate impairment in plant development, initiating by delay in seed germination until reach the grain filling process. Thus, a vicious cycle should be performed from seed to seed.

Grain filling is a fundamental process by which rice productivity and quality is determined. This process is very complex and involves many factors, such as the conversion of different metabolites and several regulatory factors in grain formation (Zhao et al., 2021). During the reproductive stage, the period of flowering induction or anthesis (R4 stage) begins. From then on, the grains are formed and quickly filled by mainly sugars and amino acids produced from photosynthesis and transformed in reserves, mainly starch and proteins (Counce et al., 2000). Therefore, when the plant has a late flowering or some delay in C and N transport from leaves its grains will present a low growth rate which can be reflected in low yield (Yang

et al., 2006). In cereals the most important steps related to the grain-filling process consists in sucrose transport from leaves to seeds and starch synthesis (Zhu et al., 2011). From 4 to 10 days after anthesis there is intense cell division, called endosperm differentiation and this process can define the final number of cells in the grain and it is related with accumulation of starch (Altenbach et al., 2003).

Starch deposition during grain filling is controlled by four key enzymes from the starch biosynthesis pathway: sucrose synthase, starch synthase, UDP - glucose pyrophosphorylase, and ADP - glucose pyrophosphorylase (Zhu et al., 2011). However, low starch synthase activity during grain filling affects grain weight (Kato et al., 2007; Tang et al., 2009). The regulation of genes responsible for starch synthesis has been elucidated over time to contribute to a better understanding of the starch synthesis pathway (Nakamura et al., 2005; Nishi et al., 2001; Fujita et al., 2003, 2006, 2007; Satoh et al., 2003; Li et al., 2009). Our results suggest that directly or indirectly OsPIF14 knockout affect gene expression and activity of some of these enzymes, but further research needs to be carried out to confirm or reject this hypothesis.

Previous studies have reported some transcription factors regulating the expression of genes involved in the starch synthesis pathways. Transcription factors have been reported to regulate AGPase activity (Zhu et al., 2003; Wuriyanghan et al., 2009; Fu & Xue., 2010; Wang et al., 2013; Schmidt et al., 2014; Morita et al., 2015). Schmidt et al., 2014, reported that the transcription factor OsSERF1 influences grain filling and starch synthesis through direct binding with the GBSSI promoter and regulates RPBF that binds directly to pGBSSI. Another reported factor is that FT OsSERF1 also downregulates the expression of AGPL2, SSI, SSIIIa, and GBSSI (Schmidt et al., 2014). Thus, it is suggested that OsPIF14 may act by regulating, directly or indirectly, some enzyme that participates in the starch synthesis during grain filling, possibly via interaction with other genes and transcription factors.

A striking feature in grain production of KO-PIF14 in this study was a lower grain weight and grain size. Generally, grain filling and agronomic characteristics determine some grain traits such as length, width and thickness which can determine the grain mass and size (Fan et al., 2019). However, it is already known that TFs OsPIL11, OsPIL13 and OsPIL15 can regulate grain size (Todaka et al., 2012; Yang et al., 2018; Ji et al., 2019. In this work, we performed a co-expression network at the protein level showing possible interactions of OsPIF14 with other transcription factors including other PIFs with roles already known in Arabidopsis and rice (Fig. S3). Besides some PIFs, another transcription factor that was shown to be regulated or induce regulation on OsPIF14 was the PGL1/PGL2 ANTAGONIST bHLH

(APG). The transcription factor POSITIVE GRAIN LENGTH REGULATOR 1 (PGL1) and (PGL2) is an atypical bHLH that presents in the lemma/palea related to grain increases length and weight in rice plants (Heang and Sassa, 2012a; Heang and Sassa 2012b).

Another protein that showed interaction with OsPIF14 from network assay by bioinformatic tool was OsPRR1 (Figure 7). PSEUDO-RESPONSE REGULATOR (PRR), also known as TIMING OF CAB EXPRESSION 1 (TOC1), controls photoperiodic flowering response in Arabidopsis (Matsushika et al., 2000; Nakamichi et al., 2010). In rice plants, OsPRR1 is already related to interacting with OsPIL11, OsPIL12 and OsPIL13, and this interaction results in phenotype response hypocotyl length of grain filling (Nakamura et al., 2007). Accordingly, we found showed that rice grain filling OsPIF14 knockout plants was a delay in their envelopment during the grain filling establishment period with less hypocotyl length.

The protein-protein interaction network analysed also evidence a possible interface between OsPIF14 and OsPRR1, a gene involved with the number of tillers in rice. Indeed, Wang et al., (2020) showed that rice plants have a minor number of tillers through OsPRR1 overexpression. OsPRR1 represses CIRCADIAN CLOCK ASSOCIATED1 (OsCCA1), a transcription factor that positively regulates the expression of TEOSINTE BRANCHED1 (OsTB1, also known as FC1), DWARF14 (D14), and IDEAL PLANTARCHITECTURE1 (IPA1, also known as OsSPL14), affecting tiller number (Wang et al., 2020). As OsPIF14 knockout strongly affected the rates of tillering in our current study, could be suggested that OsPIF14 could be involved direct or indirectly with tillering via interaction with OsPRR1.

As is summarized in Fig. 8, we are proposing here that OsPIF14 knockout affects rice development in several steps, performing a deleterious vicious cycle in a systemic perspective. As PIFs are involved in several physiological processes which are still scarcely known in the overall plant development, we suggestions are essentially speculative to date. We are initially suggesting that OsPIF14 knockout induces, direct or indirectly, constraints on grain filling and starch synthesis, inducing reduced growth and deformity in seeds as previously has been reported in rice due to lack of GIF2 gene (Wei et al. 2017). Afterwards, in the next generation, these abnormal seeds will present low germination, delay in seedling establishment, low growth rates throughout all vegetative stages and inducing low rates of tilling. This condition will directly affect the rates of panicle formation and development associated with low rates of seed growth and defective formation provably via starch synthesis.

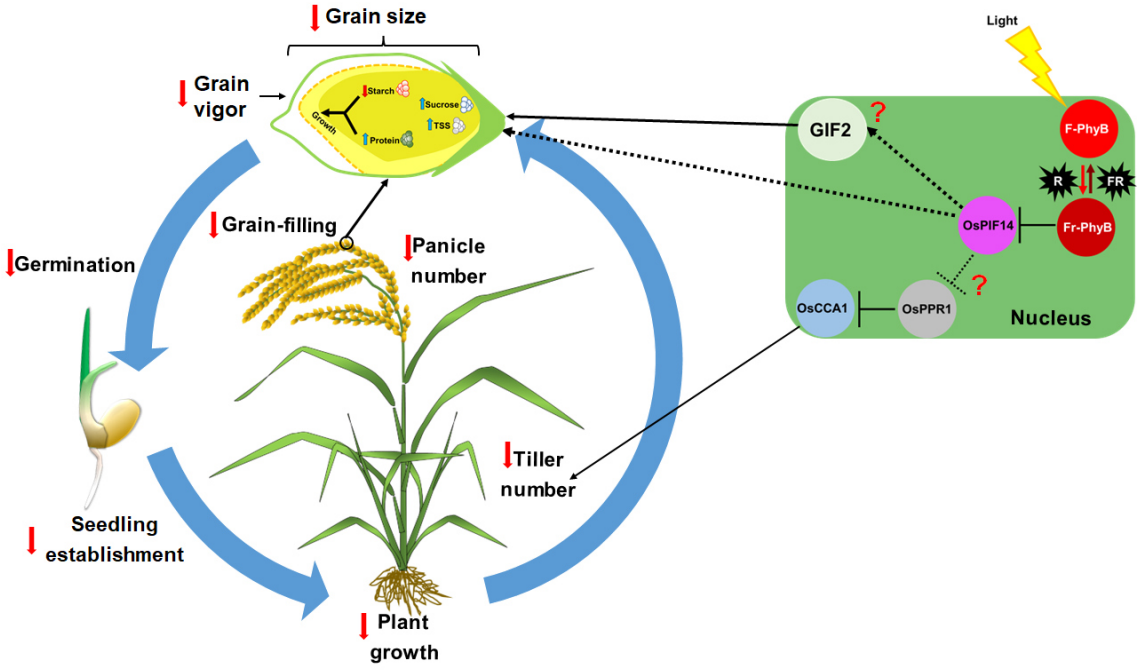

Figure 10. A schematic model summarizing the main adverse physiological effects caused by OsPIF14 knockout in rice triggering a vicious cycle in all plant development. Initially, the lack of OsPIF14 strongly decrease the grain filling associated with intense decrease in tillering. This phase intensely affects seed growth and formation producing deformity in the seeds indicated by low viability and vigor. These seeds in the next generation will show rates of germination, emergency, and seedling establishment. Afterwards, mature plants present low growth rates and low tillering rates directly affecting panicle formation and development. In the next step, the seed starch synthesis is strongly impaired inducing accumulation of sucrose and total soluble sugar and total proteins. This process is directly associated with seed deformity restarting a vicious cycle.

In conclusion, OsPIF14 knockout strongly affects various development processes in rice plants, performing a vicious cycle that is originate possibly during grain filling and seed formation. Afterwards, these constraints induce restrictions in other physiological processes during all vegetative (seed germination, seedling establishment, plant growth and tillering) and reproductive (grain filling and growth) phases, meaningfully affecting starch synthesis and seed viability. Further studies involving plant physiology associated with multi-omics, reverse genetic (overexpression), and bioinformatics approaches are needed to elucidate the direct involvement of OsPIF14 gene in specific physiological processes related to rice yield.

Therefore, the merit of this work is to broaden a perspective for elucidate more physiological roles regulated by PIF genes towards to improve crop yield from genetic management.

13.SUPPLEMENTARY MATERIAL

13.1. Supplemental figures

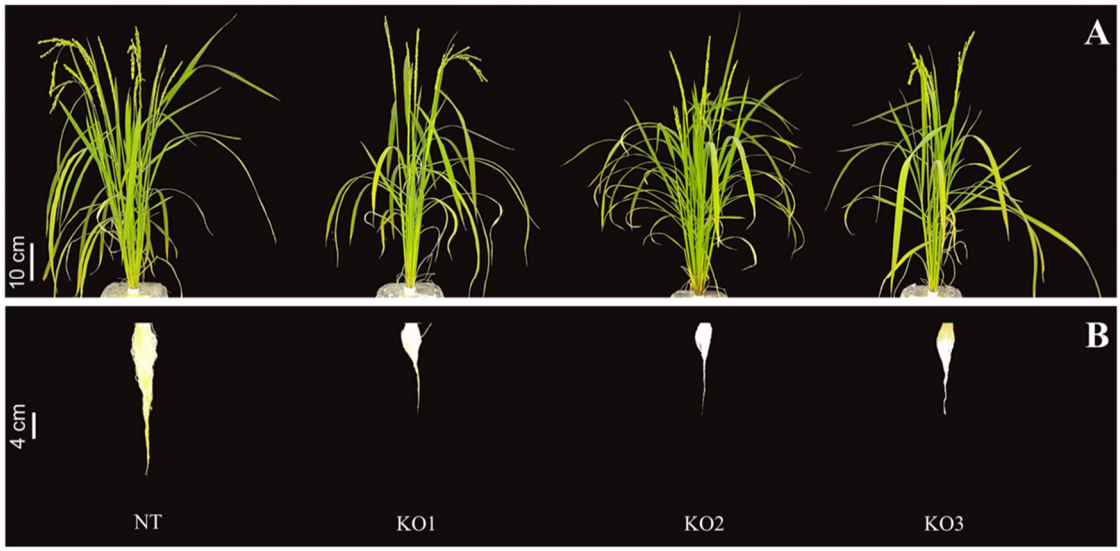

Figure 11. Characterization of KO-OsPIF14 at reproductive stage. The image shows phenotypic differences between non-transformed (NT) and three lines OsPIF14 knockout plants (KO1, KO2, and KO3). (A) Shoot and, (B) root morphology at the R8 reproductive stage. Scales are in cm and shown inside pictures.

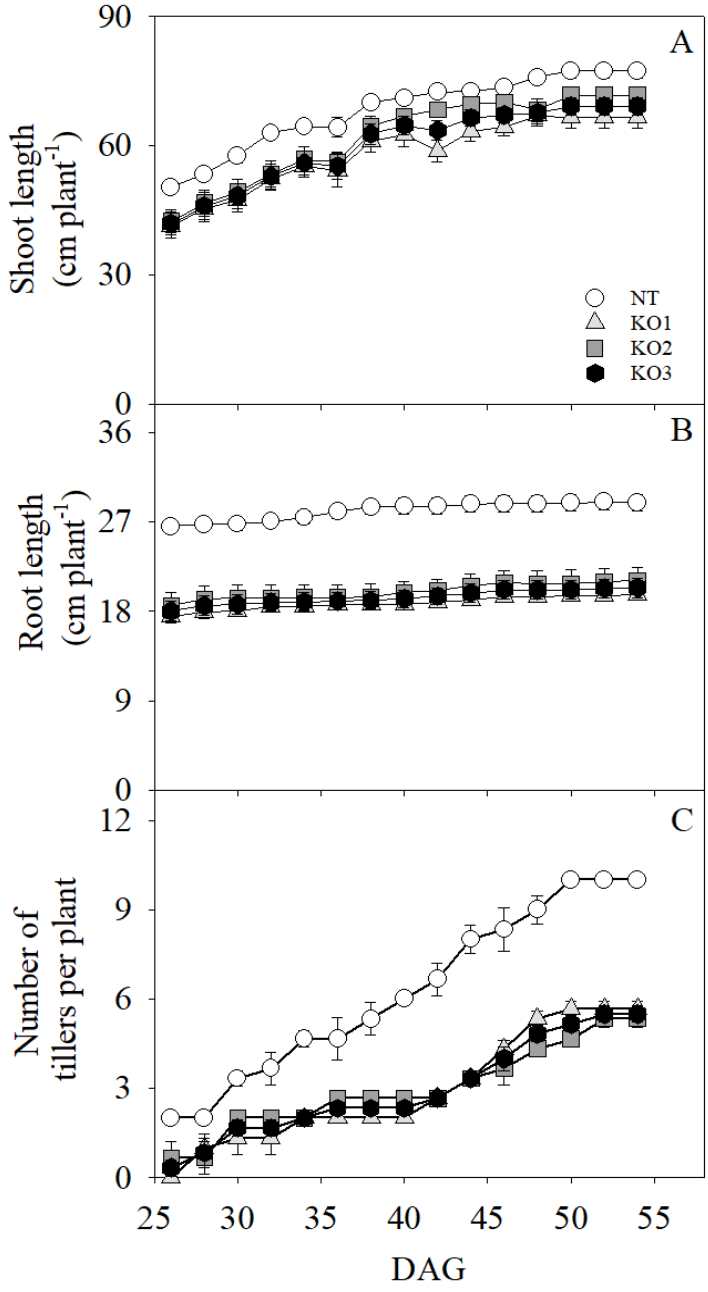

Figure 12. Changes in the rates of shoot and roots elongation of KO-OsPIF14 mature plants. KO-OsPIF14 plants (KO1, KO2, KO3) showed differences (A) shoot elongation rates, (B) root elongation rates, and (C) number of tillers from 26 to 54 days after germination compared to NT plants. Points are average (± SE) of three replicates and ($P < 0.05$) according to Tukey's test.

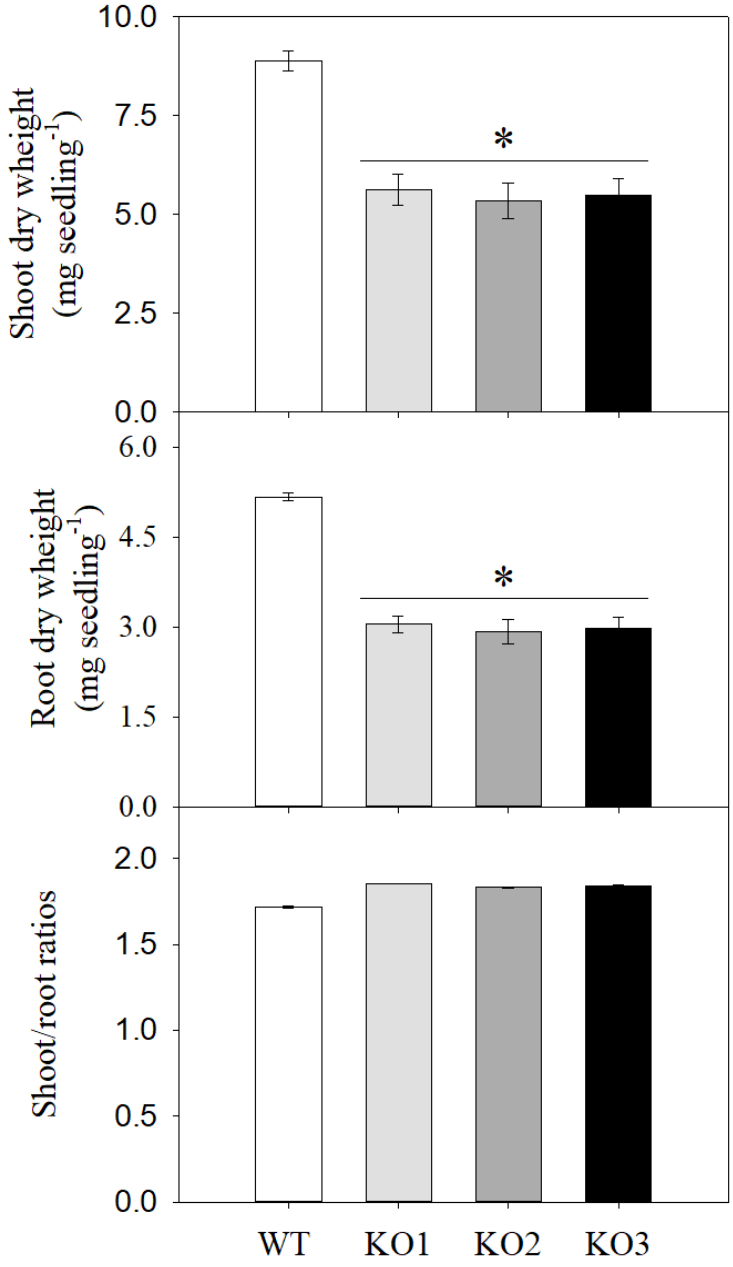

Figure 13. Growth characterization of KO-OsPIF14 seedlings by dry mass accumulation. Dry mass of seedlings at 16 days after germination: (A) shoot dry mass, (B) root and (C) shoot/root ratios in the three OsPIF14 knockout lines compared to non-transformed seedlings. Values are averages (± SE) of three replicates and * represents significant variation between NT and transformed plants ($P < 0.05$) according to Tukey's test.

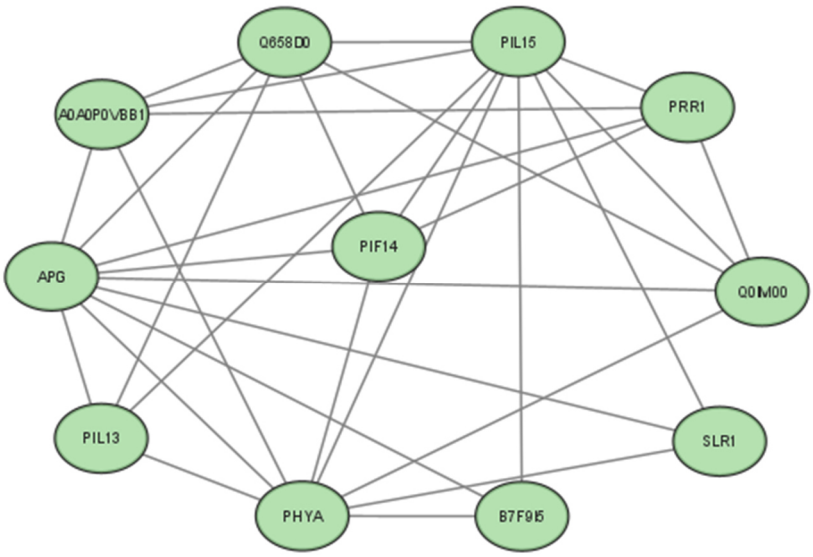

Figure 14. OsPF14 protein-protein interaction network. Predicted interactions regulated by or regulating in OsPIF14 in rice plants at protein levels. Predicted interactions of OsPIF14 (Os07g0143200) with proteins PIL13, PIL15, PHYA, DELLA protein SLR1 Q7G7J6, Helix-loop-helix DNA-binding domain containing protein Q0IM00, PRR1 Q7G7J6, Q0IM00, APG, cDNA clone J080076J05, cDNA clone B7F9I5, cDNA clone Q658D0, Os01g0885250 protein were performed using the interactions viewer (https://string-db.org/cgi/about?footer_active_subpage=content).

13.2. Supplemental tables

Table 1. Allelic mutations in PIF14 locus obtained by CRISPR/Cas9. Cas9-induced mutations were analysed in the T0 generation of transformed rice plants by Sanger sequencing. Homozygous lines were selected to perform this study. Deleted nucleotides are represented by "-". In the InDel (insertions and deletions) column, the symbol "-" represent the deletion of the represented number of nucleotides in the target region. Cas9 target site is represented in red, and the PAM motif is underlined in the wild-type sequence.

Line	Gene sequence	InDel
WT	5' -GGAGGAGACGGCGTCCGCGTGGTTCGCGGATGGCGGTGGCGGCGGCGGCGGCGACGACGA- 3'	
KO1 94S	*5' -GGAGGAGACGGCGTCCGCGT——CGCGGATGGCGGTGGCGGCGGCGGCGGCGACGACGA- 3'*	-4
KO2 85J	*5' -GGAGGAGACGGCG——————————————————————GCGACGACGA- 3'*	-37
KO3 84I	5' -GGAGGAGACGGCGTCCGCGTG–TTCGCGGATGGCGGTGGCGGCGGCGGCGACGACGA- 3'	-1

Table 2. Oligonucleotides used in this study to analyze rice lines.

Primer name	**Sequence (5' – 3')**	**Objective**
HPTII_Fw 1	AATAGCTGCGCCGATGGTTTCTACA	Detection of transformed lines
HPTII_Rv 1	AACATCGCCTCGCTCCAGTCAATG	
PIF14_Fw1	CGTTCTGTTTGCTTGCGTGG	PIF14 sequence amplification
PIF14_Rv1	GATGGAATGACAGCGCCAGAG	
PIF14_Fw2	TGGCGTAGCTCGGATGGCGA	Genotyping PIF14 locus

Table 3. Carbohydrate content of quiescent seeds. Contents of starch content, total soluble sugars, and sucrose of quiescent seeds of non-transformed plants (NT) and OsPIF14 knockout (KO1, KO2 and KO3). The carbohydrate and sugar contents were measured in the third generation of the transformation (T3). Values represented indicate the averages of three independent replications (±SE), where* represents significant difference between NT and transformed plants ($P < 0.05$) according to Tukey's test.

Carbohydrate contents	**NT** (x)	**KO1** (94.S (-4/-4))	**KO2** (85JS.0.2 (-37/-37))	**KO3** (84I.14.0.1 (-1/-1))
Starch (mmol glucose g^{-1})	42.7 ± 2.6	22.6* ± 1.1	26.0* ± 2.6	24.3* ± 1.9
Total soluble sugars (µmol glucose g^{-1})	873.0 ± 33.3	1,509.1* ± 82.8	1,412.1* ± 133.6	1,460.6* ± 108.2
Sucrose (µmol sucrose g^{-1})	4.1 ± 0.7	7.3* ± 0.1	7.7* ± 0.4	7.5* ± 0.2

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14.GENERAL CONCLUSION AND PERSPECTIVES

OsPIF14 knockout significantly affected the development of rice plants possibly initiated during grain filling and seed formation. This induces restrictions on other physiological processes during all vegetative (seed germination, seedling establishment, plant growth and tillering) and reproductive (grain filling and growth) phases. subsequently, these restrictions significantly affected some stage in the dynamics of starch storage and seed viability, originating a vicious cycle, a process that goes from seed to seed. Therefore, new studies with this transcription factor are needed to better elucidate its involvement with plant physiology through gene regulation, with a set of multiomics, reverse genetics (overexpression) and bioinformatics approaches to be able to elucidate the direct involvement of the OsPIF14 gene in specific physical processes related to rice production. With that, this work brings a perspective and amplification for future works with strong promise to explain physiological responses regulated by PIF genes to improve crop productivity from genetic management.

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APPENDIX A - PUBLISHED ARTICLES

ALENCAR, V. T. C. B.; LOBO, A. K. M.; CARVALHO, F. E. L. ; SILVEIRA, J. A. G. . High ammonium supply impairs photosynthetic efficiency in rice exposed to excess light. PHOTOSYNTHESIS RESEARCH, v. xxxxxx, p. xxxxxxxxxxxxxxx-xxxxxx, 2019.

SOUSA, RIKAELY T.; PAIVA, ANA L.S.; CARVALHO, FABRICIO E.L.; **ALENCAR, VICENTE T.C.B.**; SILVEIRA, JOAQUIM A.G. Ammonium overaccumulation in senescent leaves as a novel exclusion mechanism to avoid toxicity in photosynthetically active rice leaves. ENVIRONMENTAL AND EXPERIMENTAL BOTANY JCR, v. 186, p. 104452, 2021.

LIMA-MELO, YUGO ; **ALENCAR, VICENTE T. C. B.** ; LOBO, ANA K. M. ; SOUSA, RACHEL H. V. ; TIKKANEN, MIKKO ; ARO, EVA-MARI ; SILVEIRA, JOAQUIM A. G. ; GOLLAN, PETER J. . Photoinhibition of Photosystem I Provides Oxidative Protection During Imbalanced Photosynthetic Electron Transport in Arabidopsis thaliana. Frontiers in Plant Science JCR, v. 10, p. 1-13, 2019.

SOUSA, RACHEL H V; CARVALHO, FABRICIO E L; LIMA-MELO, YUGO ; **ALENCAR, VICENTE T C B** ; DALOSO, DANILO M ; MARGIS-PINHEIRO, MARCIA ; KOMATSU, SETSUKO ; SILVEIRA, JOAQUIM A G . Impairment of peroxisomal APX and CAT activities increases protection of photosynthesis. JOURNAL OF EXPERIMENTAL BOTANY (ONLINE) JCR, v. xxxxx, p. xxxxxxxxx-xxxxxx, 2018.

ANNEXE A - PUBLICATIONS AND CO-SUPERVISION

THESIS MANUSCRIPT SUBMISSION

Plant Science

OsPIF14 knockout triggers disturbances in starch synthesis affecting seed formation and generating a vicious cycle in rice development

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	Germination; Grain filling; Oryza sativa; Plant development; Sugar metabolism; Starch synthesis
Corresponding Author:	Joaquim Silveira Fortaleza, Ceará Brazil
First Author:	Vicente Alencar
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	Antônio Markos Silva
	Ana Luiza Paiva
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	André Cordeiro
	Nelson Saibo
	Joaquim Silveira
Abstract:	PHYTOCHROME TRANSCRIPTION FACTORS (PIFs), in addition to interacting with phytochromes inside the nucleus, are also involved, direct or indirectly, with several physiological processes which are still poorly understood. We have previously observed that OsPIF14 knockout (CRISPR/Cas9 transformation) affects seed germination and grain filling in rice. In this work, we hypothesized that this gene could be associated with grain filling and seed formation via sugar metabolism, affecting the starch accumulation and seed viability. Consequently, the generated plants should present a lower development and a progressive vicious cycle until the reproductive phase. OsPIF14 knockout induced decreases in tiller number, what was positively associated with the number of panicle formation and grain filling intensity. The seed and panicle growth rates throughout the reproductive phase were significantly reduced, resulting seeds smallest in size and amount. This growth model was paralleled by the starch accumulation rates and transformed plants seeds presented lower starch content and higher contents of sucrose and total sugars at end of the maturation phase (R9) associated with lower contents of proteins and soluble amino acids. In addition, these seeds displayed lower viability, as indicated by low germination rates and increased electrolyte leakage. Further, OsPIF14 knockout seeds presented lower seedling establishment, shoot and root elongation and delayed development in all vegetative stages. Thus, the proposed hypothesis must be accepted, evidencing that OsPIF14 knockout is capable to induce several physiological disturbances in rice development performing a vicious cycle which could be initiated during seed formation, probably involving starch synthesis and sugar metabolism.
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ARTICLE PUBLISHED AS AUTHOR

Photosynthesis Research (2019) 140:321–335
https://doi.org/10.1007/s11120-019-00614-z

ORIGINAL ARTICLE

High ammonium supply impairs photosynthetic efficiency in rice exposed to excess light

V. T. C. B. Alencar[1] · A. K. M. Lobo[1] · F. E. L. Carvalho[1] · J. A. G. Silveira[1]

Received: 9 July 2018 / Accepted: 8 January 2019 / Published online: 29 January 2019

Abstract
Mechanisms involving ammonium toxicity, excess light, and photosynthesis are scarcely known in plants. We tested the hypothesis that high NH_4^+ supply in presence of high light decreases photosynthetic efficiency of rice plants, an allegedly tolerant species. Mature rice plants were previously supplied with 10 mM NH_4^+ or 10 mM NO_3^- and subsequently exposed to 400 µmol m^{-2} s^{-1} (moderate light—ML) or 2000 µmol m^{-2} s^{-1} (high light—HL) for 8 h. HL greatly stimulated NH_4^+ accumulation in roots and in a minor extent in leaves. These plants displayed significant delay in D1 protein recovery in the dark, compared to nitrate-supplied plants. These responses were related to reduction of both PSII and PSI quantum efficiencies and induction of non-photochemical quenching. These changes were also associated with higher limitation in the donor side and lower restriction in the acceptor side of PSI. This later response was closely related to prominent decrease in stomatal conductance and net CO_2 assimilation that could have strongly affected the energy balance in chloroplast, favoring ATP accumulation and NPQ induction. In parallel, NH_4^+ induced a strong increase in the electron flux to photorespiration and, inversely, it decreased the flux to Rubisco carboxylation. Overall, ammonium supply negatively interacts with excess light, possibly by enhancing ammonium transport towards leaves, causing negative effects on some photosynthetic steps. We propose that high ammonium supply to rice combined with excess light is capable to induce strong delay in D1 protein turnover and restriction in stomatal conductance, which might have contributed to generalized disturbances on photosynthetic efficiency.

Keywords Ammonia toxicity · D1 turnover · Photosynthesis · Photoinhibition · Photosystems · *Oryza sativa*

Abbreviations

A_{max}	Maximum net CO_2 assimilation rate
Ci	Intercellular CO_2 partial concentration
ETRI	Electron transport rate at PSI
ETRII	Electron transport rate at PSII
Fm	Dark maximum fluorescence
Fm′	Light maximum fluorescence
Fo	Dark minimum fluorescence
Fo′	Light minimum fluorescence after the far-red illumination
Fs	Light steady-state fluorescence
Fv/Fm	Maximum quantum efficiency of PSII
Jc	Electron flux to Rubisco carboxylation
Jmax	Maximum electron transport rate
Jo	Electron flux to Rubisco oxygenation
NPQ	Non-photochemical quenching
OEC	Oxygen evolving complex
PPFD	Photosynthetic photon flux density
Vcmax	Maximum Rubisco carboxylation rate
Φ(NA)	Acceptor side limitation of PSI
Φ(ND)	Donor side limitation of PSI
PETC	Photosynthetic electron transport chain

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11120-019-00614-z) contains supplementary material, which is available to authorized users.

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ARTICLES PUBLISHED AS CO-AUTHOR

Journal of Experimental Botany, Vol. 70, No. 2 pp. 627–639, 2019
doi:10.1093/jxb/ery354 Advance Access publication 31 October 2018
This paper is available online free of all access charges (see https://academic.oup.com/jxb/pages/openaccess for further details)

RESEARCH PAPER

Impairment of peroxisomal APX and CAT activities increases protection of photosynthesis under oxidative stress

Rachel H. V. Sousa[1], Fabricio E. L. Carvalho[1], Yugo Lima-Melo[1,2], Vicente T. C. B. Alencar[1], Danilo M. Daloso[1], Marcia Margis-Pinheiro[3], Setsuko Komatsu[4] and Joaquim A. G. Silveira[1,*]

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Received 18 June 2018; Editorial decision 3 October 2018; Accepted 3 October 2018

Editor: Christine Foyer, Leeds University, UK

Abstract

Retrograde signalling pathways that are triggered by changes in cellular redox homeostasis remain poorly understood. Transformed rice plants that are deficient in peroxisomal ascorbate peroxidase APX4 (*OsAPX4*-RNAi) are known to exhibit more effective protection of photosynthesis against oxidative stress than controls when catalase (CAT) is inhibited, but the mechanisms involved have not been characterized. An in-depth physiological and proteomics analysis was therefore performed on *OsAPX4*-RNAi CAT-inhibited rice plants. Loss of APX4 function led to an increased abundance of several proteins that are involved in essential metabolic pathways, possibly as a result of increased tissue H_2O_2 levels. Higher photosynthetic activities observed in the *OsAPX4*-RNAi plants under CAT inhibition were accompanied by higher levels of Rubisco, higher maximum rates of Rubisco carboxylation, and increased photochemical efficiencies, together with large increases in photosynthesis-related proteins. Large increases were also observed in the levels of proteins involved in the ascorbate/glutathione cycle and in other antioxidant-related pathways, and these changes may be important in the protection of photosynthesis in the *OsAPX4*-RNAi plants. Large increases in the abundance of proteins localized in the nuclei and mitochondria were also observed, together with increased levels of proteins involved in important cellular pathways, particularly protein translation. Taken together, the results show that *OsAPX4*-RNAi plants exhibit significant metabolic reprogramming, which incorporates a more effective antioxidant response to protect photosynthesis under conditions of impaired CAT activity.

Keywords: Ascorbate peroxidase, H_2O_2 signalling, oxidative stress, photosynthetic efficiency, proteomics, redox metabolism.

Introduction

Plant peroxisomes are the most important cellular site for hydrogen peroxide (H_2O_2) production in C_3 plants exposed to light. Several studies have shown that redox changes in these organelles are able to affect metabolic regulation in other cellular compartments by cross-talk mechanisms (Nyathi and Baker, 2006; Sewelam *et al.*, 2014; Corpas, 2015). The majority of these findings have been achieved by using plants deficient in catalase (CAT, EC 1.11.1.6), indicating that such responses are associated with photorespiratory H_2O_2 accumulation and downstream oxidative signalling events (Willekens *et al.*, 1997;

ARTICLES PUBLISHED AS CO-AUTHOR

frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 July 2019
doi: 10.3389/fpls.2019.00916

Photoinhibition of Photosystem I Provides Oxidative Protection During Imbalanced Photosynthetic Electron Transport in *Arabidopsis thaliana*

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *08 March 2019*
Accepted: *28 June 2019*
Published: *12 July 2019*

Citation:
Lima-Melo Y, Alencar VTCB, Lobo AKM, Sousa RHV, Tikkanen M, Aro E-M, Silveira JAG and Gollan PJ (2019) Photoinhibition of Photosystem I Provides Oxidative Protection During Imbalanced Photosynthetic Electron Transport in Arabidopsis thaliana. Front. Plant Sci. 10:916. doi: 10.3389/fpls.2019.00916

Photosynthesis involves the conversion of sunlight energy into stored chemical energy, which is achieved through electron transport along a series of redox reactions. Excess photosynthetic electron transport might be dangerous due to the risk of molecular oxygen reduction, generating reactive oxygen species (ROS) over-accumulation. Avoiding excess ROS production requires the rate of electron transport to be coordinated with the capacity of electron acceptors in the chloroplast stroma. Imbalance between the donor and acceptor sides of photosystem I (PSI) can lead to inactivation, which is called PSI photoinhibition. We used a light-inducible PSI photoinhibition system in *Arabidopsis thaliana* to resolve the time dynamics of inhibition and to investigate its impact on ROS production and turnover. The oxidation state of the PSI reaction center and rates of CO_2 fixation both indicated strong and rapid PSI photoinhibition upon donor side/acceptor side imbalance, while the rate of inhibition eased during prolonged imbalance. PSI photoinhibition was not associated with any major changes in ROS accumulation or antioxidant activity; however, a lower level of lipid oxidation correlated with lower abundance of chloroplast lipoxygenase in PSI-inhibited leaves. The results of this study suggest that rapid activation of PSI photoinhibition under severe photosynthetic imbalance protects the chloroplast from over-reduction and excess ROS formation.

Keywords: photosystem I, photosynthesis, ROS, CO_2 fixation, photoinhibition, P700, redox

INTRODUCTION

Light is vital for photosynthesis, but when supplied in excess it can damage the photosynthetic apparatus and cause photo-oxidative stress. This condition occurs during states of photosynthetic imbalance, when the electron pressure in the photosynthetic electron transport chain exceeds the capacity of reducing power consumption by sink pathways, which is usually associated with stressful environmental conditions. As a result, transient or sustained production of reactive oxygen species (ROS) can occur. Excessive accumulation of ROS can impair metabolic homeostasis through oxidative damage to cells because of their high reactivity with lipids, proteins, and nucleic acids (McCord, 2000; Apel and Hirt, 2004; Munns, 2005; Sharma et al., 2012). On the other hand,

ARTICLES PUBLISHED AS CO-AUTHOR

Environmental and Experimental Botany 186 (2021) 104452

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Ammonium overaccumulation in senescent leaves as a novel exclusion mechanism to avoid toxicity in photosynthetically active rice leaves

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ARTICLE INFO

Keywords:
Leaf senescence
Nitrogen metabolism
NH_4^+ tolerance
Plant nutrition

ABSTRACT

High ammonium concentration is toxic for most plant species, but some rice cultivars are tolerant by mechanisms not understood yet. We tested the hypothesis that NH_4^+-tolerance could be associated with an exclusion mechanism involving ammonium deposition on the oldest leaves after triggering localized senescence. An ammonium-tolerant cultivar was exposed to increasing ammonium (NH_4^+) concentrations for two weeks and afterward subjected to 15 mM throughout 4 weeks, to evaluate toxicity and resistance mechanisms. Plants exposed to similar nitrate (NO_3^-) concentrations were taken as control. In presence of high NH_4^+ rice displayed similar nitrogen (N) root influx compared to nitrate-supplied roots. In parallel, these plants exhibited a strong impairment in root growth even after exposure to mild NH_4^+ concentration (3.75 mM). This response was related to increased activity of type III peroxidases, but no evidence for oxidative stress and tissue damages on roots were apparent. Intriguingly, high NH_4^+-supplied plants displayed a prominent increase in dry mass of senescent oldest leaves, which were 5-fold higher than that observed in nitrate-supplied ones. Ammonium-treatment induced strong NH_4^+ accumulation on the dead leaf tissues (by 2.8 mmol g^{-1} DW) representing 75 % of the total-N in this part. This high deposition rate of potentially toxic NH_4^+ associated with a "self-destruction" strategy should indicate an exclusion mechanism. In contrast, mature leaves presented low NH_4^+ content, which was associated with no alterations in both CO_2 assimilation and photosystem II activity. Our data suggest that rice displays an unusual NH_4^+ exclusion mechanism by triggering fast and intense senescence on its older leaves. Afterwards, this process is followed by the death of these leaves and deposition of extremely high amounts of this toxic component but preserving younger photosynthetically active tissues.

1. Introduction

High ammonium concentration is toxic for the majority of plant species and this problem might affect the yield of several crops. Ammonium toxicity decreases plant growth by many direct and indirect mechanisms some of which have been well characterized in different species. The most common reported adverse constrain is the induction of stunted root growth (Araya et al., 2016; Liu et al., 2013), oxidative stress (Li et al., 2019; Podgórska et al., 2013; Xie et al., 2015), earlier leaf senescence (Wu et al., 2016), reduction in photosynthetic efficiency (Alencar et al., 2019) and intensified respiratory metabolism (Hachiya et al., 2010). However, despite plant tolerance to excess ammonium varies largely among species, the involved underlying mechanisms or genetic traits are still poorly understood (Esteban et al., 2016). In this context, the use of rice as a plant model is interesting because some cultivars are adapted to paddy soil conditions, where commonly they face high NH_4^+ concentrations (Wang et al., 2020).

Although several studies have attempted to investigate how plants can deal with high ammonium exposure, paradoxically, the physiological mechanisms that might confer tolerance are only partially known to date (Esteban et al., 2016; Liu and Von Wirén, 2017). Often, studies aiming to investigate such questions have adopted fragmented approaches, which are not enough to provide robust mechanistic conclusions. Some works have suggested that tolerant species could have developed an efficient enzymatic system associated with NH_4^+-assimilation represented by the GS/GOGAT cycle and/or

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https://doi.org/10.1016/j.envexpbot.2021.104452
Received 11 February 2021; Received in revised form 9 March 2021; Accepted 12 March 2021
Available online 13 March 2021

CO-SUPERVISION OF GRADUATION WORK

FEDERAL UNIVERSITY OF CEARÁ

CENTER OF SCIENCES

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

UNDERGRADUATE COURSE IN BIOTECHNOLOGY

AYRTON MARKOS DA SILVA

OsPIF14 **GENE KNOCKOUT DELAYS SEED GERMINATION, SEEDLING DEVELOPMENT AND GRAIN FILLING IN RICE**

FORTALEZA

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ANNEXE B - UNPUBLISHED RESULTS

GERMINATION TEST IN DIFFERENT LIGHT SPECTRUM

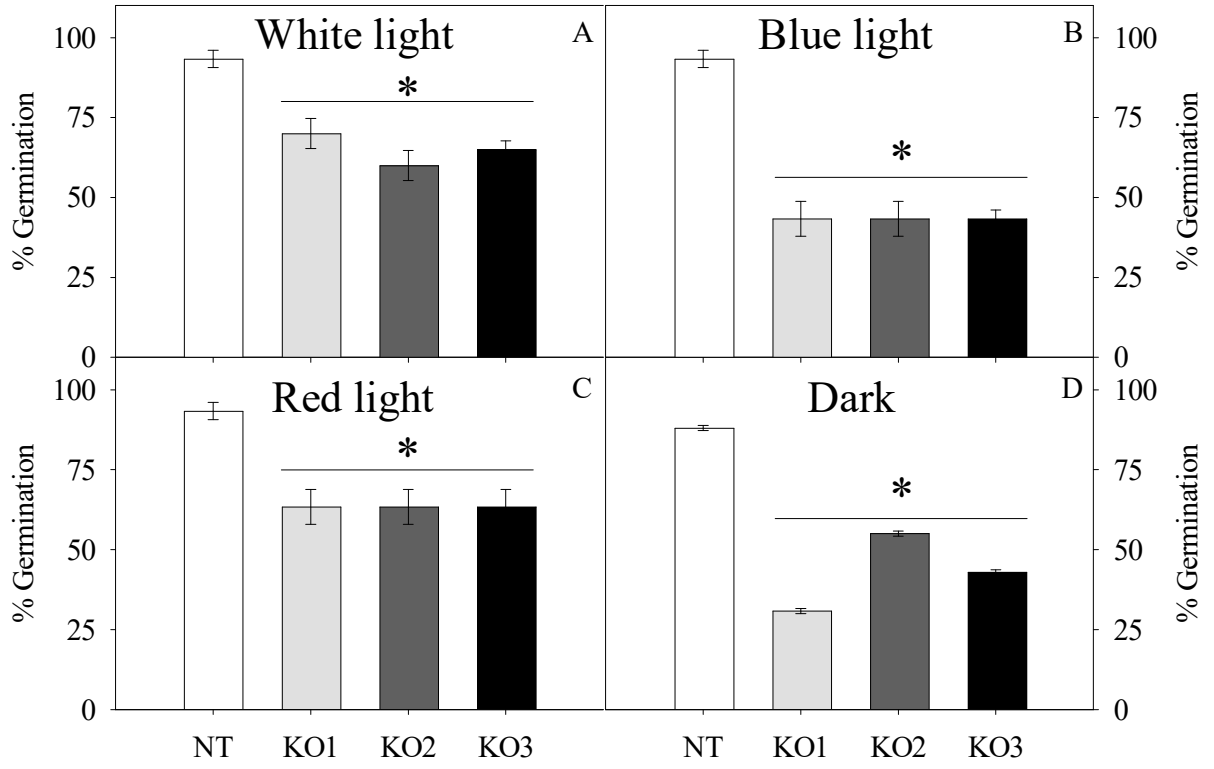


Figure 1. Germination test in different light spectrum. Transformed plants (KO-OsPIF14) showed delayed in the germination under different light spectrum at 100 µmol m^{-2} s^{-1}) A) white, B) blue, C) red, and dark in compared to non-transformed plants (NT).

DIFFERENT GROWTH STAGES OF KO-OsPF14 AND NT

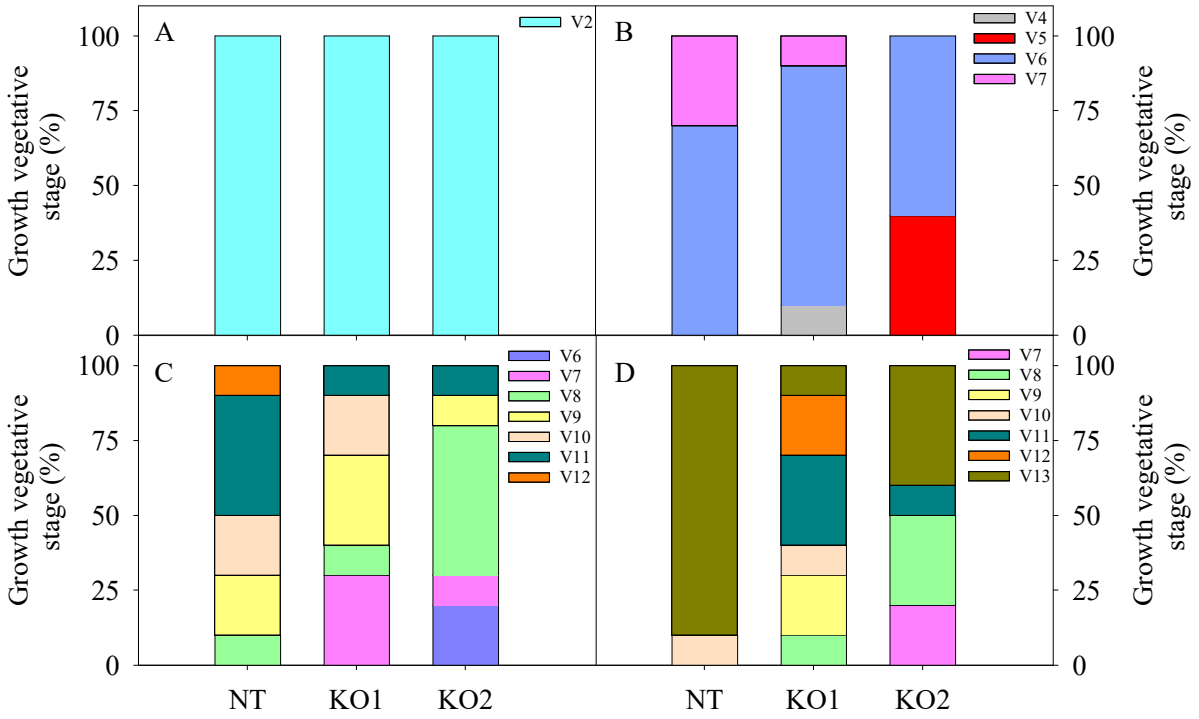


Figure 1. Rate growth stage. Transformed (KO-OsPIF14) showed delayed developmental stage compared to non-transformed plants (NT) in different days A) 12, B) 28, C) 48 and 56 day-olds.

DIFFERENT GROWTH STAGES OF OX-OsPIF14 AND NT

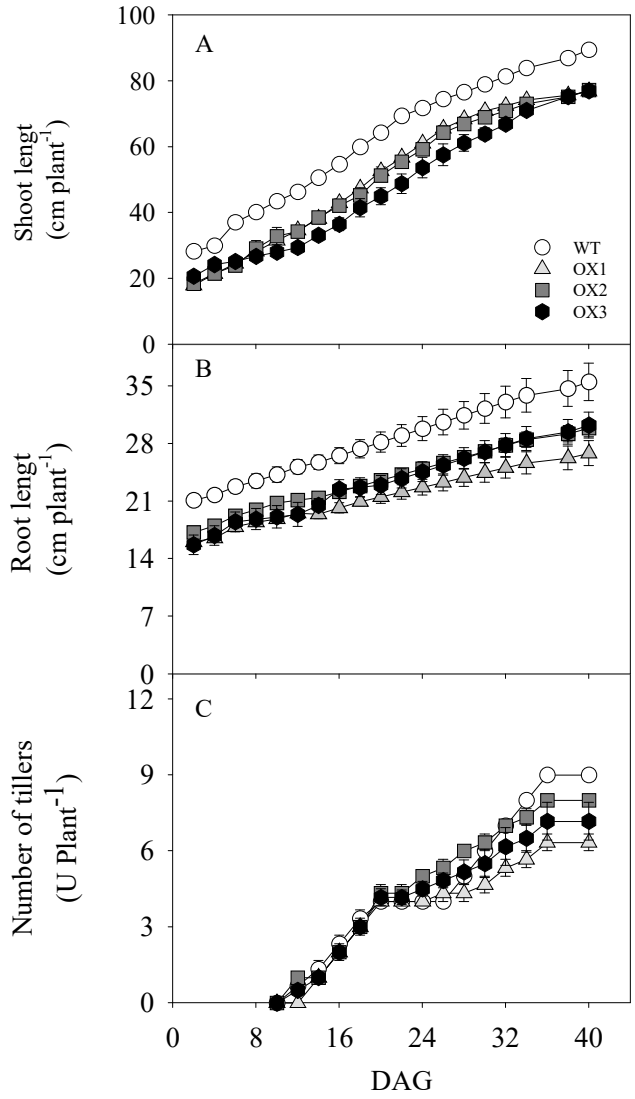


Figure 1. Plants overexpressing OsPIF14 gene showed no difference in development A) Shoot length (cm plant^{-1}), B) root length (cm plant^{-1}), and C) number of tillers (U plant^{-1}) in compared with non-transformed plants (NT).

DRY BIOMASS OF OX-OsPIF14 AND NT

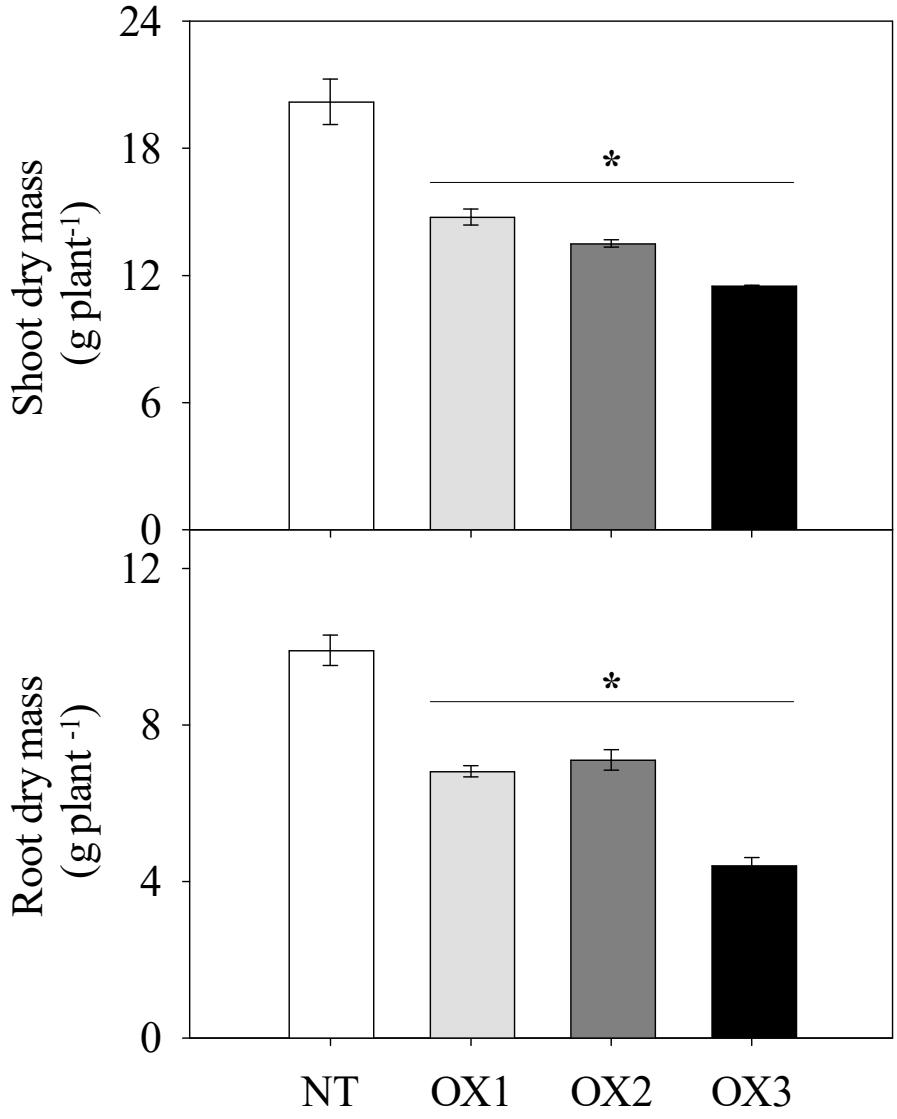


Figure 1. Plants overexpressing OsPIF14 gene showed minor dry biomass, A) Shoot dry mass (g plant^{-1}), and B) root dry mass (g plant^{-1}) in compared with non-transformed plants (NT).

LIGHT CURVE OF KO-OsPIF14 AND NT (NET PHOTOSYNTHESIS)

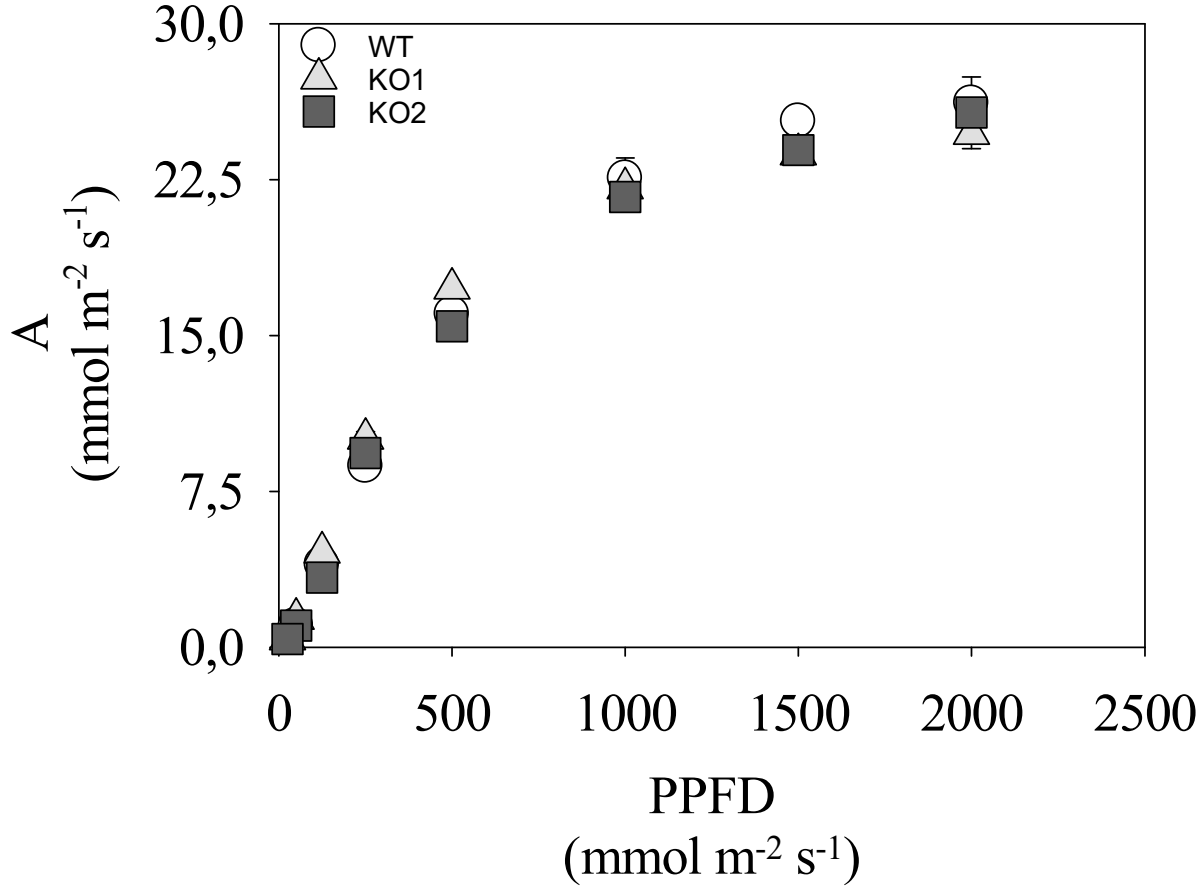


Figure 1. Light curve showed no difference in net photosynthesis (A – mmol m^{-2} s^{-1}) between transformed (KO-OsPIF14) and non-transformed plants (NT).

LIGHT CURVE OF KO-OsPIF14 AND NT (STOMATIC CONDUCTANCE)

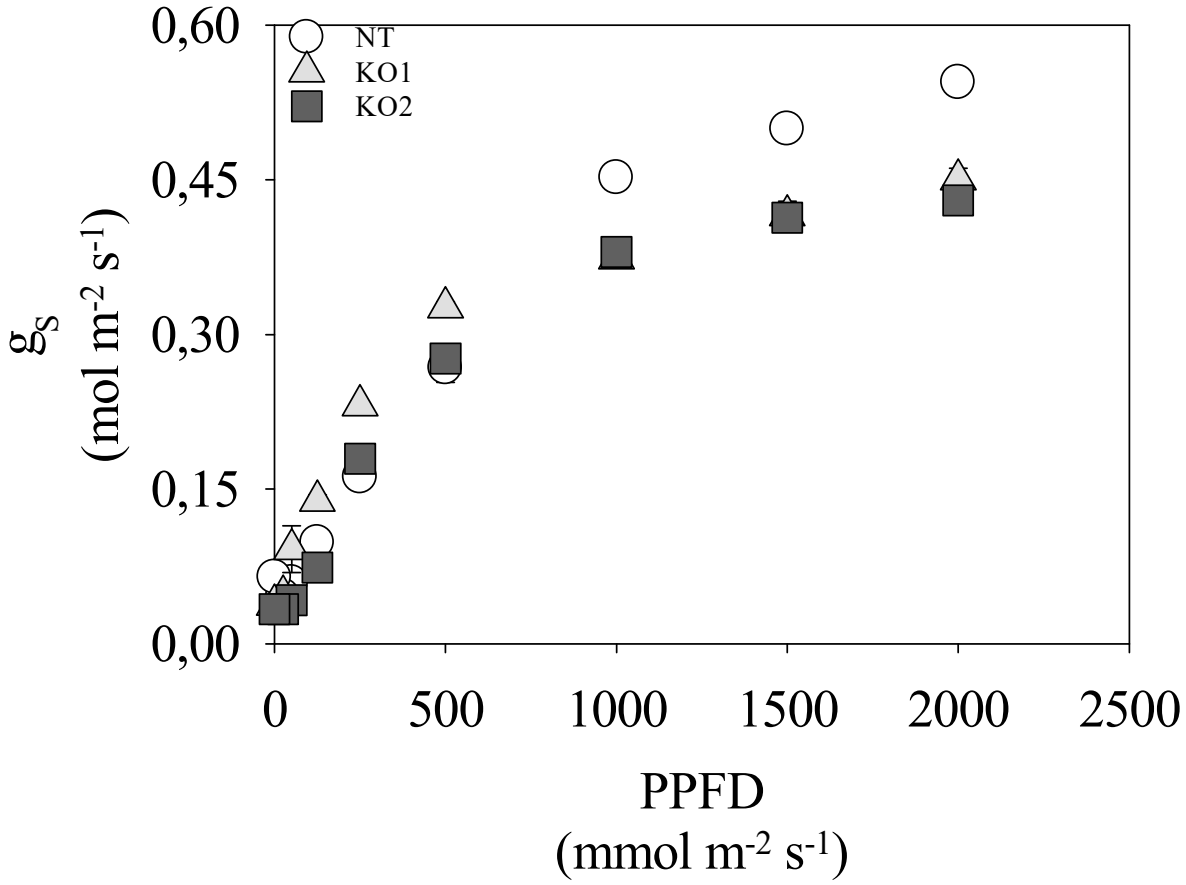


Figure 2. Light curve showed that transformed has less stomatal conductance (g_S – mol m^{-2} s^{-1}) in greater light intensity in compared to non-transformed plants (NT).

A-CI CURVE OF KO-OsPIF14 AND NT (NET PHOTOSYNTHESIS)

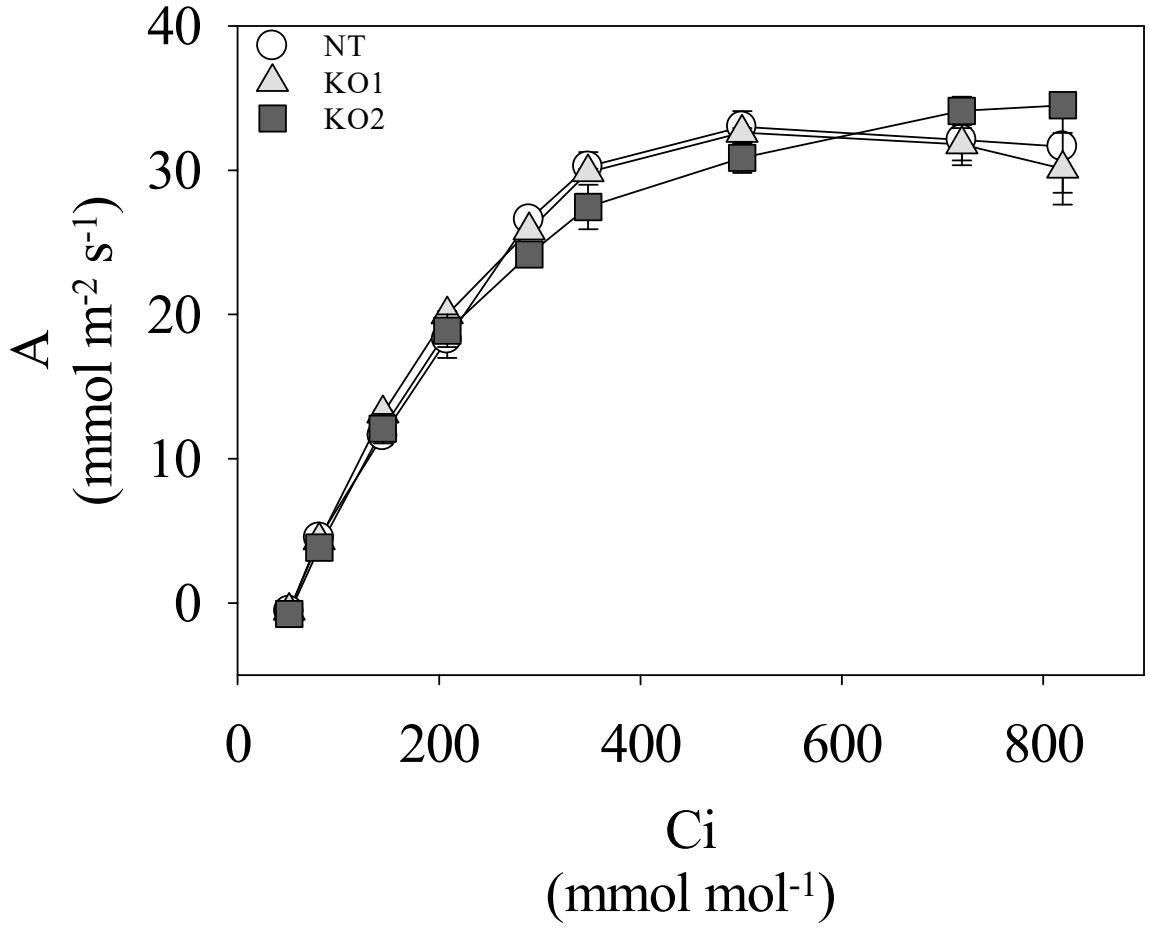


Figure 1. A-Ci curve showed no difference in net photosynthesis (A – mmol m^{-2} s^{-1}) between transformed (KO-OsPIF14) and non-transformed plants (NT).

A-CI CURVE OF KO-OsPIF14 AND NT (STOMATIC CONDUCTANCE)

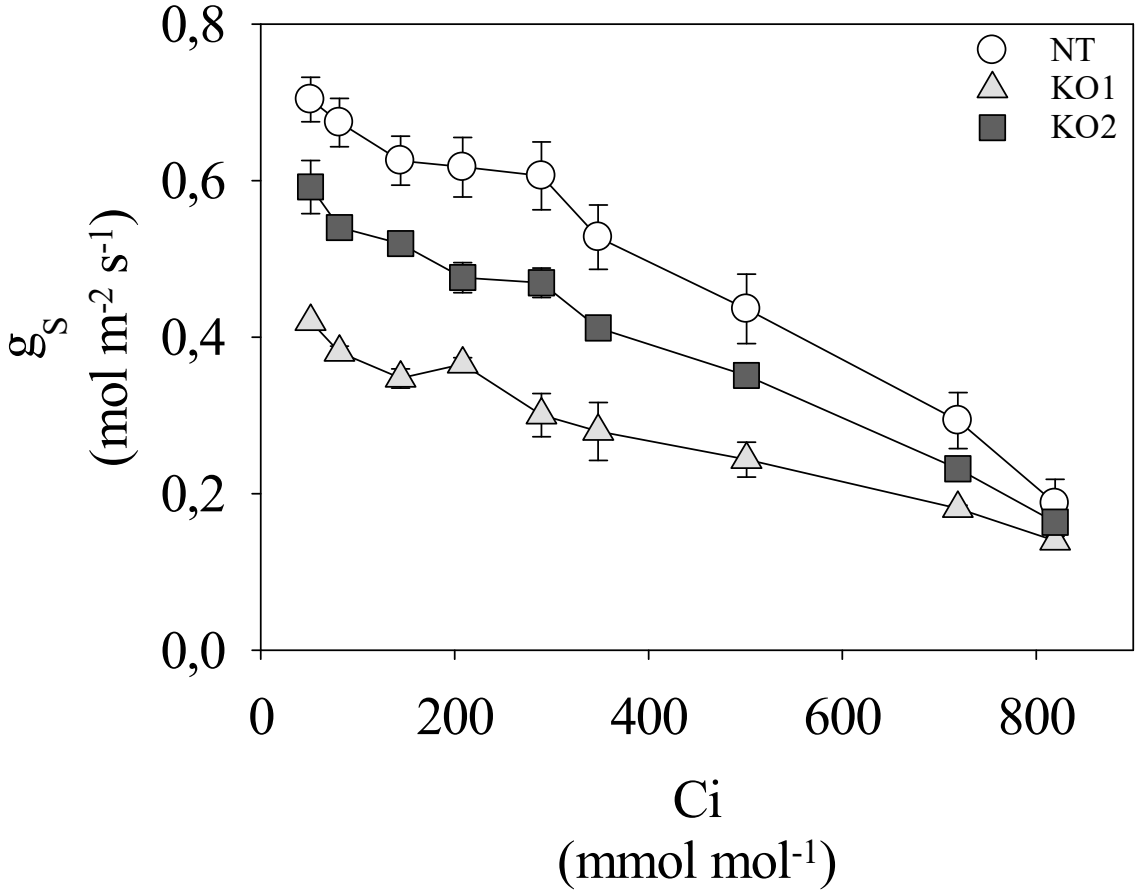


Figure 2. A-Ci curve showed that transformed has less stomatal conductance (g_S – mol m^{-2} s^{-1}) in minor CO_2 concentrations in compared to non-transformed plants (NT).

SEED HISTOLOGY (EMBRYO)

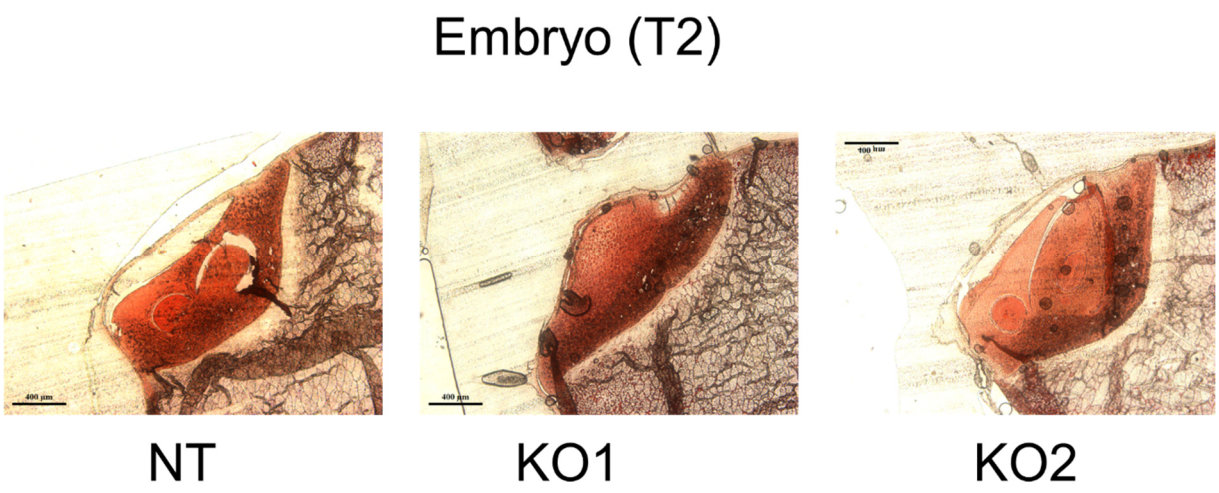


Figure 1. Analyses seed histology (T2 generation) showed that apparently no there is difference between embryo in the transformed (KO-OsPIF14) in compared to non-transformed plants (NT).

SEED HISTOLOGY (STARCH GRANULES)

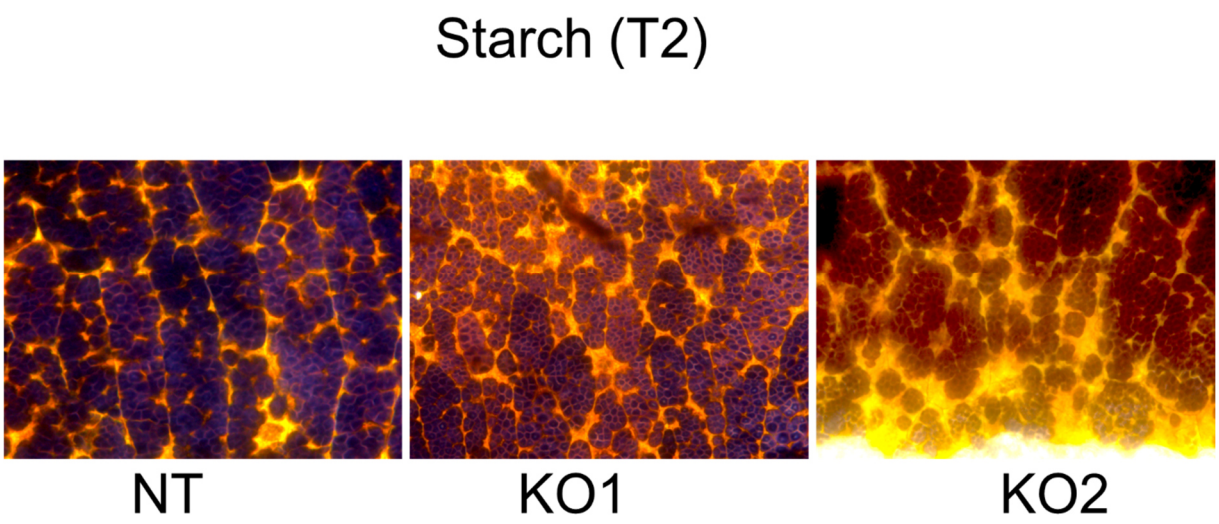


Figure 2. Analyses seed histology (T2 generation) showed less starch granules in the transformed (KO-OsPIF14) in compared to non-transformed plants (NT).

GERMINATION TEST AT DIFFERENT LIGHT INTENSITIES

Table 1 - Germination rate (%)

Lines	Generation	Genotype	CRIPSR	Time (h)		Light (µmol m^{-2} s^{-1})
				24	48	
WT	n	x	----------	22	88	Dark
KO1	T2	94.S	(-4/-4)	3	31	Dark
KO2	T2	85JS.0.2	(-37/-37)	8	55	Dark
KO3	-------	84I.14.0.1	(-1/-1)	-------	-------	Dark
WT	n	x	----------	92	100	100
KO1	T2	94.S	(-4/-4)	8	36	100
KO2	T2	85JS.0.2	(-37/-37)	12	44	100
KO3	-------	84I.14.0.1	(-1/-1)	-------	-------	100
WT	n	x	----------	90	100	100
KO1	T2	94.S	(-4/-4)	33	40	100
KO2	T2	85JS.0.2	(-37/-37)	27	43	100
KO3	T2	84I.14.0.1	(-1/-1)	0	13	100
WT	n	x	----------	88	92	290
KO1	T3	94.S	(-4/-4)	44	60	290
KO2	T3	85JS.0.2	(-37/-37)	48	88	290
KO3	T2	84I.14.0.1	(-1/-1)	64	64	290
WT	n	x	----------	96	100	290
KO1	T2	94.S	(-4/-4)	72	80	290
KO2	T2	85JS.0.2	(-37/-37)	92	100	290
KO3	T2	84I.14.0.1	(-1/-1)	64	100	290

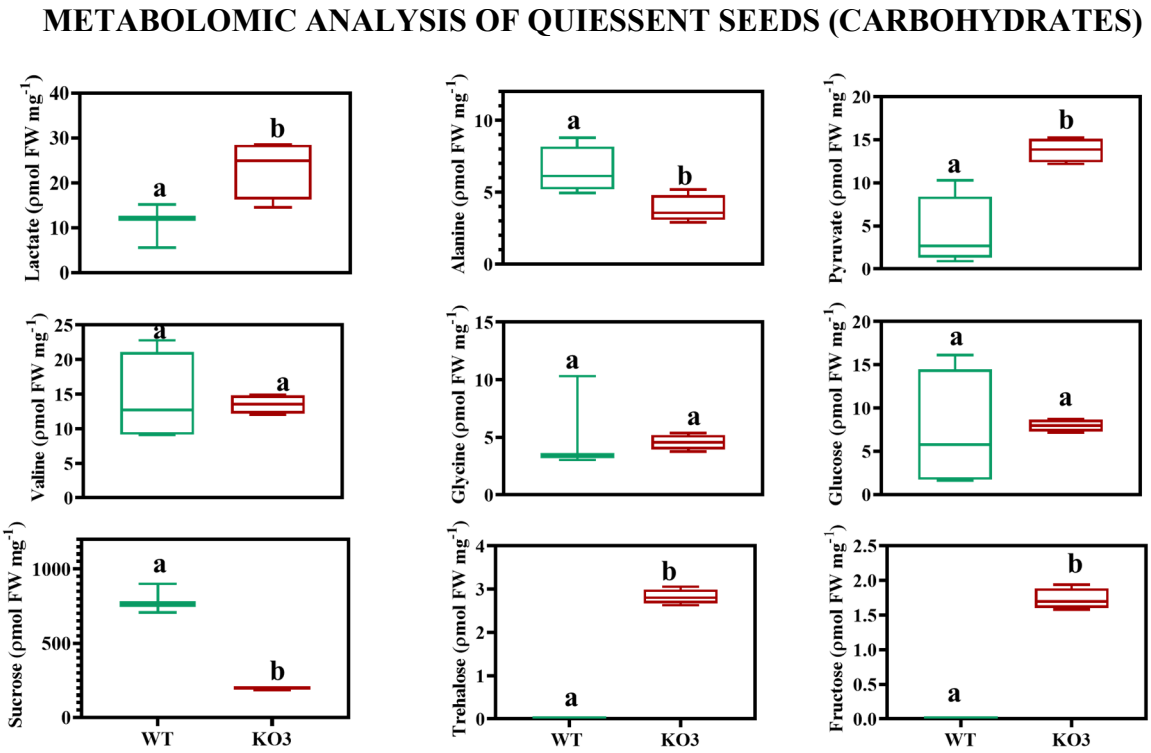


Figure 1. Differences metabolic between transformed KO3 line in compared to wild type (WT).

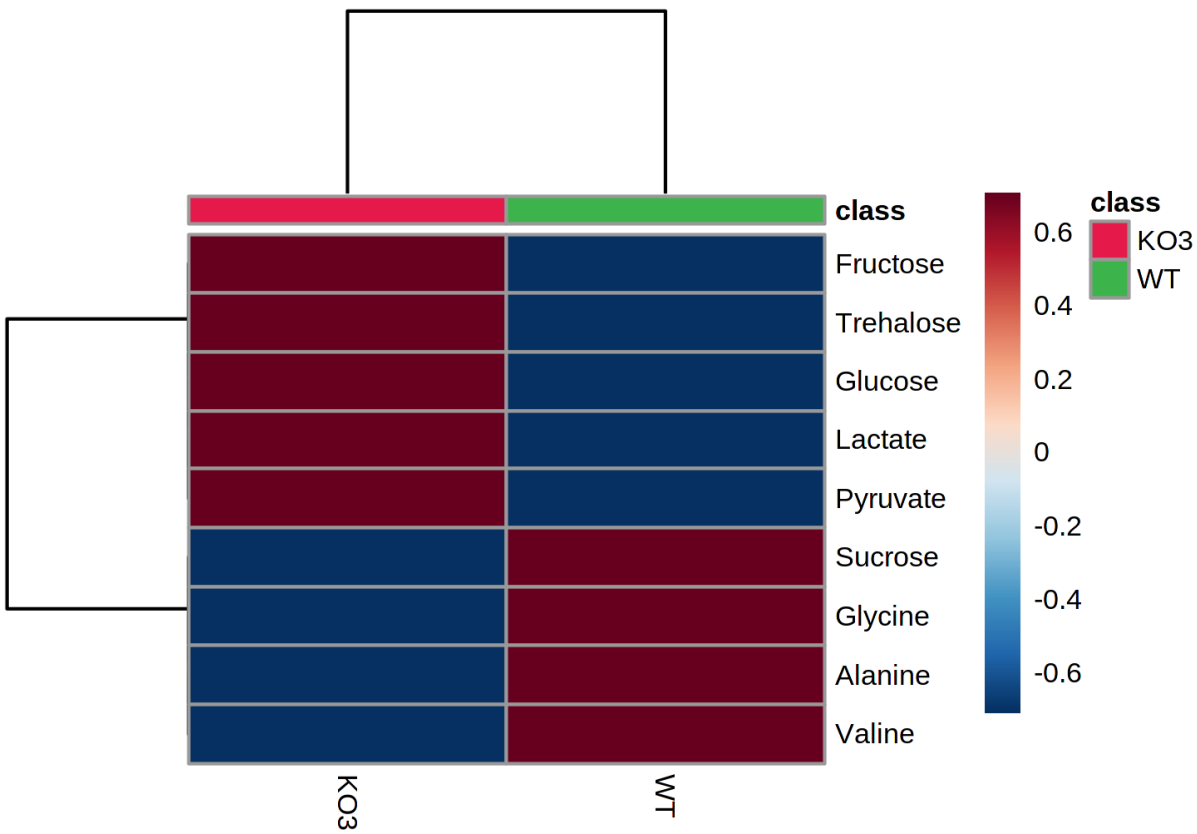
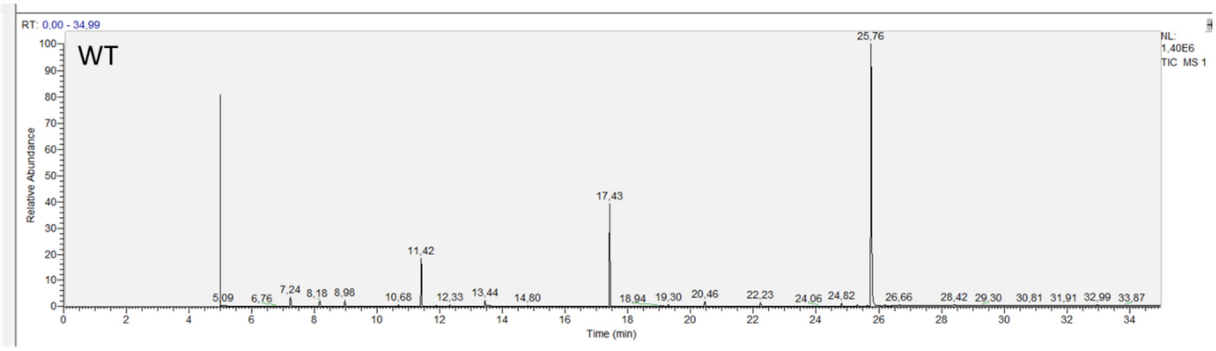
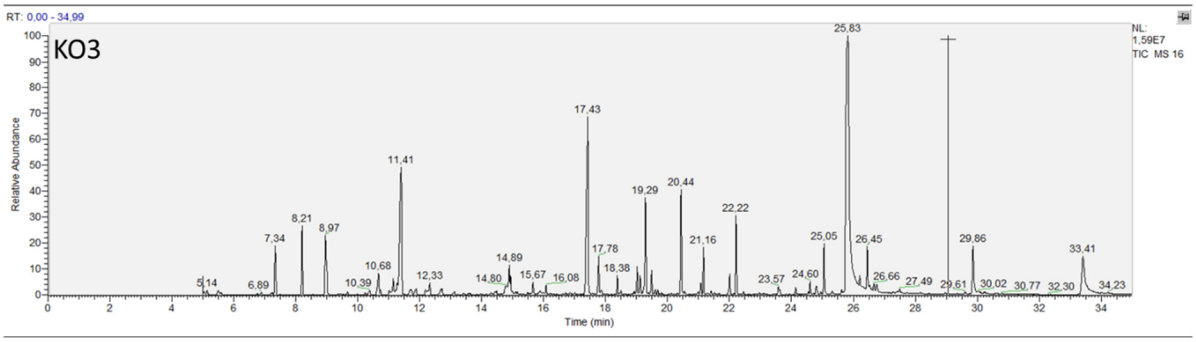


Figure 1. HEATMAP from KO-OsPIF14 quiescent seeds. Transformed (OK-OsPIF14) showed differences in metabolites compared to wild-type (WT).

CHROMATOGRAMS





EMERGENCY INDEX OF OX-OsPIF14

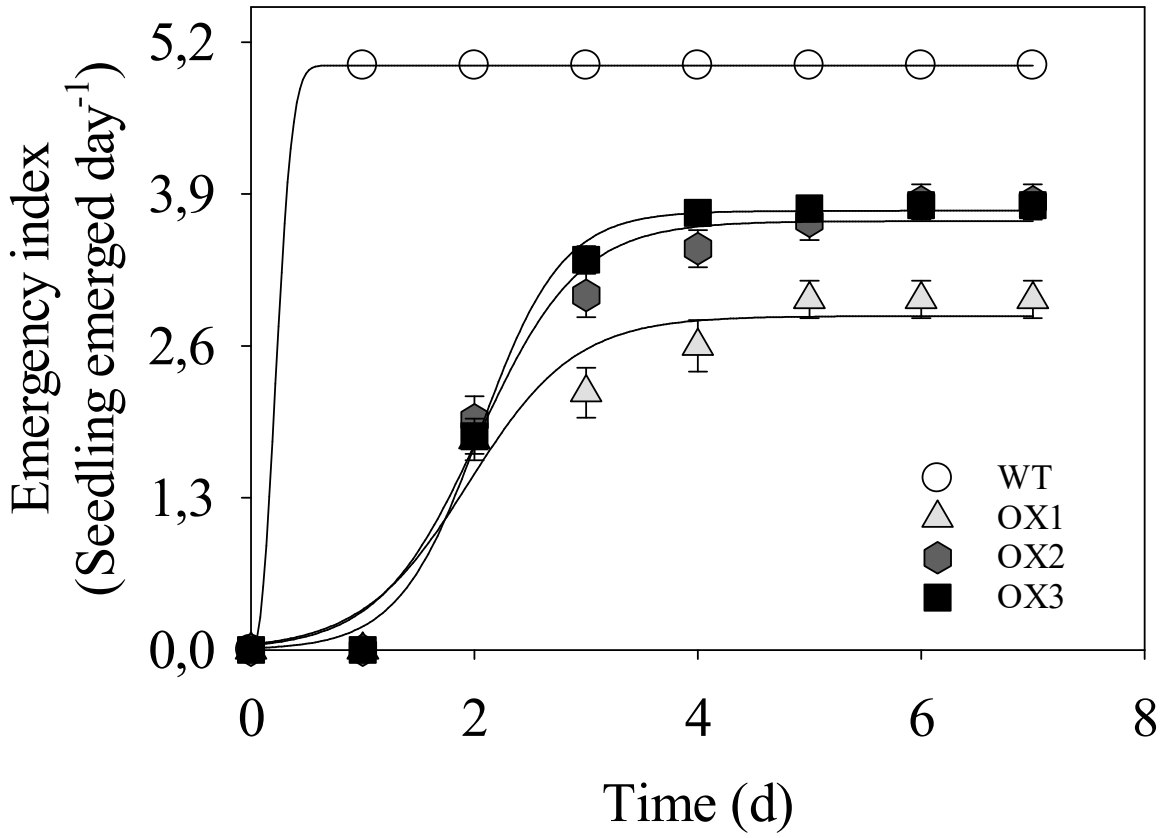


Figure 1. Emergency index from OX-OsPIF14. Transformed (OX-OsPIF14) showed delayed developmental in germination compared to wild-type (WT) during eight days.

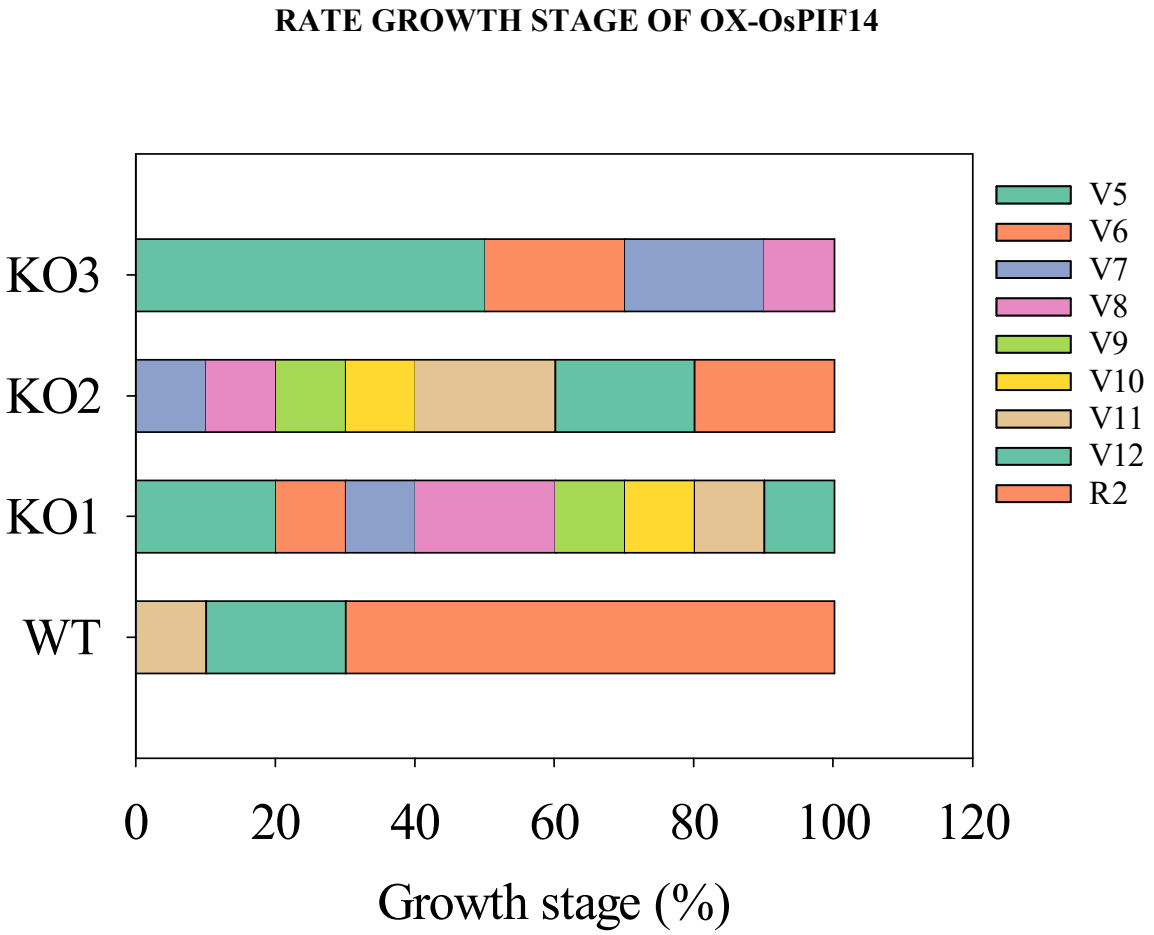


Figure 1. Rate growth stage. Transformed (KO-OsPIF14) showed delayed developmental stage compared to wild-type (WT) during all growth stages.

CULTIVATION OF PLANTS TRANSFORMED INTO VEGETATION AND HYDROPONICS HOUSE

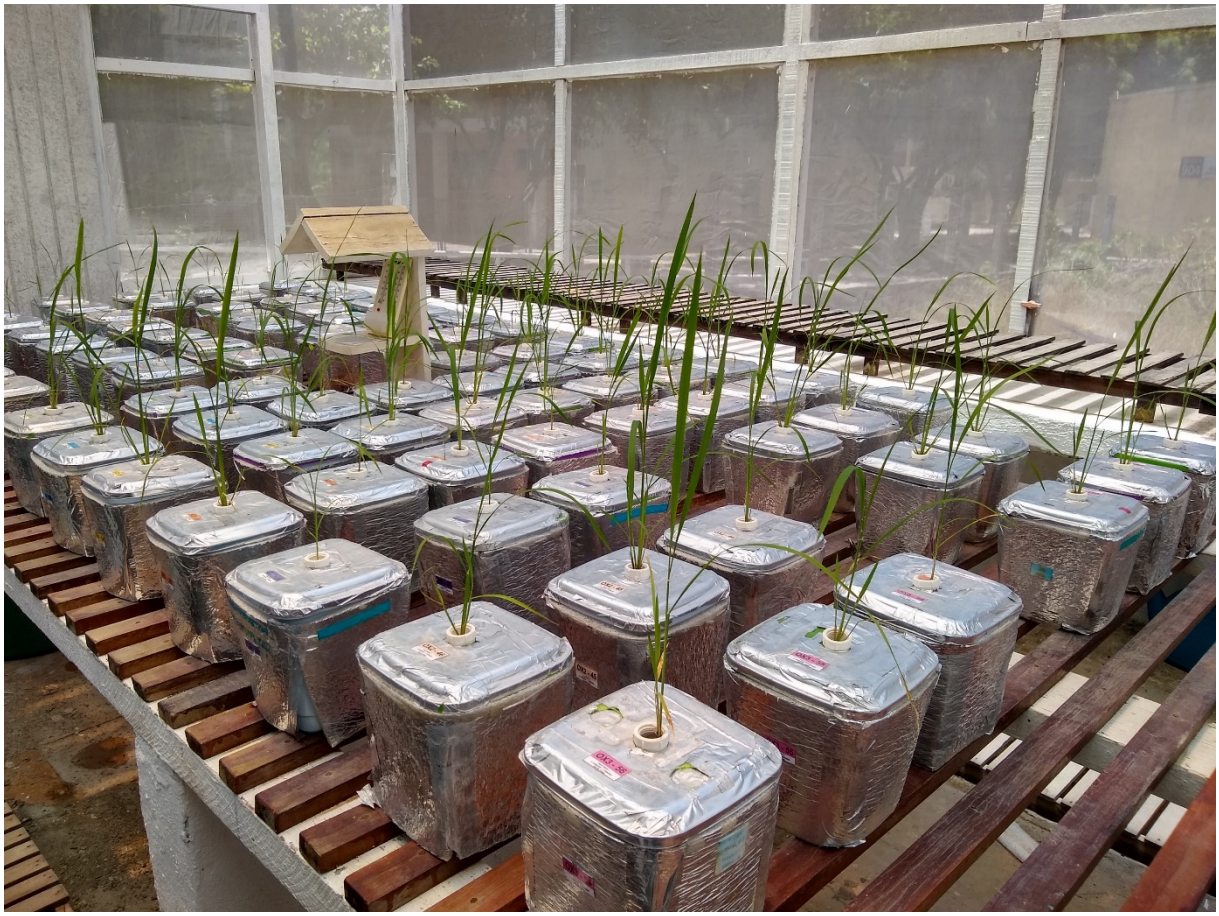


Figure 1. Transformed (KO-OsPIF14) and non-transformed (NT) plants cultivated in a hydroponic medium in a greenhouse.

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

Figure 1 Germination test on hygromycin agar medium to select transformed plants. KO and OX - OsPIF14. Positive (+) and negative (-) symbols indicates medium with and without hygromycin.

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

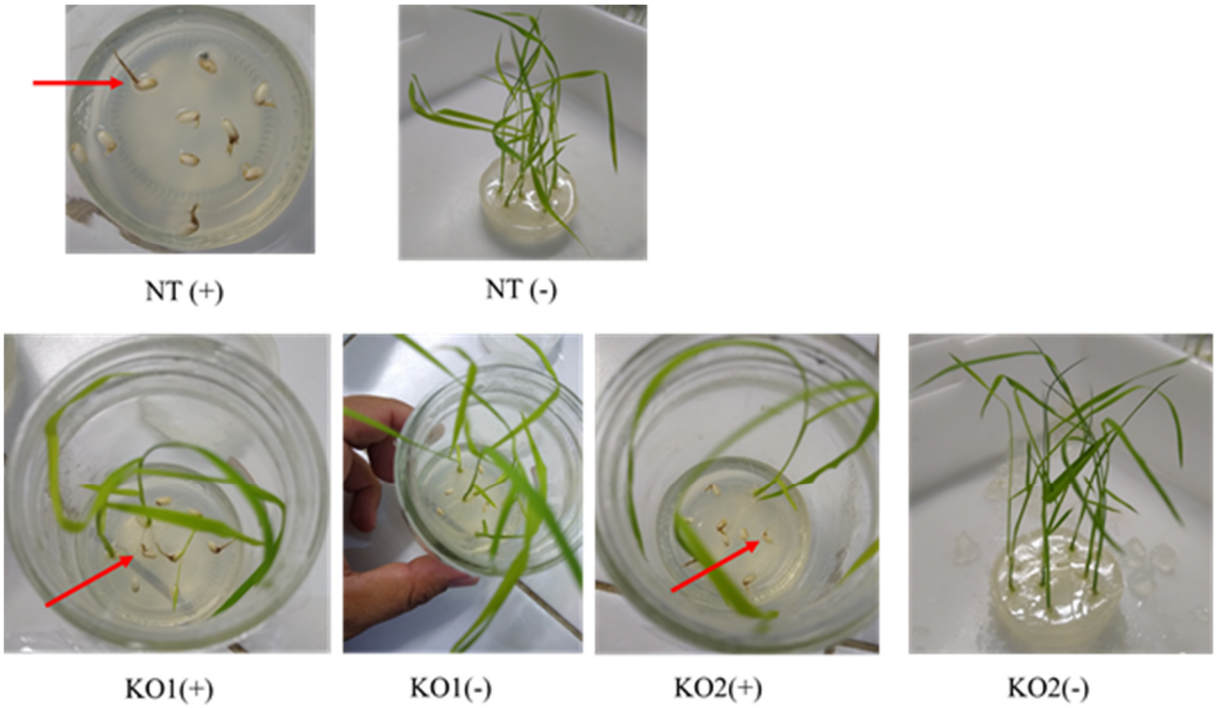


Figure 2 Germination test on hygromycin agar medium to select transformed plants. KO-OsPIF14. Positive (+) and negative (-) symbols indicates medium with and without hygromycin. Red arrows show ungerminated seeds.

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

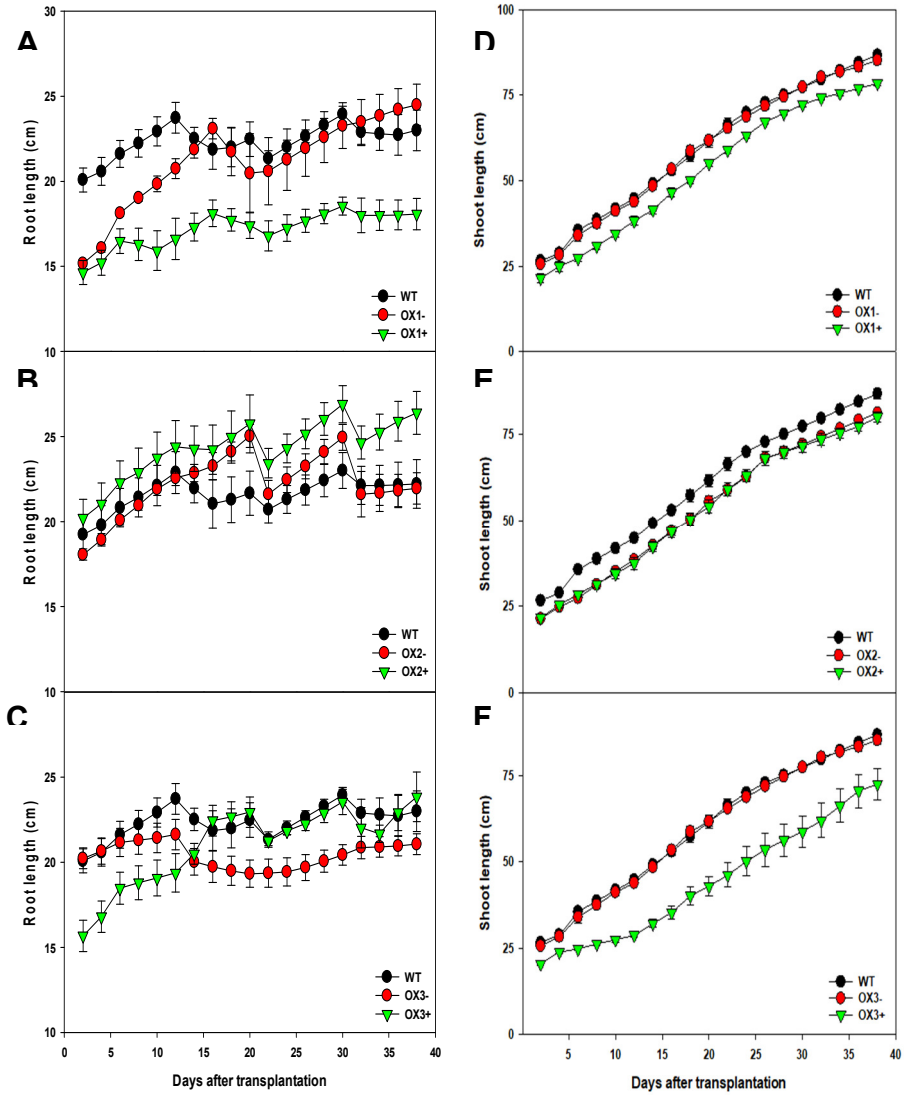


Figure 1 Root and shoot length (cm) of three lines overexpressing OsPIF14 gene (OX1, OX2, OX3) and wild type (WT) of intact rice plants 35 days old. The lineages are identified by black circles (WT), red (negative) and green triangle (positive). Represented values indicate the average of four independent replicates (± SE).

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

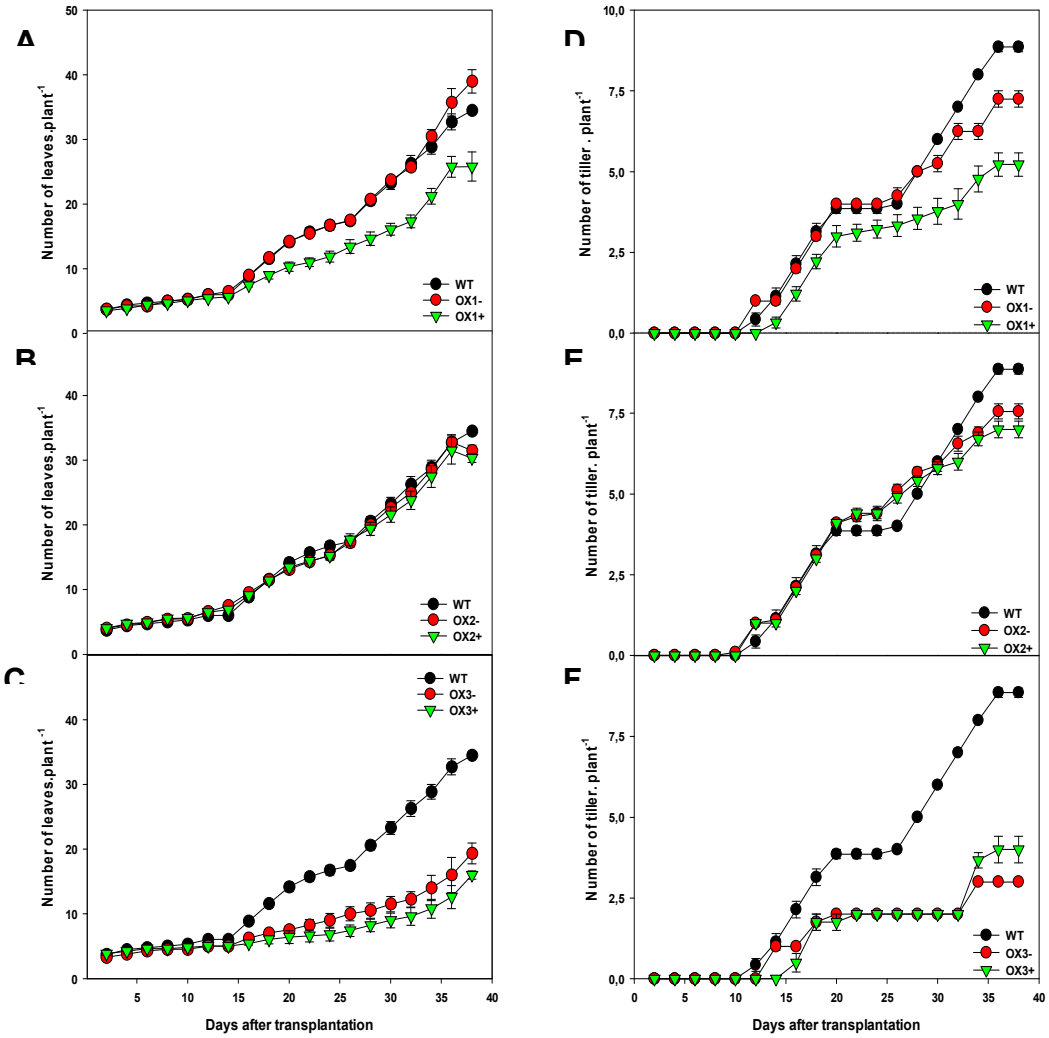


Figure 2 Number of leaves and tiller (plant $^{-1}$) of three lines overexpressing OsPIF14 gene (OX1, OX2, OX3) and wild type (WT) of intact rice plants 35 days old. The lineages are identified by black circles (WT), red (negative) and green triangle (positive). Represented values indicate the average of four independent replicates (± SE).

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

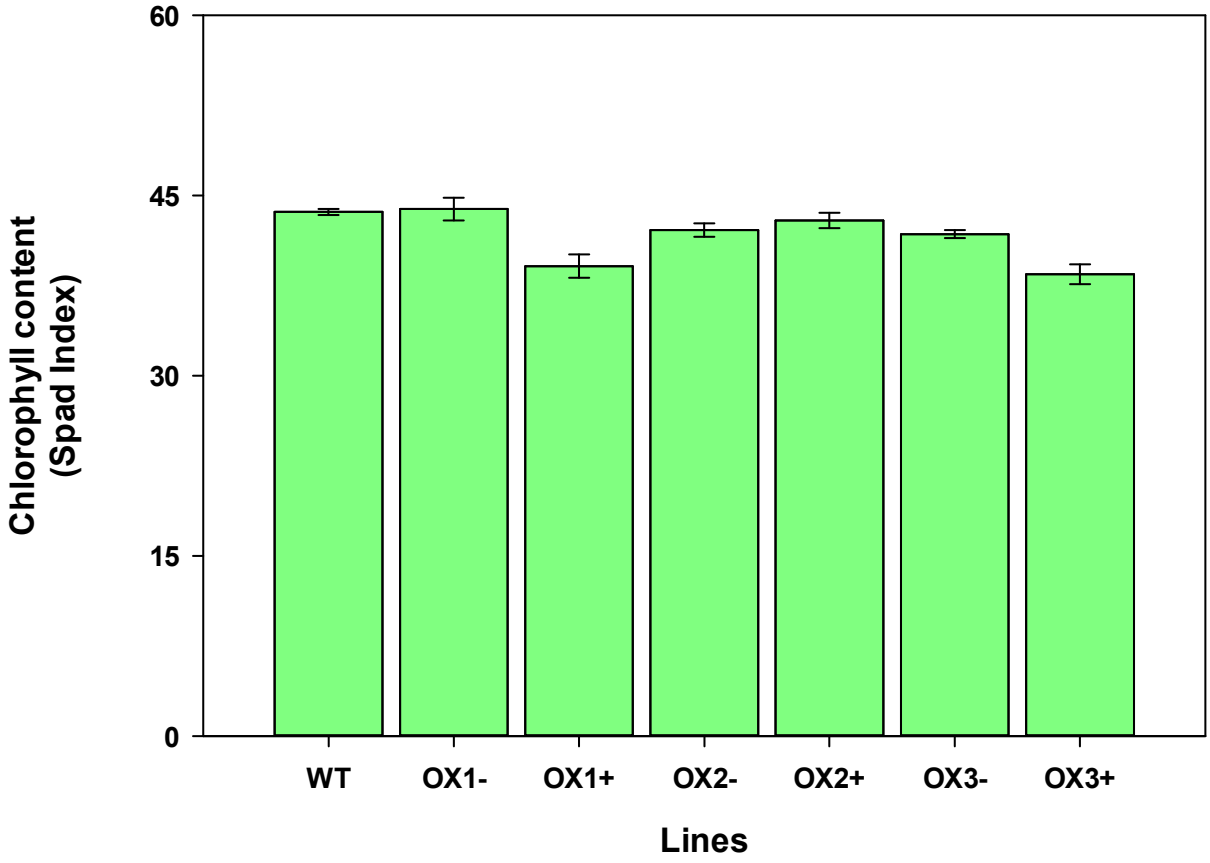


Figure 3 Chlorophyll content measured from SPAD-502 Plus, in leaves from three lines overexpressing OsPIF14 gene (OX1, OX2, OX3) and wild type (WT) of intact rice plants 35 days old. Represented values indicate the average of four independent replicates (± SE).

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

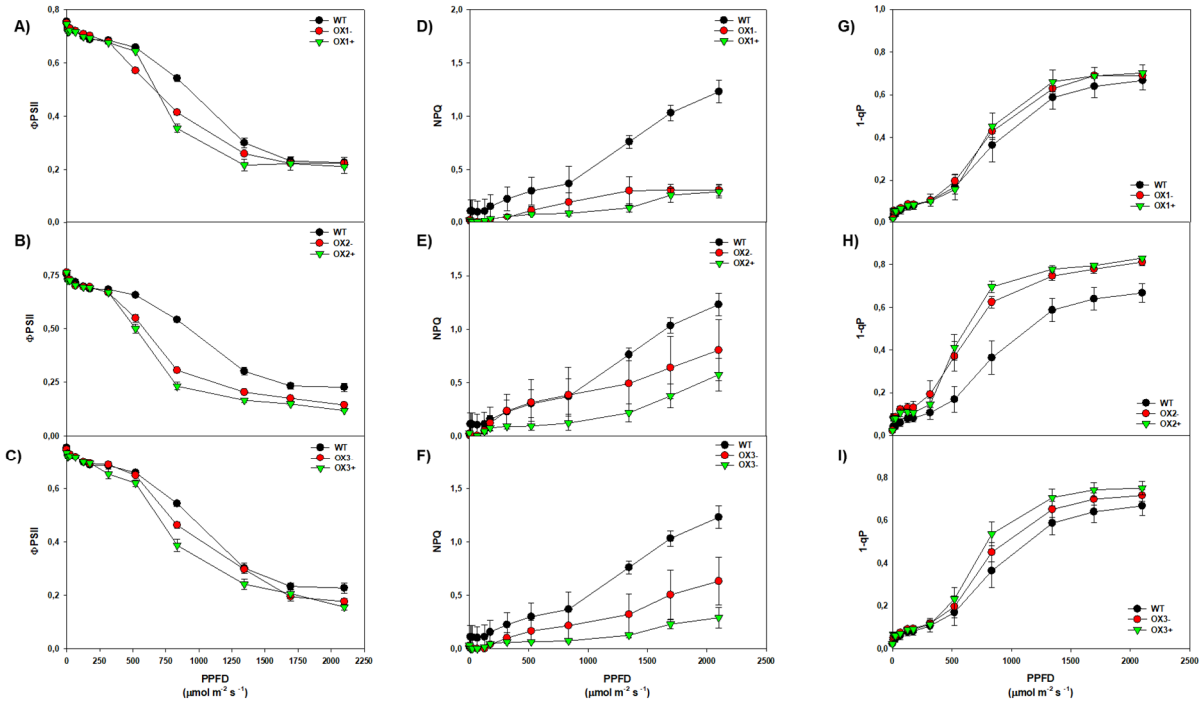


Figure 4 Light-response curve (PPFD). Photochemical measures in leaves from three lines overexpressing *OsPiF14* gene (OX1, OX2, OX3) and wild type (WT) of intact rice plants 35 days old. The lineages are identified by black circles (WT), red (negative) and green triangle (positive). The measures are effective quantum efficiency of PSII – ϕPSII (A-C); non-photochemical quenching – NPQ (D-F) and pool of PSII acceptor redox state – 1- qP (G-I). Represented values indicate the average of four independent replicates (± SE).

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

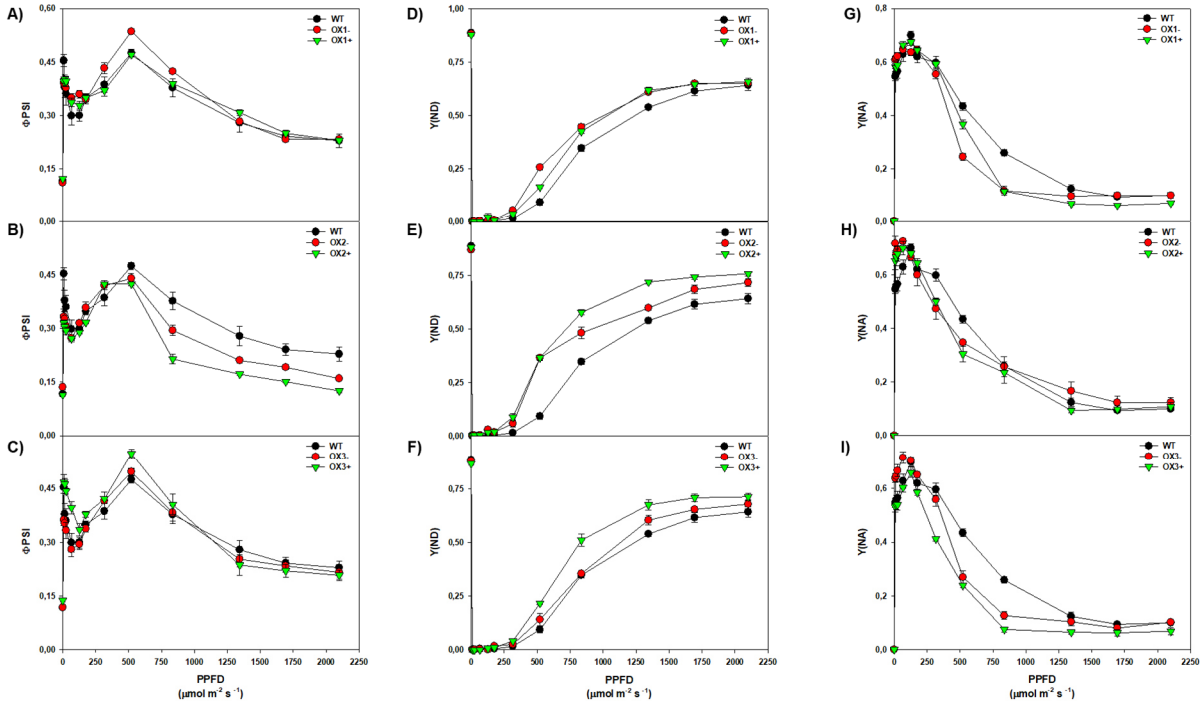


Figure 5 Light-response curve (PPFD). Photochemical measures in leaves from three lines overexpressing OsPiF14 gene (OX1, OX2, OX3) and wild type (WT) of intact rice plants 35 days old. The lineages are identified by black circles (WT), red (negative) and green triangle (positive). The measures are effective quantum efficiency of PSI – ϕPSI (A-C); PSI donor side limitation – Y(ND) (D-F) and PSI acceptor side limitation – Y(NA) (G-I). Represented values indicate the average of four independent replicates (± SE).

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

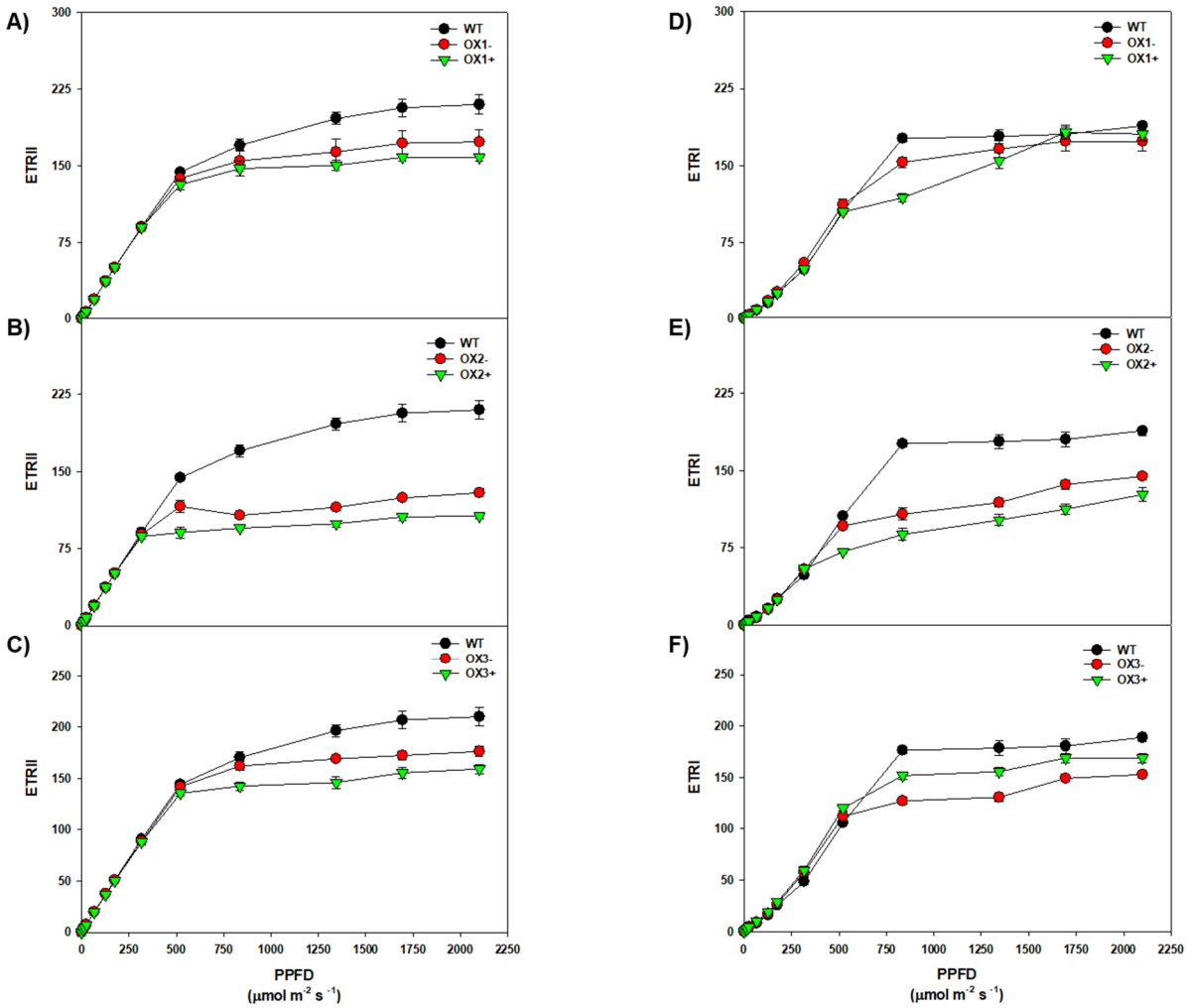


Figure 6 Light-response curve (PPFD). Photochemical measures in leaves from three lines overexpressing OsPiF14 gene (OX1, OX2, OX3) and wild type (WT) of intact rice plants 35 days old. The lineages are identified by black circles (WT), red (negative) and green triangle (positive). The measures are: Electrons transport rate of PSII – ETRII (A-C); electrons transport rate of PSI – ETRI (D-F). Represented values indicate the average of four independent replicates (± SE).

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

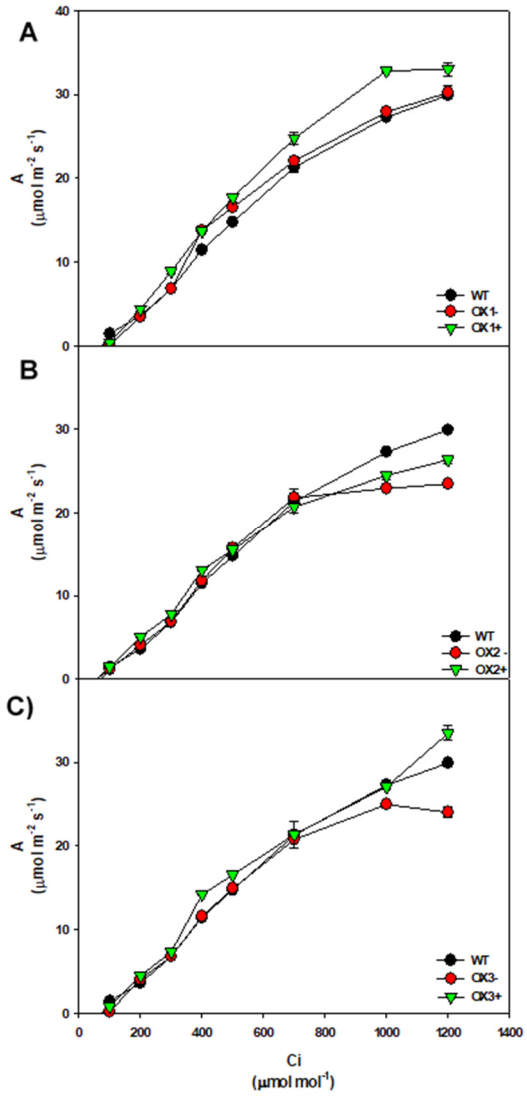


Figure 7 Intercellular CO_2 partial pressure-response curves of net CO_2 assimilation (A–Ci) in leaves from three lines overexpressing OsPiF14 gene (OX1, OX2, OX3) and wild type (WT) of intact rice plants 35 days old. The lineages are identified by black circles (WT), red (negative) and green triangle (positive). Represented values indicate the average of four independent replicates (± SE).

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

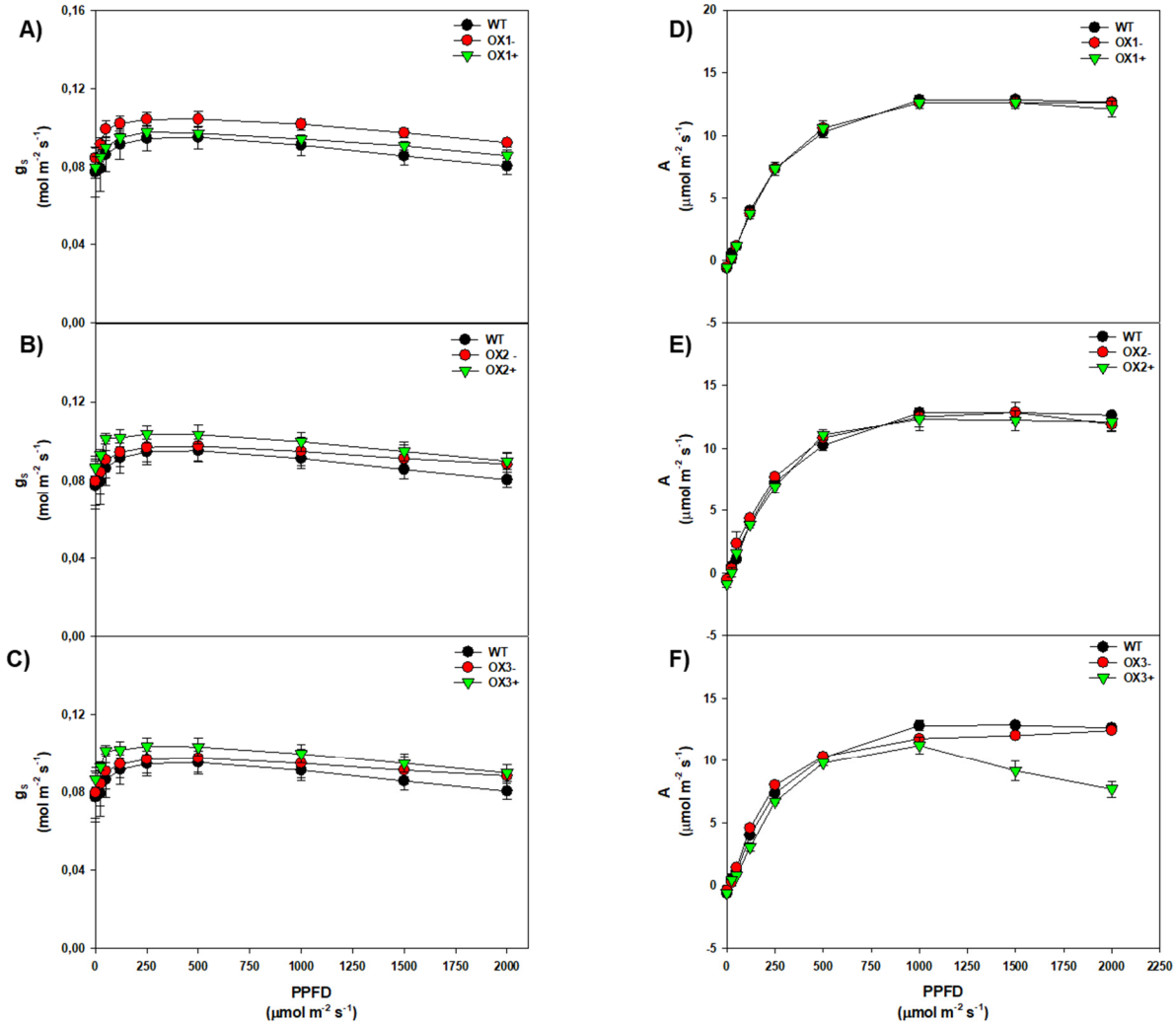


Figure 8 Light-response curves of net CO_2 assimilation (A-PPFD) (A-C), stomatal conductance (g_S - PPFD) (D-F) in leaves from three lines overexpressing OsPiF14 gene (OX1, OX2, OX3) and wild type (WT) of intact rice plants 35 days old. The lineages are identified by black circles (WT), red (negative) and green triangle (positive). Represented values indicate the average of four independent replicates (± SE)

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

Table 1: Dry mass root, shoot and total and relation root / shoot of three lines OsPIF14 overexpression and wild type rice plants.

	Dry mass (g)			Root:Shoot
	Root	Shoot	Total	
Wild type	0.91(0.10)	16.61 (1.36)	17.52 (1.46)	0.50 (0.01)
OX1-	1.17(0.20)	21.98 (2.45)	23.16 (2.63)	0.49 (0.02)
OX1+	0.79 (0.07)	16.41 (1.34)	17.20 (1.41)	0.55 (0.01)
OX2-	0.75 (0.03)	12.76 (0.44)	*13.50 (0.45)	0.54 (0.01)
OX2+	0.71 (0.05)	11.15 (0.89)	*11.86 (0.95)	0.67 (0.01)
OX3-	*0.51 (0.09)	10.91 (2.96)	*11.42 (3.04)	0.44 (0.04)
OX3+	*0.41 (0.07)	*7.80 (1.68)	*8.22 (1.70)	0.61 (0.05)

*Statistical difference according to WT Dunnett's Method (P < 0,050) (P = 0,001).

HYGROMYCIN TEST WITH TRANSFORMED PLANTS

Table 2: Seeds biomass and number of three lines of OsPIF14 overexpression and wild type rice plants. Seed/plant, seed/panicle, 100 seeds, viable and total biomass; viable and total seed number; and harvest index.

Line	Seeds biomass (g)					Seed number		Harvest index (%)
	Seed/Plant	Seed/Panicle	100	Viable	Total	Total	Viable	
Wild type	0.73 (0.07)	0.81 (0.04)	2.06 (0.06)	10.58 (0.84)	12.34 (0.61)	856.71 (45.67)	600.28 (49.90)	0.50 (0.01)
OX1-	0.55 (0.05)	0.85 (0.04)	1.57 (0.24)	11.05 (0.87)	12.56 (1.24)	1044.25 (151.63)	642.20 (36.21)	0.49 (0.02)
OX1+	*0.71 (0.03)	0.72 (0.06)	1.90 (0.04)	*6.16 (1.23)	*7.90 (1.15)	854.22 (86.63)	*310.67 (61.57)	0.55 (0.01)
OX2-	0.71 (0.03)	0.76 (0.02)	2.23 (0.10)	7.96 (0.57)	9.55 (0.54)	835.30 (43.65)	*373.70 (24.24)	0.54 (0.01)
OX2+	0.76 (0.08)	0.79 (0.03)	2.17 (0.05)	8.00 (1.20)	9.22 (1.15)	716.27 (57.21)	*374.20 (57.62)	0.67 (0.01)
OX3-	0.64 (0.05)	0.78 (0.05)	1.89 (0.02)	6.30 (1.60)	*7.02 (1.91)	579.75 (147.30)	*332.25 (85.18)	0.44 (0.04)
OX3+	*0.44 (0.08)	0.80 (0.03)	2.07 (0.04)	*4.50 (1.50)	*4.56 (1.17)	*324.60 (82.75)	*199.20 (44.22)	0.61 (0.05)

*Statistical difference according to WT Dunnett's Method ($P < 0,050$) ($P = 0,001$).