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SHAHID AZIZ

EXPLORING A ROLE FOR ROS-EQUILIBRATING ENZYMES IN PLANT STRESS TOLERANCE AND SARS-COV-2-INFECTED HUMAN CELLS – AN INTERDISCIPLINARY APPROACH THATTARGETS RESILIENCE PREDICTION

FORTALEZA

2023

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Thesis presented to the Graduate Program in Biochemistry of the Federal University of Ceara as a partial requirement to obtain the title of the doctorate in Biochemistry. Concentration area: Molecular Biology
Supervisor: Prof. Dr Jose Helio Costa

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Life is too short and uncertain, don’t be damn sure that your day is next, do your best now and enjoy every flash.

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SHAHID AZIZ

ABSTRACT

This thesis is organized into three chapters comprising comprehensive *in silico* studies on public experimental data from diverse species to strengthen our insights into *alternative oxidase* (*AOX*) and its complementary candidate genes as functional markers involved in reprogramming and host cell resilience prediction. The first chapter elucidates the unique expression pattern and organization of the *AOX* family from *Vitis vinifera*, which has been declared a model plant to validate the *AOX* family as a functional marker. We explored the expression of the *AOX* gene family across developmental stages and in response to several biotic and abiotic stress conditions associated with the hidden regulatory potential of large introns. Transcriptomic expression of *AOX2* was constitutively or developmentally regulated throughout the experiments. However, we identified *AOX2* alternative splicing (intron-1 dependent) that changes exon1 of normal *AOX2* and is effectively linked to increasing temperature. *AOX1a* and *1d* are associated with biotic and abiotic stress. In addition, the *AOX* intronic sequence assessment identified 16 microRNA (miRNA) candidates only in the *AOX2* introns, including the master regulator of plant development and stress responses mir-398. Among them, nine were conserved and validated in other plant species, whereas seven were considered potential novel miRNA candidates from *V. vinifera*. Enrichment analysis of the miRNA targets revealed the hierarchical functions of miRNAs in gene regulatory networks. The target genes mainly encode transcription factors, enzymes, and DNA-binding proteins and have a potential role in biological and metabolic pathways, including plant growth and developmental processes, pathogen defense mechanisms, and stress-responsive processes. Chapter two begins with validating the defined sets of genes complemented by *AOX*, called `ReprogVirus,` potentially involved in early cell reprogramming and host cell resilience prediction. To investigate the role of cell reprogramming initiation in the defined sets of genes, we performed transcript-level expression analyses of the most critical genes in the transcriptomic data of the two rice cultivars with contrasting responses to salt stress (Pokkali-tolerant and IR29 susceptible) over a short period of 24 h. Our data support the involvement of alternative pathways and glycolysis/fermentation in the more efficient stress response observed in a salt stress-tolerant rice genotype. This response is primarily associated with adaptive ROS balancing by *AOX* (via *AOX1a* expression), effective tuning of the antioxidant system, and rapid energy production (via fermentation). Both contribute to sustaining and optimizing respiration. Chapter three proceeds with validating the transcriptome-level profiles of the most critical marker candidates from the 'ReprogVirus' gene sets, identifying the major

complex trait for early de novo programming 'CoV-MAC-TED' in cultured human nasal epithelial cells infected by two SARS-CoV-2 variants differing in disease severity. Significant upregulation of the candidate genes revealed virus-induced ROS/RNS de-balancing, differential glycolysis, fermentation, and cell cycle regulation, essential for cell survival and escape from stress. In short, this thesis elucidates the diverse potential of *AOX* as a functional marker gene for predicting cell resilience and mediating cell reprogramming under stress. In parallel, this study justified the function of genes potentially substituting the role of plant *AOX* in virus-infected human nasal epithelial cells (NECs) with promising therapeutic strategies.

Keywords: functional marker; cell reprogramming; ROS/RNS equilibration; miRNA; SARS-CoV-2.

RESUMO

Esta tese está organizada em três capítulos que abrangem estudos *in silico* a partir de dados experimentais públicos de diversas espécies com o objetivo de avançar no conhecimento sobre a *oxidase alternativa* (*AOX*) e os seus genes complementares, candidatos a marcadores funcionais envolvidos na reprogramação e na predição da resiliência na célula hospedeira sob fatores estressores. O primeiro capítulo elucida o padrão único de expressão e de organização da família multigênica da *AOX* de *Vitis vinifera,* planta modelo usada para validar esta família como um marcador funcional. A expressão dessa família multigênica foi explorada em todos os estágios de desenvolvimento e em resposta a várias condições de estresses, bióticos e abióticos, associadas ao potencial regulador desconhecido de grandes íntrons no gene *AOX2*. Entre os membros da família, a expressão de *AOX2* foi regulada de forma constitutiva ou de acordo com o estágio de desenvolvimento ao longo dos experimentos. No entanto, nós identificamos um *splicing* alternativo em *AOX2* (dependente do íntron 1) que altera o éxon 1 da *AOX2* e está efetivamente ligado ao aumento da temperatura. *AOX1a* e *1d* foram associados ao estresse biótico e abiótico. Além disso, a avaliação dos íntrons da *AOX* identificou 16 candidatos a microRNA (miRNA) exclusivamente nos íntrons da *AOX2*, incluindo o principal regulador do desenvolvimento da planta e de respostas ao estresse: o mir-398. Dentre os miRNAs, verificou-se que nove são conservados e, anteriormente validados em outras espécies vegetais, enquanto sete foram considerados potenciais novos candidatos a miRNA de *V. vinifera*. A análise de enriquecimento dos miRNAs alvos revelou a hierarquia das funções dos miRNAs dentro das redes de regulação gênica. Os genes-alvo codificam, principalmente, fatores de transcrição, enzimas e proteínas de ligação ao DNA e têm um papel potencial em vias biológicas e metabólicas, incluindo crescimento e desenvolvimento de plantas, mecanismos de defesa a patógenos e processos responsivos ao estresse. O capítulo dois começa com a validação do conjunto de genes associados a função substitutiva da *AOX* em humanos chamados `ReprogVirus`. Estes genes estão potencialmente envolvidos na reprogramação celular precoce e na predição da resiliência da célula hospedeira. Para investigar o papel da iniciação da reprogramação celular, nos conjuntos de genes definidos, realizamos análises de expressão dos genes mais relevantes utilizando dados transcriptômicos de dois cultivares de arroz com respostas contrastantes ao estresse salino (Pokkali e IR29, genótipos tolerante e sensível à salinidade, respectivamente) em um curto período de 24 horas. Nossos dados reforçam o envolvimento da via alternativa e da glicólise/fermentação em uma resposta ao estresse mais eficaz no genótipo de arroz tolerante

ao estresse salino. Essa resposta está associada, principalmente, ao equilíbrio adaptativo das EROS pela *AOX* (via expressão da *AOX1a*), ao ajuste efetivo do sistema antioxidante e à rápida produção de energia (via fermentação). Portanto, ambos contribuem para sustentar e otimizar a respiração. O capítulo três prossegue com a validação dos perfis no nível do transcriptoma dos candidatos a marcadores mais críticos dos conjuntos de genes 'ReprogVirus', identificando o principal traço complexo para a programação inicial de novo 'CoV-MAC-TED' em células epiteliais nasais humanas cultivadas infectadas por dois Variantes de SARS-CoV-2 que diferem na gravidade da doença. A significativa regulação positiva desses genes revelou um desbalanceamento das ROS/RNS induzido por vírus e uma diferencial glicólise, fermentação e regulação do ciclo celular, essenciais para a sobrevivência celular e para a mitigação do estresse. Em suma, esta tese explora o potencial diversificado de *AOX* como um gene marcador funcional para prever a resiliência celular e mediar a reprogramação celular sob estresse. Paralelamente, este estudo revelou a função de genes/proteínas substituindo a ausência da *AOX* em células epiteliais nasais humanas infectadas por vírus (NECs), o qual indicou estratégias terapêuticas promissoras.

Palavras-chave: marcador funcional; reprogramação cellular; balanço de ERO/ERN; miRNA; SARS-CoV-2.

LIST OF FIGURES

Figure 1 Sketch of the unbranched typical mitochondrial ETC. e is an electron; QH_2 is ubiquinol; and C is cytochrome c……………………………………………… 14

Figure 2 Depiction of the ETC comprising the AOX pathway.………………………. 15

Figure 3 Depiction of AOX response to environmental threats. Under normal conditions, NADH oxidation by complex I is coupled to proton transport from matrix to IMS, and the electron flow from ubiquinol to complex III and then to complex IV to reduce O_2 to H_2O. On exposure to stress, ETC shows resistance to electron flow which induces AOX. AOX puts a branch in ETC after the ubiquinol pool and directly reduces O_2 to H_2O with heat production………………………………………………………………… 20

LIST OF ABBREVIATIONS

AOX	Alternative oxidase
APX	Ascorbate oxidase
ARP	Alternative respiratory pathway
AsA-GSH	Ascorbate–glutathione cycle
ASMT	N-acetylserotonin O-methyltransferase
ATP	Adenosine triphosphate
CAT	Catalase
cDNA	Complementary DNA
COX	Cytochrome oxidase
DHAR	Dehydroascorbate reductase
EST	Expresses sequence tag
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide
FM	Functional marker
GPX	Glutathione peroxidase
GR	Glutathione reductase
imiRNA	Intronic miRNA
IMM	Inner mitochondrial membrane
LECA	Last eukaryotic common ancestor
MDHAR	Monodehydroascorbate reductase
miRISC	miRNA-induced silencing complex
miRNA	MicroRNA
MREs	miRNA response elements
NADH	Nicotinamide adenine dinucleotide
NAT	Serotonin N-acetyltransferase
NCBI	National Center for Biotechnology Information
NECs	Nasal epithelial cells
NO	Nitric oxide
PFK	Phosphofructokinase
PQ	Plastoquinone
PTOX	Plastid terminal oxidase
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
RPKM	Reads per kilo base per million mapped reads
SD	Standard deviation
SE	Somatic embryogenesis
SHAM	Salicylhydroxamic acid
SOD	Superoxide dismutase
SRA	Sequence Read Archive
TCA	Tricarboxylic acid
UCP	Uncoupling protein
UTR	Untranslated region

CONTENTS

1	**THEOTETICAL BACKGROUND**..	13
1.1	**Cellular Respiration**...	13
1.2	**Alternative oxidase**..	14
1.3	**Genomic design of AOX**..	16
1.4	**Taxonomic distribution of AOX**..	17
1.5	**AOX presents *Vitis vinifera* specific profile**…………………………………	18
1.6	**AOX as a naturally evolved rescue mechanism in living cells**…………….	19
1.7	**AOX as a learning tool for designing human virus-defense strategies** …..	23
2	**ARTICLE 1: *IN SILICO* ANALYSES OF THE ALTERNATIVE OXIDASE (AOX) FAMILY FROM *Vitis vinifera* INDICATE THAT INTRON-DERIVED MIRNA-REGULATED MECHANISMS MIGHT GOVERN HOST GENE DIVERSE POTENTIAL**………………..............	26
3	**ARTICLE 2: TRANSCRIPTOME ANALYSES IN A SELECTED GENE SET INDICATE ALTERNATIVE OXIDASE (AOX) AND EARLY ENHANCED FERMENTATION AS CRITICAL FOR SALINITY TOLERANCE IN RICE**……………………….…………………………….	66
4	**ARTICLE 3: MAJOR COMPLEX TRAIT FOR EARLY DE NOVO PROGRAMMING ‘COV-MAC-TED’ DETECTED IN HUMAN NASAL EPITHELIAL CELLS INFECTED BY TWO SARS-COV-2 VARIANTS IS PROMISING FOR DESIGNING THERAPEUTIC STRATEGIES**…..	98
5	**CONCLUSION**…………………………………………………………….	123
	REFERENCES …………………………………………………………….	124

1. THEORETICAL BACKGROUND

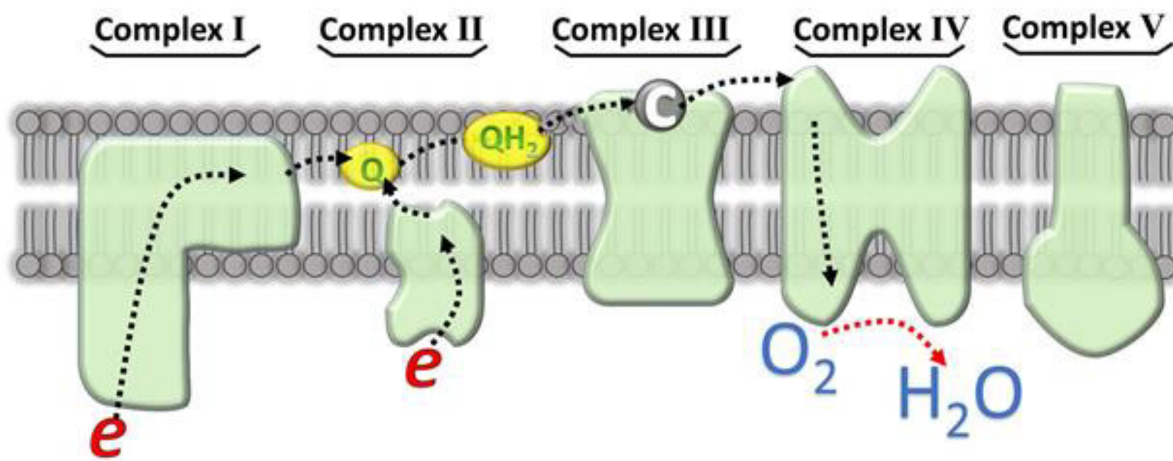
1. 1. Cellular Respiration

In early evolution, approximately 2 billion years ago, during the endosymbiotic event, bacteria were phagocytosed by primitive eukaryotes for their nutrient value. According to endosymbiotic theory, a hydrogen-supplying archaeal host and a hydrogen-consuming α-proteobacterial partner are adapted to life under anoxia, possibly giving birth to the supposed "last eukaryotic common ancestor" (LECA) (MALLEY et al., 2019). This endosymbiotic bond resulted in the evolution of mitochondria, primarily known as energy factories found in the cytoplasm of almost all eukaryotic cells except oxymonad *Monocercomonoides* species (KARNKOWSKA et al., 2019).

Mitochondria are intracellular organelles surrounded by a double membrane. The outer and inner membranes enclose the intermembrane space and mitochondrial matrix. The inner membrane harbors the mitochondrial respiratory chain or electron transport chain (ETC) that creates a proton gradient across the inner mitochondrial membrane, which drives the synthesis of the final energy source adenosine triphosphate (ATP) in a complete system named oxidative phosphorylation (BRAUN, HANS-PETER. 2020). The ETC is responsible for the transfer of electrons from reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), produced in the tricarboxylic acid (TCA) cycle, to oxygen across complexes I-IV. The electrons pass through in a series of donors and acceptors in the complexes, releasing helpful energy. The energy released forms a proton gradient, used in chemiosmosis to make a proportional amount of ATP by the action of complex ATP-synthase, taken together called cellular respiration (GUO et al., 2013). Regarding tissues and whole plants, respiration is vital to growth and productivity (TCHERKEZ et al., 2021). A diversity of organisms, except insects and vertebrates, possess an enzymatic mechanism that confers resistance to respiratory stress conditions by transferring an electron from ubiquinol to oxygen molecules, bypassing the

complexes of the ETC so-called alternative respiratory pathway (ARP) (McDONALD et al., 2008).

Figure 1. Sketch of the unbranched typical mitochondrial ETC. e is an electron; QH_2 is ubiquinol; and C is cytochrome c.



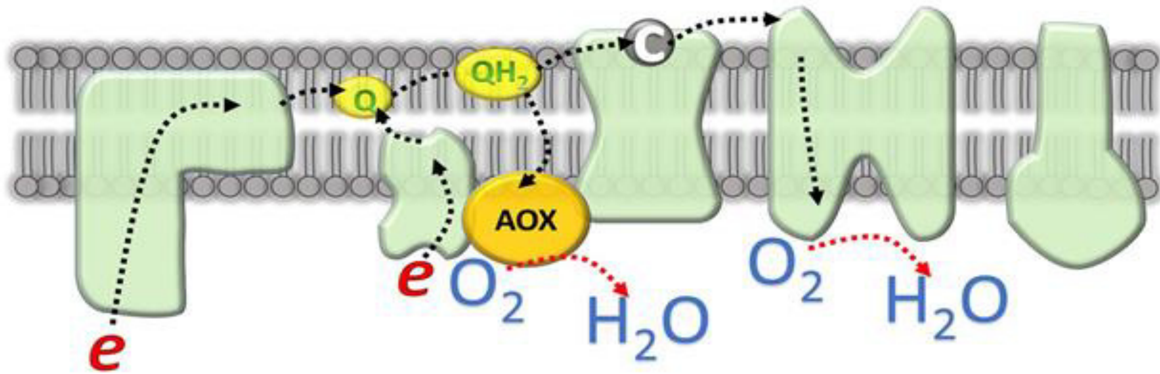
Taken from WEAVER et al. (2019).

1.2. Alternative Oxidase (AOX)

Many eukaryotic organisms and most plants possess ARP as part of total respiration (McDONALD et al., 2008). The key regulatory enzyme of this pathway is multigene nucleus-encoded alternative oxidase (*AOX*) (SLUSE et al., 1998). In eukaryotes, *AOX* is located at the substrate side of the cytochrome bc1 complex, forming an integral part of the electron transport chain in the mitochondria (Figure 2) (SMITH et al., 2012). *AOX* introduces a branch point at ubiquinone within the respiratory chain system in the inner mitochondrial membrane protein.

Under normal physiological conditions, electrons move through the ETC channel, generating proton gradients to make ATP by another transmembrane protein complex, ATP synthase (McDONALD et al., 2008).

Figure 2. Depiction of the ETC comprising the *AOX* pathway.



Taken from WEAVER et al. (2019).

When the cytochrome pathway shows resistance to electron flow in unfavourable conditions, *AOX* transfers electrons directly from the ubiquinone pool to oxygen, bypassing complexes III and IV, thus preventing cellular oxidative stress. Therefore, since this alternative pathway bypasses complexes III and IV, complex I is left as the only site of proton pumping. As a result, ATP production still occurs but at a lower rate (ELHAFEZ et al., 2006). The *AOX* pathway is insensitive to all known inhibitors of the last two complexes of the ETC, and as a result, it is referred to as 'cyanide-resistant' respiration (McDONALD and VANLERBERGHE, 2006). In most plant species, under normal physiological conditions, *AOX* is not expressed but expressed and localized to mitochondria when the function of the ETC is disrupted by chemical inhibitors or mutations (SWEETMAN et al., 2022; YOSHIDA et al., 2009). For example, antimycin A (AA) inhibits electron flow at Complex III, while cyanide (CN) inhibits complex IV

(WYATT et al., 2004). *AOX* is inhibited by salicyl hydroxamic acid (SHAM), and n-propyl gallate inhibits *AOX* by blocking the flow of electrons through *AOX* (SLUSE et al., 1998; MALLO et al., 2014).

1.3. Genomic design of AOX

In higher plants, *AOX* is nuclear encoded by a small multigene family comprising one to six gene members distributed by two subfamilies, *AOX*1 and *AOX2* (CARDOSO et al., 2015). In monocots, only genes belonging to the *AOX*1 subfamily have been identified, whereas *AOX2* existed in the most ancient monocot ancestor and was lost during speciation events of many monocots (COSTA et al., 2017), while in dicots, genes from both subfamilies are present (CARDOSO et al., 2015). The number of genes belonging to each subfamily varies considerably with the plant species (CARDOSO et al., 2015). In carrots, one gene of the *AOX*1 subfamily (Dc*AOX*1, COSTA et al., 2009) and two genes belonging to the *AOX2* subfamily (Dc*AOX2*a and Dc*AOX2*b) were identified (COSTA et al., 2009). In recent years, based on advanced deep sequencing data, the *AOX*1 and *AOX2* subfamilies have expanded (including *AOX1a*-e and *AOX2*a-d) in the majority of angiosperm species (COSTA et al., 2014).

The predominant structure of genomic *AOX* sequences consists of four exons interrupted by three introns at well-conserved positions (SAISHO et al., 2001; CONSIDINE et al., 2002; SAIKA et al., 2002; POLIDOROS et al., 2009; CAMPOS et al., 2009). Evolutionary intron loss and gain have resulted in the variation of intron numbers in some *AOX* members that may harbour two to four introns and three to five exons in their sequence (CARDOSO et al., 2014; CONSIDINE et al., 2002;). *AOX* genes are highly polymorphic in exon regions and even more pronounced in the non-exon areas (POLIDOROS et al., 2009). In general, causative polymorphic sites within a gene were found to have a low degree of conservation, and phenotypic variation in a target trait can be linked to diverse sequence polymorphisms (CARDOSO et al., 2013). High variability in intron size has also been reported within members of *AOX* genes from a single species and across plant species (CARDOSO et al., 2009).

Furthermore, it was found that *AOX* polymorphisms could be used to distinguish individual plants from the same species (FERREIRA et al., 2009). Nevertheless, high conservation in the protein-coding sequence leads to peptides with approximately 300 amino acid residues with highly conserved regions. Two highly conserved cysteine residues located toward the N-terminal hydrophilic domain mark the target site of redox and α-keto acid regulation (RHOADS et al., 1998; HOLTZAPFFEL et al., 2003). *AOX* proteins contain a four-helix bundle coordinating the di-iron center (BERTHOLD et al., 2002; MOORE et al., 2013). Iron-binding motifs within the four helical regions rich in histidine and glutamate (4 glutamates and two histidines) that coordinate the di-iron center and those that interact with *AOX* inhibitors such as SHAM have been identified in *AOX* across kingdoms (MOORE et al., 2013).

1.4. Taxonomic distribution of AOX

The *AOX* gene was transferred into eukaryotes from prokaryotes via primary endosymbiosis (ATTEIA et al., 2004). At first, *AOX* attracted attention in thermogenic plant species and was first discovered in plants due to the interesting observation of thermogenesis in the reproductive tissues of members of the *Araceae* family (MEEUSE. 1975). Initially, *AOX* was long thought to exist only in plants and fungi. Advanced research has identified *AOX* in all kingdoms except *Archaebacteria* (Matus et al., 2011; McDONALD and VANLERBERGHE, 2004; McDonald and Vanlerberghe, 2006; SIEDOW and UMBACH. 2000; MERCY et al., 2015; McDONALD et al., 2009). In plants, *AOX* is considered a ubiquitous enzyme; however, in algae, fungi, protozoa and several invertebrate species, it is pretty sporadic. (ÜNLÜ et al., 2019; LAMBOWITZ et al., 2013; LUÉVANO et al., 2018; JARMUSZKIEWICZ et al., 1997; MALLO et al., 2013). In the animal kingdom, *AOX* identification has been extended to 28 animals representing nine phyla (Porifera, Placozoa, Cnidaria, Molluska, Annelida, Nematoda, Echinodermata, Hemichordata and Chordata (McDONALD and VANLERBERGHE, 2004; McDONALD et al., 2009). However, *AOX* is absent in vertebrates and arthropods, and most bacteria lost *AOX* during evolution (VICENTE et al., 2015).

1.5. AOX presents *Vitis vinifera* specific profile

Information regarding *AOX* gene organization presents an almost universal pattern in plant species except for grapevine (*Vitis. vinifera*). The *AOX* family in *V. vinifera* revealed some peculiarities not yet found in other species (COSTA et al., 2009). The structure of the *Vv-AOX1a* and *Vv-AOX2* genes of Pinot Noir and PN40024 (a genotype of *V. vinifera*) consists of four exons interrupted by three introns, as previously noted in the majority of plant *AOX* genes. However, *Vv-AOX1a* revealed an additional intron in the 3'-UTR, and this is the first example of a *V. vinifera AOX* gene. The existence of UTR introns remains quite mysterious, and a retrotransposon element is integrated into the ubiquitously expressed *Vv-AOX2* (COSTA et al., 2009).

The popular notion that genes exhibiting high constitutive expression have short introns is not justified for *AOX* in *V. vinifera* plants, where the constitutive gene *AOX2* has an intron whose length is more than 60% of the gene length. On the other hand, the introns of inducible *AOX*1 genes expressed in response to oxidative stress have a total length of less than 15% of the gene length (COSTA et al., 2009). The mechanism of Vv-*AOX2* ubiquitous expression in the presence of the mysteriously large intron is poorly understood. Alternative splicing events or miRNA-related mechanisms may have evolved to constitute a network of controlling molecules that coordinately regulate gene expression through multiple interactions with other molecules, such as DNA, RNA and proteins. Introns mediate complex gene regulation by generating intronic microRNA (miRNA). An increasing body of literature addresses several microRNAs (miRNAs) that appear to be located within introns of protein-coding and noncoding genes (BHASKARANM et al., 2014; STATELLO et al., 2021; AXTELL et al., 2011). The genes in which these miRNAs are embedded are called host genes, and the miRNAs are called intronic or intragenic miRNAs (BOIVIN et al., 2018). miRNAs are vital in plant development and posttranscriptional regulation of genes involved in growth and development across eukaryotic organisms. We recommend a recent review by DONG et al. (2022) for more detailed information about RNAi mechanisms and their application in plant defense and development.

1.6. AOX as a naturally evolved rescue mechanism in living cells

AOX ensures electron transport along the alternative nonphosphorylating cyanide-resistant pathway in the mitochondrial ETC and plays an essential role in the maintenance of redox balance in the cell and defense against almost all environmental constraints (Figure 3) (JAYAWARDHANE et al., 2020; OH et al., 2022; MA et al., 2020). The involvement of *AOX* in plant responses to stress mitigation, development and growth processes by displaying altered expression levels has been established over the last decade (ZHANG et al., 2022; VANDERBURGH et al., 2016; DAHAL et al., 2014). Approximately two decades ago, Arnholdt-Schmitt et al. (2006) proposed *AOX* as a functional marker (FM) gene for genetic variation in cell reprogramming and yield stability. It shows a strong correlation with adaptive behaviour conferring stress alleviation and can be used in plant breeding to identify genotypes with high-yield stabilities in response to environmental plasticity. It is a truism to write that *AOX* influences the response of plant defense against hostile conditions and is involved in normal plant growth and development. Plants face numerous adverse biotic and abiotic conditions during their growth cycle. These stress conditions are known to induce *AOX*, and the induction may regulate the effect of those stressors (GARMASH et al., 2022). In the last decade, *AOX* has been explored in all kingdoms of life and plays significant roles in countering adverse growth conditions and maintaining host cell health (MOHANAPRIYA et al., 2019; ZHANG et la., 2012; VIANELLO et al., 2007; GALLUZZI et al., 2012). The majority of work done on the regulation of *AOX* has been in plants such as Arabidopsis and in fungi (GARMASH et al., 2021; SWEETMAN et al., 2022; HO et al., 2008; LANKINEN et al., 1991; WESTRICK et al., 2022; EBILOMA et al., 2019).

Figure 3- Depiction of *AOX* response to environmental threats. Under normal conditions, NADH oxidation by complex I is coupled to proton transport from matrix to IMS, and the electron flow from ubiquinol to complex III and then to complex IV to reduce O_2 to H_2O. On

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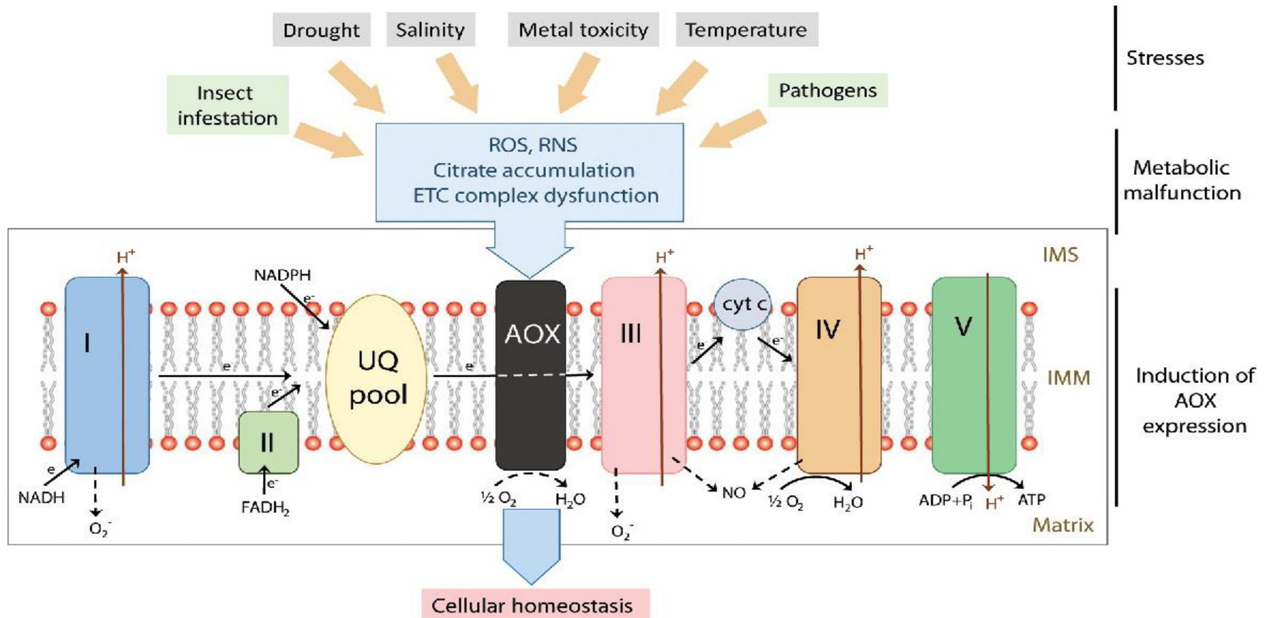

Taken from SAHA et al. (2016).

Stress conditions are known to induce *AOX*, and induction, in turn, may regulate the effect of those stressors (WESTRICK et al., 2022; EBILOMA et al., 2019). Many previous studies have shown that *AOX* genes are either constitutively expressed at specific stages, organs or tissues during development or are induced by several different stress factors and other outside stimuli (POLIDOROS et al., 2009). In general, plant *AOX* families are generally considered inducible (*AOX*1 group) or constitutive or developmentally regulated (*AOX2*) (COSTA et al., 2014; BORECKÝ et al., 2006). Although *AOX* is a multigene family, many organisms have one *AOX* gene of the family that is most responsive to stress and other stimuli. For example, in an Arabidopsis study, plants were exposed to five different abiotic stresses: heat, drought, salt, cold, or intense light. Microarray analysis confirmed that all stresses, except intense light, induced *AOX1a* expression (ATKINSON et al., 2013). Some studies have shown that the *AOX1d* transcript predominates during early rosette development and flowering (CLIFTON et al., 2006),

while *AOX2* is constitutively expressed and more associated with developmental stages (MOHANAPRIYAET al., 2019; MARELJA et al., 2014).

AOX has been extensively studied in higher plants, and its involvement in the plant response to stress mitigation by displaying altered expression levels upon external and internal stimuli has been established over the last decade. It has been shown in various kingdom organisms (ROGOV et al., 2014; FERNANDEZ et al., 2018). *AOX* controls respiration, photosynthesis and chlorophyll synthesis during drought to maintain overall homeostasis and enhance plant lifespan (VANLERBERGHE et al., 2016). *AOX* has also been reported to be responsible for developing cold resistance in winter wheat seedlings, whereas at temperatures below zero, *AOX* activity slightly decreases (FERNANDEZ et al., 2018). Phosphorous deficiency leads to a shortage of adenylates and Pi, which are substrates for oxidative phosphorylation, and hence, *AOX* is induced to maintain electron flow (VANLERBERGHE et al., 2013). The role of At*AOX1a* in maintaining cellular redox homeostasis, protecting cells against oxidative damage, improving the chance of survival and sustaining growth under oxidative stress in *Saccharomyces cerevisiae* was reported (VISHWAKARMA et al., 2016). *AOX2*a in carrots was connected to earlier or later stages of embryo development (FREDERICO et al., 2009). *AOX2* upregulation was observed during de novo growth induction from quiescent root phloem tissue (CAMPOS et al., 2016). Another study examined the variation in *AOX* expression in stress-sensitive and stress-tolerant genotypes of *Vigna unguiculata* (cowpea) (COSTA et al., 2007). The study examined the transcript and protein expression of Vu*AOX*1, Vu*AOX2*a, and Vu*AOX2*b in the roots of the two strains after exposure to salt and osmotic stress. Vu*AOX*1 and Vu*AOX2*a remained unchanged in both cultivars. Vu*AOX2*b was expressed at low levels under salt stress conditions but was induced under osmotic stress in the tolerant cultivar. On the other hand, in the sensitive cultivar, Vu*AOX2*b was overexpressed under salt stress conditions but not under osmotic stress (COSTA et al., 2007).

Several studies have indicated that *AOX* may also resist biotic stress, such as infection by pathogens. For example, *AOX* protein levels increased in local and systemic *Nicotiana tabacum* and tomato (*Solanum lycopersicum*) leaves infected with the tobacco mosaic virus (LENNON et al., 1997; LIAO et al., 2012). The overexpression of the *AOX* protein resulted in smaller hypersensitive response lesions, suggesting that *AOX* may suppress programmed cell death

(PCD) in virus-infected leaves (ORDOG et al., 2002). The induction of *AOX* in leafy mustard (*Brassica juncea*) during infection with turnip mosaic virus was suppressed in the presence of the ethylene biosynthesis inhibitor aminoethoxyvinylglycine, suggesting that ethylene and *AOX* are likely involved in the systemic resistance of green mustard to virus infection (ZHU et al., 2011).

To evaluate the function of *AOX* in the genus of fungi *Annulohypoxylon stygium*, *AsAOX* was cloned from *A. stygium*, and As*AOX*-silenced strains were constructed. The As*AOX*-silenced strains exhibited increased relative growth inhibition rates, higher oxidative stress and decreased melanin content compared with the wild-type strain (LIU et al., 2022). SHI et al. (2017) reported RNA interference (RNAi) functional analyses of *AOX* in *Ganoderma lucidum* with two silenced strains (*AOX*i6 and *AOX*i21). The *AOX*-silenced strains showed significant decreases of approximately 60 and 50% in alternative pathway respiratory efficiency compared to the wild type. In algae, *AOX* enables the survival of *Chlamydomonas reinhardtii* in high light. After characterizing *Chlamydomonas reinhardtii* mutants lacking the mitochondrial alternative terminal respiratory oxidases Cr*AOX*1 and Cr*AOX2*, a recent study (KAYE et al., 2019) suggested that when photosynthetic electron carriers are highly reduced, chloroplast-mitochondria coupling allows safe dissipation of photosynthetically derived electrons via the reduction of O_2 through *AOX* (KAYE et al., 2017). In addition, Liu et al. (2021) summarized that the mitochondrial *AOX* pathway is vital in sustaining algal photosynthetic performance. Treating *Auxenochlorella protothecoides* with an *AOX* inhibitor delayed chlorophyll accumulation, lagged reorganization of chloroplast structure, altered PSI/PSII stoichiometry, and decreased photosynthetic activities, indicating that the impairment in *AOX* activity dramatically hindered the development of functioning chloroplasts in algal cells (LIU et al., 2021). In the bacterial species *Vibrio fischeri*, Anne and Dunn (2018) identified that *AOX* reduces stress levels in cells exposed to oxygen and nitric oxide (NO). In previous analyses, the same authors reported NO-induced *AOX* expression in *V. fischeri*. The NsrR protein, a known NO-responsive regulatory protein, prevents this induction without NO. A binding site for the protein was identified upstream of the coding sequence of *AOX* via bioinformatics. Deletion of NsrR further showed that the protein negatively regulates *AOX* expression (DUNN et al., 2010).

In tardigrades, WOJCIECHOWSKA et al. (2021) recently confirmed *AOX*'s active role in tardigrade revival after anhydrobiosis using *Milnesium inceptum* as a model. Certain other

animals found to contain *AOX* have been investigated concerning *AOX* expression. *AOX* transcripts were found in the gill, heart, adductor muscle, hemolymph and mantle tissues of *Crassostrea gigas* (Pacific oyster) (McDONALD et al., 2009). Express sequence tag (EST) data have indicated that *AOX* transcripts can be detected in *C. gigas* exposed to bacterial or sewage challenges (SCHMITT et al., 2012; ROBERTS et al., 2008). Furthermore, *AOX* transcripts have been found in some tissues of some gastropod species, particularly in the central nervous system (McDONALD et al., 2009). Other animals where *AOX* transcripts have been found include some annelids and two members of Ecdysozoa (protostome animals) (McDONALD et al., 2009).

Recently, a significant increase in the expression of *AOX* after the addition of cytochrome pathway inhibitors in pathogen-infected *Scophthalmus maximus* suggested the important physiological functions of *AOX* in respiration under hypoxia and in protecting against oxidative stress generated during infection in the host (FOLGUEIRA et al., 2020). In mice, overexpressed tunicate *AOX* maintained electron flux when respiratory complexes III and IV were inhibited (NATASCHA SOMMER et al., 2020). However, mammals did not evolve *AOX* genes as integral to their complex metabolic and multiorganism networks. Currently, *AOX* is being developed to understand its beneficial mechanism functionality in mammals that naturally do not contain *AOX*. In *AOX*-overexpressing transgenic mice, the presence of *AOX* enhanced mitochondrial respiratory rates through forward electron transport from succinate dehydrogenase (cII) both under phosphorylating (presence of ADP) and nonphosphorylating (absence of ADP) conditions (SZIBOR et al., 2017). In a nut shell, as a functional marker gene, *AOX* sustains cell life by inducing resilience capacity on exposure to environmental threats. Furthermore, transcriptome-level profiling of *AOX* in plant and other AOX-encoding species with contrasting tolerances to biotic and abiotic stimuli can further validate the resilience prediction role of the *AOX* family genes.

1.7. AOX as a learning tool for designing human virus-defense strategies

Focusing on plant *AOX* as an FM gene is becoming increasingly interesting research in

plant breeding and across-species identification of candidate genes potentially substituting *AOX* (COSTA et al., 2021). *AOX* plays a significant role in homeostasis, cell reprogramming, plant growth and adaptation to diverse abiotic and biotic stresses, including viral infection LIAO et al., 2012). It is believed that *AOX* is primarily involved in regulating sugar-dependent fermentation, a vital feature for the efficient reprogramming of infected cells (AZIZ et al., 2022; ARNHOLDT-SCHMITT et al., 2022). By controlling ROS/RNS levels, *AOX* adjusts respiration, which at the same time is associated with the induction and regulation of cell division growth. Identifying differentially expressed ROS/RNS equilibrating *AOX* genes primarily involved in cell reprogramming has led to breakthroughs in host cell resilience prediction under multiple field conditions. De novo reprogramming effectively reverts stress-infected mature specialized cells into induced pluripotent stem cells, giving rise to cells with specialized functions and promoting organism survival (KUMAR et al., 2015). Plant somatic embryogenesis (SE) is the earliest and most studied model for de novo programming upon severe stress that promotes individual cell and organism survival (FEHÉR, ATTILA et al., 2015). *AOX* is a target for developing FMs for efficient cell reprogramming to assist in breeding robust plants with unique or multi stress tolerance linked to traits such as yield stability (ARNHOLDT-SCHMITT et al., 2006; POLIDOROS et al., 2009; ARNHOLDT-SCHMITT, 2015).

While learning from plant *AOX*, in recent years, attempting to understand the severity of COVID-19 infection and the deprived tolerance of the human defense system, our research group took an interdisciplinary approach to explore potentially *AOX* substituting gene networks with the same beneficial role (ARNHOLDT-SCHMITT et al., 2021). To analyze the *AOX* counteracting genes or genes with the same expression profile from human COVID-19-infected cell transcriptome data, we selected a standard set of genes named ReprogVirus connected to SE and virus-induced mechanisms. The ReprogVirus set of genes linked to ROS/RNS equilibration, antioxidant activities, NO production, lactic fermentation, structural cell organization, energy status signalling, cell cycle regulation, and regulation of apoptosis/programmed cell death and from other interconnected networks are detailed by Arnholdt-Schmitt et al. (2021). This approach identified a major complex trait for early de novo programming during SARS-CoV-2 infection, called 'CoV-MAC-TED' (major complex trait for early de novo programming during SARS-CoV-2 infection). It consists of unbalanced ROS/RNS levels, which are connected to increased aerobic fermentation linked to alpha-tubulin-based cell restructuring and progression of the cell

cycle (COSTA et al., 2021).

Although *AOX* is not present in humans, in search for similarities between the beneficial role of *AOX* in plants concerning adaptive oxidative stress level equilibration relevant for virus tolerance and that of natural agents in human cells, melatonin seems to be a strong candidate (COSTA et al., 2021). Accordingly, melatonin interacts with other enzymes involved in ROS/RNS balance and is present in most plants involved in stress mitigation rather than growth regulation. In addition, melatonin has been reported to enhance adventitious root induction by interacting with auxin-mediated signalling, suggesting a role for melatonin in early reprogramming (ARNAO et al., 2018). When all the information is considered, melatonin may be recommended to replace *AOX*'s early antioxidant function during reprogramming (COSTA et al., 2021). The identification of `CoV-MAC-TED' links to alpha-tubulin-based cell restructuring and cell cycle control is promising for the standardized identification of anti-SARS-CoV-2 targets. It is necessary to test various virus types and other pathogens infected cells from multiple origins to examine the use of this marker/host cell system for targeting anti-viral strategies.

2. ARTICLE 1

IN SILICO ANALYSES OF THE ALTERNATIVE OXIDASE (AOX) FAMILY FROM V_itis vinifera_ INDICATE THAT INTRON-DERIVED miRNA-REGULATED MECHANISMS MIGHT GOVERN HOST GENE DIVERSE POTENTIAL

In silico analyses of the alternative oxidase (_AOX_) family from _Vitis vinifera_ indicate that intron-derived miRNA-regulated mechanisms might govern host gene diverse potential

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All main figures, supplementary data and figures, and additional files are currently available in the link below. https://drive.google.com/drive/folders/1kAnPVwezg_NbzXn17b20khGZguE9yLFE?usp=share_link.

Abstract

The *V. vinifera* alternative oxidase (*AOX*) gene family has been previously identified with two *AOX1* genes (*AOX1a* and *1d*) and an exceptional *AOX2* endowed with large introns. Surprisingly, this large *AOX2* intron was associated with constitutive expression bucking the central dogma of gene expression in eukaryotes, indicating *V. vinifera* as a research model species to validate the *AOX* family as a functional marker. Thus, in the present study, we explored the expression of the *AOX* gene family across developmental stages and in response to several biotic and abiotic stress conditions associated with the hidden regulatory potential of large introns. Transcriptomic expression of *AOX2* was constitutively or developmentally regulated throughout the experiments. However, we identified *AOX2* alternative splicing (intron-1 dependent) that changes exon1 of normal *AOX2* and is effectively linked to increasing temperature. *AOX1a* and *1d* are associated with biotic and abiotic stress. In addition, the *AOX* intronic sequence assessment identified 16 microRNA (miRNA) candidates only in *AOX2*, including the master regulator of plant development and stress responses mir-398. Among them, nine were conserved and validated in other plant species, whereas seven were considered potential novel miRNA candidates. Enrichment analysis of miRNA targets revealed the hierarchical functions of miRNAs in gene regulatory networks. The target genes mainly encode transcription factors (TFs), enzymes, and DNA-binding proteins and have a potential role in biological and metabolic pathways, including plant growth and developmental processes, pathogen defense mechanisms, and stress-responsive processes. Furthermore, transcriptomic-level expression profiles of the targets and a comprehensive view of the negative regulatory relationship between miRNAs hosting the *AOX2* gene and their targets were acquired based on inverse expression levels. Overall, these findings clarified *AOX* expression in *V. vinifera* and indicated that miRNA induced enormous plasticity in *AOX2* gene regulation during stress alleviation and physiological improvement in *V. vinifera* cultivars.

Keywords: intron-derived miRNA; alternative splicing; gene regulation; stress alleviation

1. Introduction

All plants examined to date have a terminal oxidase enzyme known as alternative oxidase (*AOX*). In plants, *AOX* provides an alternate route when the cytochrome pathway shows resistance to the flow of electrons, thus preventing cellular oxidative stress (Jayawardhane et al., 2020; Oh et al., 2022; Ma et al., 2020). The *AOX* gene was transferred into eukaryotes from prokaryotes via primary endosymbiosis (Atteia et al., 2004). At first, *AOX* attracted attention in thermogenic plant species and was first discovered in plants due to the interesting observation of thermogenesis in the reproductive tissues of members of the Araceae family (Meeuse. 1975). Initially, *AOX* was long thought to exist only in plants and fungi. Advanced research has identified *AOX* in all kingdoms except *Archaebacteria (*Matus et al., 2011; McDonald and Vanlerberghe, 2004; McDonald and Vanlerberghe, 2006; Siedow and Umbach, 2000; Mercy et al., 2015; McDonald et al., 2009). In higher plants, *AOX* is nuclear encoded by a small multigene family comprising one to six gene members distributed by two subfamilies, *AOX*1 and *AOX*2 (Cardoso et al., 2015; Borecky et al., 2006). The flow of electrons through *AOX* does not build a proton electrochemical gradient. Therefore, alternative systems considered the energy-dissipating process. It plays a role in protecting plant cells against oxidative stress and provides plant metabolic flexibility, mediating the levels of energetic molecules (NAD(P)H and ATP/ADP) and messenger molecules such as ROS (Pu et al., 2015) (Yoshida et al., 2009; Maia et al., 2019).

Typically, *AOX* genes fall into two discrete subfamilies: *AOX*1a has members *AOX*1b and *AOX*2 (Polidoros et al., 2009; Costa et al., 2014). Various signals trigger AOX expression, indicating that it is a typical respondent to adverse conditions and displays patterns of variability across developmental stages and upon exposure to stimulants (Sweetman et al., 2021; Mohanapriya et al., 2021). Perturbation in *AOX* activity causes a decrease in plant productivity, including development and growth. In the presence of *AOX* inhibitors, embryogenic cells cannot develop an embryogenic structure with a diminished growth rate (Frederico et al., 2009). Subsequently, a study by Liu et al. (2021) demonstrated that treatment with the *AOX* inhibitor salicyl hydroxamic acid (SHAM) increased cellular ROS levels and antioxidant enzyme activities. In response to *Botrytis cinerea*, *AOX* deletion resulted in defects in mycelial growth, sporulation, spore germination virulence and increased fungicide and oxidative stress sensitivity (Lin et al., 2019). Aziz et al. (2022) reported an *AOX*-marked salt stress-tolerant rice genotype.

Polymorphisms in *AOX* favor ecotype adaptation to a particular environment and indicate more efficient *AOX* performance (Thiers et al., 2022). Considering the background knowledge, it is a truism to write that *AOX* influences the plant defense response against hostile conditions and is involved in plant growth and development.

Information regarding *AOX* gene organization presents an almost universal pattern in plant species except for grapevine (*V. vinifera*) (Costa et al., 2009). The *AOX* family in *V. vinifera* revealed some peculiarities not yet found in other species. The structure of the Vv*AOX*1b and Vv*AOX*2 genes of Pinot Noir and PN40024 (genotypes of *V. vinifera*) consists of four exons interrupted by three introns, as previously noted in the majority of plant *AOX* genes. However, Vv*AOX*1a revealed an additional intron in the 3'-UTR and a retrotransposon element (TE) integrated with the ubiquitously expressed *VvAox2* that is 5028 bp in size; the function/regulation is yet unknown (Costa et al., 2009). TE are involved in gene regulation and can potentially induce alternative splicing, resulting in a relatively rapid change in a gene's function and contributing to its host's adaptive fitness (Arnholdt-Schmitt 2004; Xu and Ramakrishna., 2008). The popular notion that genes exhibiting high constitutive expression have short introns is not justified for *AOX* in *V. vinifera* plants, where the constitutive gene *AOX*2 has an intron whose length is more than 60% of the gene length. On the other hand, the introns of *AOX*1 genes expressed in specific tissues/conditions have a total length of less than 15% of the gene length (Costa et al., 2009).

Introns are ubiquitous in eukaryotic genomes and have long been considered 'junk RNA,' but the vast energy expenditure of their transcription, elimination and degradation implies that they will have functional significance and might provide evolutionary advantages (Mukhopadhyay et al., 2021). Introns mediate complex gene regulation by generating intronic microRNA (miRNA). An increasing body of literature addresses several microRNAs (miRNAs) that appear to be located within introns of protein-coding and noncoding genes (Bhaskaranm et al., 2014; Statello et al., 2021; Axtell et al., 2011). The genes in which these miRNAs are embedded are called host genes, and the miRNAs are called intronic or intragenic miRNAs (Boivin et al., 2018). miRNAs are vital in plant development and posttranscriptional regulation of genes involved in plant growth and development across eukaryotic organisms (Dong et al.,

2022).

Approximately half of all currently identified plant miRNAs are derived from introns of protein-coding genes and other noncoding RNAs. For instance, the *Arabidopsis thaliana* genome contains 37 protein-coding genes with intronic miRNAs (imiRNAs), and the rice genome contains 181 protein-coding genes with imiRNAs (Yang et al., 2012; Knop et al., 2017; Wang et al., 2019). Recent findings indicate that plants assign miRNAs as critical gene-expression regulators in a sequence-specific manner to attenuate plant growth and development under numerous adverse conditions they face during their growth cycle (Dong et al., 2022; Yadav et al., 2020; Begum et al., 2022; Asefpour et al., 2020). The miRNA-induced silencing complex (miRISC) actions of mechanisms such as slicing target mRNA or miRISC-mediated translational inhibition and target mRNA decay depend on the degree of miRNA response element (MRE) complementarity. The complete complement-targeted region induces AGo2 endonuclease activity and degrades targeted mRNA, while partial complementarity mediates translational inhibition (Rani et al., 2022; Xu et al., 2019; Naeli et al., 2022).

Characterization of miRNA is a fast-evolving topic regarding computational and experimental methods. Computational strategies provide a valuable method to predict miRNA genes and their targets and have been successfully applied in mango (Moh et al., 2021), rice and *Arabidopsis thaliana* (Jones-Rhoades et al., 2004; Munusamy et al., 2017), sugar cane (Thiebaut et al., 2012), *Phaseolus vulgaris* (Nithin et al., 2015), and *Helianthus petiolaris* (Sahu et al., 2013). It is necessary to identify their targets to understand the biological function of miRNAs in plant development. No high-throughput experimental techniques for target site identification have yet been reported. However, several computational methods have been designed for miRNA target identification in plants, including the frequently used tool psRNAtarget, which also reports the inhibition pattern of cleavage (Dai et al., 2018). In plants, the perfect base pairing of miRNAs with their target sites is expected, which results in endonucleolytic cleavage by AGO (Argonaut protein) of the RISC complex (RNA-induced silencing complex) (Li et al., 2018). The minimal element needed to engage a target mRNA is the pairing of nucleotides (nts) 2–7 of the miRNA, called the seed regions, which are often solely responsible for targeting specificity (Chipman and Pasquinelli, 2019). Thus, the above findings indicate that plants assign miRNAs as critical gene-expression regulators in a sequence-specific manner to attenuate plant growth

and development under numerous adverse conditions they face during their growth cycle.

Undoubtedly, the study of the regulatory roles of noncoding RNAs uncovered a new field in plant biology as efficient regulators of biological processes. Debates on miRNA function, identification, and role association are emerging, and considerable work has been documented, but there are still enigmas to be uncovered regarding the mechanistic details of miRNA biology. Nevertheless, no extensive studies have assessed how the expression of *AOX* genes varies across the developmental stages, upon exposure to stress conditions in different tissues, and the functional role of introns in the *AOX*-Research-Model plant *V. vinifera*. In this work, we characterized the *AOX* gene, including the noncoding regions, to advance the understanding of the mechanisms of *AOX* gene regulation by *in silico* analysis of the finest SRA-Seq data (Sequence Read Archive) available in the public database NCBI. Our approach enables the complete identification of each gene function and uncovers the fascinating hidden potentials of the mysterious large introns that offer enormous plasticity in host gene regulation. This effort is promising to help further investigate the molecular mechanism underlying the interaction of miRNAs and other pathways through wet lab experiments. The main findings and future perspectives are discussed.

2. Materials and Methods

2.1. Searches for *AOX*-encoding genes and alternative splicing events in *V. vinifera*

The *V. vinifera* alternative oxidase genes *AOX1a*, *AOX1d,* and *AOX2* were obtained according to Costa et al. (2009) from the available reference genome of grapevine cultivar "Pinot Noir" clone PN40024 deposited in the GenBank database (NCBI) (http://www.ncbi.nlm.nih.gov). Alternative splicing events were analyzed using *AOX* genes in BLAST searches against *AOX* proteins and transcripts. These splicing events were validated by searching the alternative transcripts in *V. vinifera* transcriptome databases (TSA and SRA).

2.2 Selection of transcriptomic data and read quality check

We retrieved the finest transcriptomic data of *V. vinifera* with three biological replicates for each sample associated with developmental stages and stress conditions deposited in the public database SRA-NCBI https://www.ncbi.nlm.nih.gov/sra. Bioprojects and experimental conditions are detailed in Supplementary Table 1. The raw read quality was evaluated using FastQC v.0.11.7 software (Leggett et al., 2013). The raw reads containing adapters, low-quality bases (< Q20), and short-size sequences (≤ 50 bp) were trimmed and filtered using the fastp tool (Chen et al., 2018) and re-evaluated for quality conformation.

2.3 Transcriptomic profiling of *AOX* genes in *V. vinifera* RNA-seq data

The expression analysis of target genes *AOX1a*, *1d* and *2*, as well as alternative splicing in transcriptomic data of *V. vinifera,* was performed in four steps: (1) mapping of reads by Magic-Blast software (Boratyn et al., 2019); (2) quantification of mapped reads using the HTseq program (Andres et al., 2015); and (3) normalization of read amount in all samples. Thus, the target cDNA was aligned against RNA-seq data. After quantification of the mapped reads, the normalization of reads among different samples was carried out using the reads per kilobase of transcript per million mapped reads (RPKM) method (Mortazavi et al., 2008), according to the following equation: RPKM = (number of mapped reads × 109)/(number of sequences in each dataset × number of nucleotides of each gene). (4) Finally, the RPKM means of biological replicates were presented as a heat map using each transcript's log2-fold-change (Log2FC) in a given developmental stage or stress condition. In tissue development, data were compared to the

first stage, while in stress conditions, data were compared to the control to assess/estimate differential gene expression.

2.4 Identification of intron derived miRNA candidates in *AOX* gene

To explore the potential noncoding regulatory miRNAs, we subjected all introns of the *AOX* genes to the publicly available fast miRNA precursor and hairpin structure predictor software miRNA fold https://evryrna.ibisc.univ-evry.fr/miRNAFold using default parameters (Tav et al., 2016; Tempel et al., 2012). The repeated sequences were removed to avoid overlapping precursor sequences, and the remaining sequences were selected for further analyses. Following Zhang et al. (2013), more stable structures with minimum free energy above −30 dG were used as a query for homology/sequence similarity-based miRNA sequence identification. Using BLASTn, we subjected the precursor sequence to high throughput miRNA Seq-data PRJNA433719, PRJNA601829, PRJNA431619, PRJNA256276, PRJNA814871 and PRJEB42777 of *V. vinifera* available in SRA-NCBI to identify the potential miRNA sequences [Supplementary Table 1]. To ascertain the validity of the sequence identity-based detected miRNAs, we subjected the identified sequences to the previously reported mature miRNAs deposited in miRBase https://www.mirbase.org/ (current version (release 22, March 12, 2018). Sequences identical to known miRNAs from the miRBase database were selected as the miRNA dataset of *V. vinifera* only if they fit the following criteria: (1) at least 18 nt length was adopted between the predicted/mature miRNAs and (2) 0-4 nt mismatches in sequence with all previously known plant mature miRNAs were allowed (Meyers et al., 2008; Zhang et al., 2013).

2.5 Prediction of miRNA targets

The targets for mature miRNAs were predicted using the psRNATarget server https://www.zhaolab.org/psRNATarget/home and RNAhybrid tool https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid by submitting the mature miRNAs as a query and *V. vinifera* all transcript sequences from the JGI genomic project, Phytozome 13, Version: 457_v2.1 as a subject. The psRNATarget predicts miRNA targets by reverse complementary matching between miRNA and target transcripts and evaluates the target site accessibility by calculating unpaired energy required to open secondary structure around the small RNA's target site (Dai et al., 2018; 2011). It also reports translational inhibition or cleavage degradation by the presence/absence of a

mismatch in the central complementary region of the small RNA sequence. The following default parameters under Schema V2 (2017 release) were followed: the number of top target mRNAs for each small RNA was set to 100; the expectation was set to 3.0; the seed region was set to 2-13 nt; the maximum number of mismatches in the seed region was set to 2; the high-scoring segment pair (HSP) size (the complementarity scoring between small RNA and target mRNA) was set to 19; the expectation value was set to 3.0; and the range of central mismatch leading to translation inhibition was between 10-11 nt (Dai et al., 2018; Dai et al., 2011a). The RNAhybrid predicts hits with free energies up to a user-defined energy or p-value threshold, using the 5′-part of the miRNA, for example, from nucleotides 2–7 seed. The miRNA is hybridized to the target in an energetically optimal way (i.e., yielding the minimum free energy, MFE), forbidding intramolecular base pairings and branching structures (multiloops) (Krüger et al., 2006).

2.6 Validation of the miRNA targets

We selected five well-characterized unique target regions of each miRNA to ascertain the posttranscriptional regulatory role (target cleavage) of the identified miRNAs. We performed BLASTn (following step 2.3) using the miRNAs exhibiting gene (*AOX*2) and the target regions against the transcriptome data of *V. vinifera*, evaluating their expression level under changing environments and developmental stages.

2.7 Gene ontology of the target regions and transcription factor identification

To better comprehend the biological role of the miRNAs, we subjected the target regions to non-redundant (N.R.) and Swiss-Prot databases (2022-01-05) from the National Center for Biotechnology Information (NCBI) using BLASTX with an E-value of 10^{-5} and with a max target of 5 for homology searches of mRNA sequences (miRNA target genes). All identified unique targets regulated by the annotated miRNAs were uploaded to the AgriGO toolkit analysis http://systemsbiology.cau.edu.cn/agriGOv2/ to investigate gene ontology (Tian et al., 2017). In addition, we performed enrichment analyses of KEGG pathways via the KOBAS-I program. Hypergeometric statistical tests and the Benjamini and Hochberg FDR collection method were applied to identify significant pathways (Bu et al., 2021). The TFs were identified by searching all target regions against T.F. datasets from PlantTFDB http://planttfdb.gao-lab.org/, and the

default filtered results were kept (Jin et al., 2017).

2.8 Statistical analysis

Data were analyzed by ANOVA (one-way), and the significance analysis among different treatments was performed by Bonferroni's test or t-test ($p<0.05$). The value is the mean ± standard deviation (S.D.) of at least three replicates.

3. Results

3.1 Intron-dependent alternative splicing in *AOX* genes

The *AOX2* exon/intron structure indicating the *AOX2* alternative splicing identified in *V. vinif*era is shown in Figure 1. This splicing event is characterized by an alternative exon 1 in *AOX2* intron 1 (Figure 1A). Analyses in transcriptome databases revealed this alternative splicing in practically all experiments, despite the lowest mRNA level compared to normal *AOX2* (data not shown). A full-length mRNA representing the *AOX2* splicing alternative is found in the TSA database (accession: GAKH01019062.1). Analyses of deduced protein sequences of normal and alternative *AOX*2 revealed 46.5% identity, comparing the 95 with 87 amino acids derived from exon 1 (Figure 1B). Normal and alternative *AOX2* proteins were observed to have pre-sequences detected in the Mitoprot II webserver (Claros and Vincens, 1996) with a 99.4 and 98.3% probability of being exported to mitochondria (Figure 1B).

(A)

Aox2 PN40024 5' Promoter Exon 1 Alternative Exon 1 Exon 2 Retrotransposon 5028 bp Exon 3 Exon 4 3'

Intron 1 Intron 2 Intron 3 500 bp

Splicing events

Normal *AOX2* mRNA

Alternative *AOX2* mRNA

(B)

```
Normal_AOX2    MNQLVARSLMRGLINGAKCNRSILCSVTRVELRDE-KAAFGRMMSTETALAEKNQKEKSV 59
Altern_AOX2    ---------MQGRCNGGNCRRCIFSTTVVRSLRFRNRAAFWRAMTSKMAFPQKEIKEKSV 51
                        *:*  **.:*.*.*:.:..  .** . :*** * *::: *:.:*: *****

Normal_AOX2    EEVEKQSKVVFSSYWGIQRPKITREDGSPWPWNCFMPWETYHADTAIDLSKHHVPKTFVD 119
Altern_AOX2    SKTEKNGEVDVSSYCGISRPKIVRKDGTEWPWNSFVPWETYHADTAIDLSKHHVPKTFVD 111
               .:.**:.:* .*** **.****.*:**: ****.*:************************

Normal_AOX2    KVAYRTVKLLRIPTDIFFQRRYGCRAMMLETVAAVPGMVGGMLLHLRSLRKFEHSGGWVK 179
Altern_AOX2    KVAYRTVKLLRIPTDIFFQRRYGCRAMMLETVAAVPGMVGGMLLHLRSLRKFEHSGGWVK 171
               ************************************************************

Normal_AOX2    ALLEEAENERMHLMTMVELVRPKWYERLLVLTVQGVFFNAFFVLYVLSPKAAHRVVGYLE 239
Altern_AOX2    ALLEEAENERMHLMTMVELVRPKWYERLLVLTVQGVFFNAFFVLYVLSPKAAHRVVGYLE 231
               ************************************************************

Normal_AOX2    EEAIHSYTEFLKDIDSGAIENVPAPAIAIDYWRLPKDATLKDVITVIRADEAHHRDVNHF 299
Altern_AOX2    EEAIHSYTEFLKDIDSGAIENVPAPAIAIDYWRLPKDATLKDVITVIRADEAHHRDVNHF 291
               ************************************************************

Normal_AOX2    ASDIHFQGKKLNEAPAPIGYH 320
Altern_AOX2    ASDIHFQGKKLNEAPAPIGYH 312
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Figure 1. (A) *AOX*2 intron/exon structure and alternative splicing. (B) Peptide chain deduced from the normal and alternatively spliced exons.

In addition, normal *AOX*2 showed the conserved first cysteine (C) known to be involved in 2-oxo acid activation (Selinski et al., 2017), while alternative *AOX*2 revealed a serine (S) at this position (Figure 1B). Another alternative splicing event was found in *AOX1a,* constituted by the retention of unusual intron 4 in the 3' UTR. Interestingly, this intron targets eight miRNAs, as shown in Table 1.

Table 1. *AOX*1a 3'UTR additional intron targeted by eight miRNAs.

S. N0	miRNA Acc.	Target Acc.	Expect	Alignment	Action
1	osa-miR5542	*AOX*1a_3\`UTR	5.0	miRNA 21 UAGAGUACUAUGGAAGAGUUU 1 :.:.:::::::..: .:..:: Target 99 UUUUUAUGAUAUUUGUUUGAA 119	Cleavage
2	ptc-miR7840	*AOX*1a_3\`UTR	5.0	miRNA 21 CUACAGUGAUUAAUGAGGAAC 1 :::::.::::: :::.:: Target 31 CUUGUCAUUAAUU-CUCUUUU 50	Cleavage
3	smo-miR1114	*AOX*1a_3\`UTR	5.0	miRNA 21 GACGAGUUACAAAACAGGGAA 1 : .: : :::::::::...:: Target 53 GUUUUGAUUGUUUUGUUUUUU 73	Cleavage
4	gma-miR5379	*AOX*1a_3\`UTR	4.5	miRNA 24 CUAUAGUA-UUACUUACUAAAAGUA 1 : .:.:: ::: ::::::::::.:: Target 1 UAAGUUAUUAAUCAAUGAUUUUUAU 105	Cleavage
5	gra-miR8707	*AOX*1a_3\`UTR	4.5	miRNA 24 UGACUUAAUAAGUUUUCAAAAAUA 1 :::: :::: . ::::::::..... Target 82 AAGUUAUUAAUCAAUGAUUUUUAU 105	Translation
6	mtr-miR5244	*AOX*1a_3\`UTR	4.5	miRNA 21 UGGUUGUUAGAAGUACUCUAU 1 :.:::..::.::.:::: :: Target 91 AUCAAUGAUUUUUAUGAUAUU 111	Cleavage
7	ptc-miR828b-3p	*AOX*1a_3\`UTR	4.5	miRNA 21 AUUAUAAAGAACGAGCUUACU 1 : ::. : .:::.:.:::::. Target 102 UUAUGAUAUUUGUUUGAAUGG 122	Cleavage
8	rgl-miR5142	*AOX*1a_3\`UTR	4.5	miRNA 19 UAGUGAAUAGUUAGUUAUA 1 .: : ::::.:::::::. Target 80 GUAAGUUAUUAAUCAAUGA 98	Cleavage

3. 2 Expression profile of *AOX1a*, *1d*, *2* genes and alternative splicing events (*AOX1a-alt* and *AOX2-alt*) in different tissues of *V. vinifera* in an ordinary environment

The expression analysis of target genes *AOX1a*, *1d* and *2*, as well as alternative splicing (*AOX1a-alt* and *AOX2-alt*), were evaluated in all mRNA experiments. For *V. vinifera* tissues, the expression profiles were evaluated in transcriptomic data of leaves, stems, and tendrils from two cultivars, Yan73 and Muscat Hamburg (male parent), grown under the same conditions (Figure 2). Significant gene expression profile differences were observed by comparing tissues and cultivars. *AOX2* presented more stable expression in different tissues and cultivars. For *AOX1a* and *1d*, despite lower expression in Yan73, these genes were more highly expressed 5 to 25 times in the Muscat Hamburg cultivar in different tissues. *AOX2-alt* was detected at low levels compared with normal *AOX2*. However, it also presented stable expression when comparing the same tissue between cultivars. *AOX1a-alt* was also detected at low levels. However, higher expression was observed in the Muscat Hamburg cultivar in different tissues.

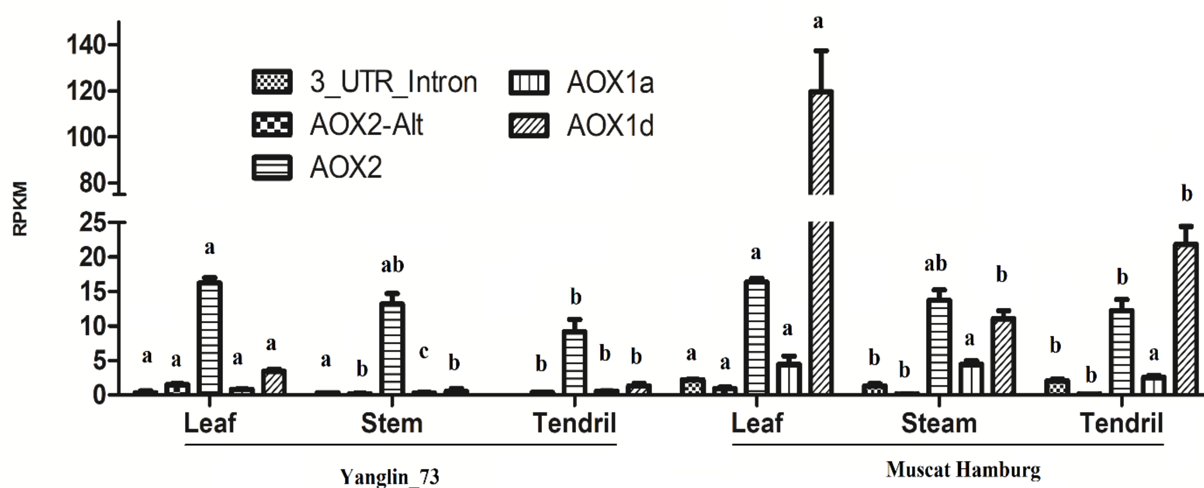

Figure 2. Expression profiles of *AOX* family genes and *AOX*1a 3'UTR intron in different tissues (leaf, stem, and tendril tissues) of *V. vinifera* genotypes Yan_73 and its male parent Muscat Hamburg. Data represent RPKM means with standard deviations from 3 biological replicates. For each gene, different letters indicate significant differences ($p < 0.05$) between tissues of both genotypes according to Bonferroni's test.

3.3 Transcriptome level expression profile of the selected genes in *V. vinifera* varieties during

physiological disorder

To understand the dynamics in *AOX* gene expression induced by the physiological disorder shrivel, we evaluated transcriptomic data in a time course experiment (PRJNA436693) 72 days after anthesis (DAA) (30, 44, 51, 58, 65, and 72 days). The differential gene expression was evaluated in shrivel compared to the control during berry development, as shown in Figure 3. Most of the evaluated genes showed no significant changes in expression level compared to their control. Nevertheless, some shrivel disorder responses appeared from 58 DAA. In this sense, a reduction in the mRNA levels of *AOX1a*, *AOX1a-alt*, and *AOX2* was observed at this point (p<0.05_ Supplementary Table 2). In contrast, *AOX1d* was upregulated at 58 DAA and downregulated at 30 DAA (Figure 3).

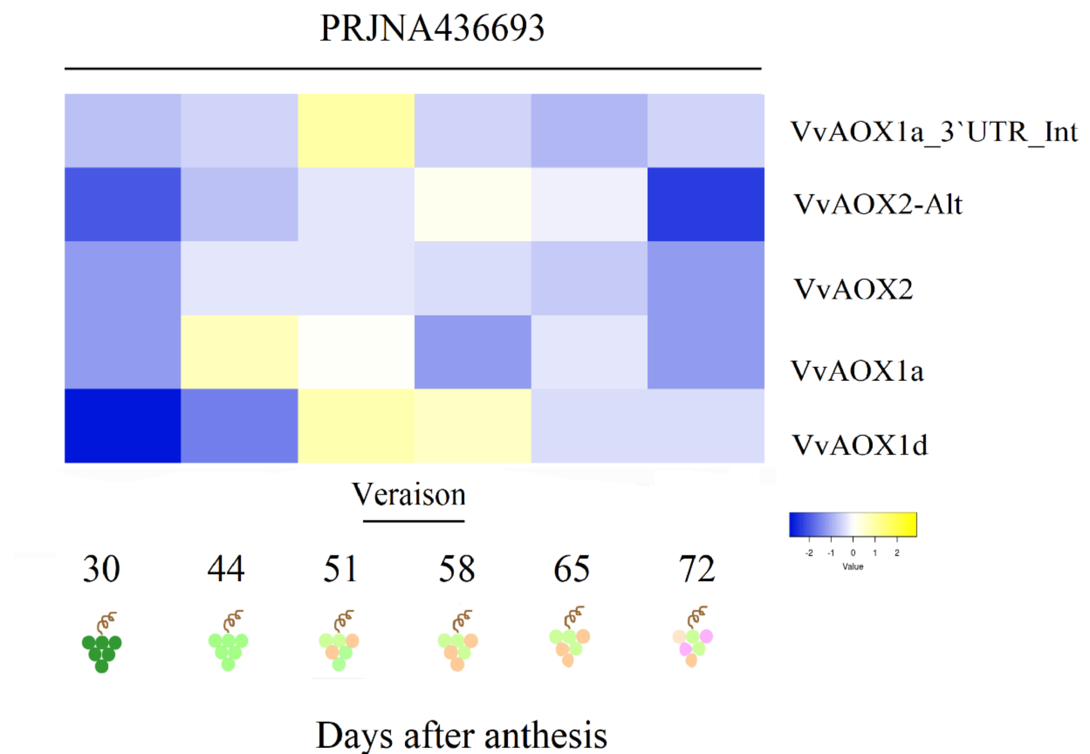

Figure 3. The heat map presents *AOX* family genes and *AOX*1a 3'UTR intron expression levels across developmental stages during physiological disorder shriveling. The data represent log2-fold changes Of RPKM + 1 in shrivel disorder in relation to the respective control in each developmental stage at 30, 44, 51, 58, 65, and 72 days after anthesis. In the heat map, yellow and blue represent up and downregulated genes, respectively.

3.4 Transcriptome level expression profile of the selected genes in *V. vinifera* varieties across berry developmental stages

Transcriptome level comparison of *AOX* genes and alternative splicing in the four fruit developmental stages, pea, touch, soft, and ripe fruit stage, among five genotypes (Sangiovese, Barbera, Negro Amaro, Refosco, and Primitivo) of *V. vinifera* is shown in Figure 4. Except for Barbera, all genotypes presented increasing *AOX1a-alt* transcription across the developmental stages, while *AOX2-alt* significantly increased at the final stages of ripening in all genotypes. *AOX2* was the most highly expressed gene and showed significant upregulation at the final stages of ripening in all cases. On the other hand, *AOX1a* generally decreased expression at the touch stage, followed by an abrupt increase in the late ripening stages. *AOX1d* showed variable expression profiles among genotypes, reducing in Sangiovese and Barbera or increasing in the late stages of Negroamaro, Refosco, and Primitivo. Significant differences are indicated by * at $p < 0.05$, Supplementary Table 3a.

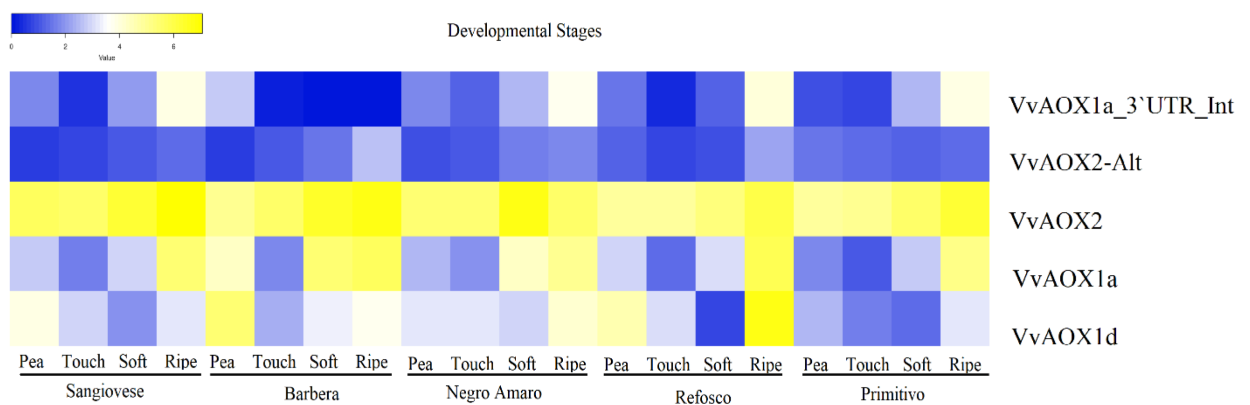

Figure 4. Heat map showing the expression level of the selected genes in four developmental stages of five *V. vinifera* cultivars. The data represent log2 (RPKM + 1). In the heat maps, blue and yellow represent low and high gene expression, respectively.

Other analyses were performed on berry skins at four specific °Brix (soluble sugar) levels of 20°, 22°, 24°, and 26° in seven genotypes, including Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot Noir, Chardonnay, Sauvignon Blanc, and Semillon, as shown in Figure 5. Unlike berries, many genes related to alternative respiratory pathways showed a cultivar-specific pattern in the skin at late-ripening stages, with some exceptions. Notably, *AOX1a* was upregulated through ripening in different genotypes ($p < 0.05$). The main transcriptional alterations occurred between the berry's skin with 20 and 24 °Brix. *AOX1a*_alt

transcription was upregulated across increasing sugar levels. *AOX1d* expression was markedly reduced in most of the genotypes in the late stages. Supplementary Table 3b shows that the significant differences from 20 ° Brix are indicated by * at $p < 0.05$.

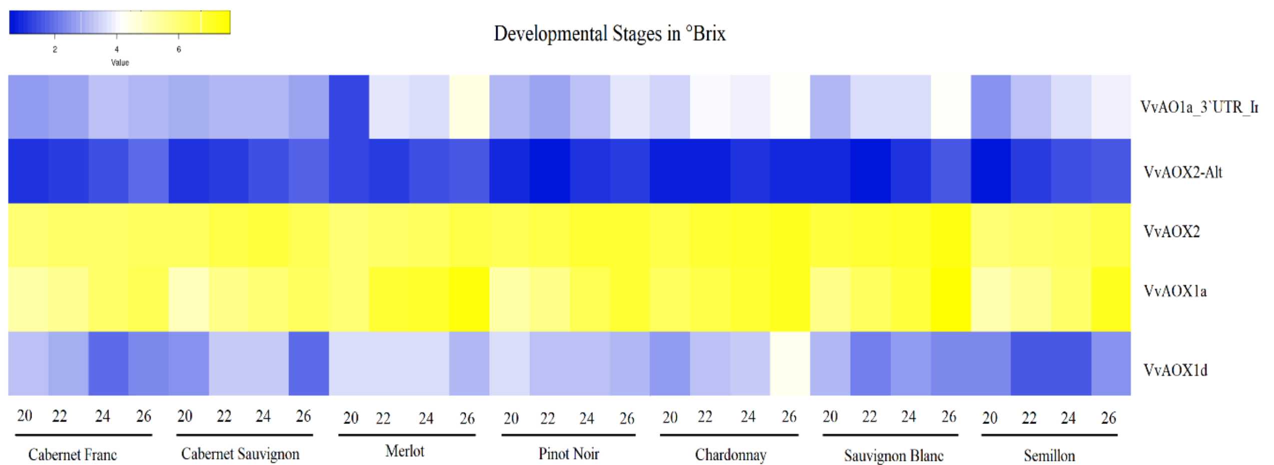

Figure 5. The heat map depicts the expression levels of the *AOX* family and alternative genes in the transcriptomic data of berry skin at four specific °Brix levels (20°, 22°, 24°, and 26°) in seven genotypes of *V. vinifera.* The data represent log2 (RPKM + 1). In the heat map, blue and yellow represent low and high gene expression, respectively.

In addition, similar analyses were performed during abscisic and auxin1-naphthaleneacetic acid-induced treatments in berries at stages E-L 34 to E-L 38 (Figure 6). *AOX1d* differentiated the treatment with significant upregulation during abscisic acid treatment, while a severely reduced level was observed in the auxin1-naphthaleneacetic acid treatment. High *AOX2*-alternative transcript accumulation was observed only in the case of auxin1-naphthaleneacetic acid treatment at phenological stage 36. *AOX2* was downregulated in both cases (Figure 6). These differential expressions were significant at $p < 0.05$, Supplementary Table 3c.

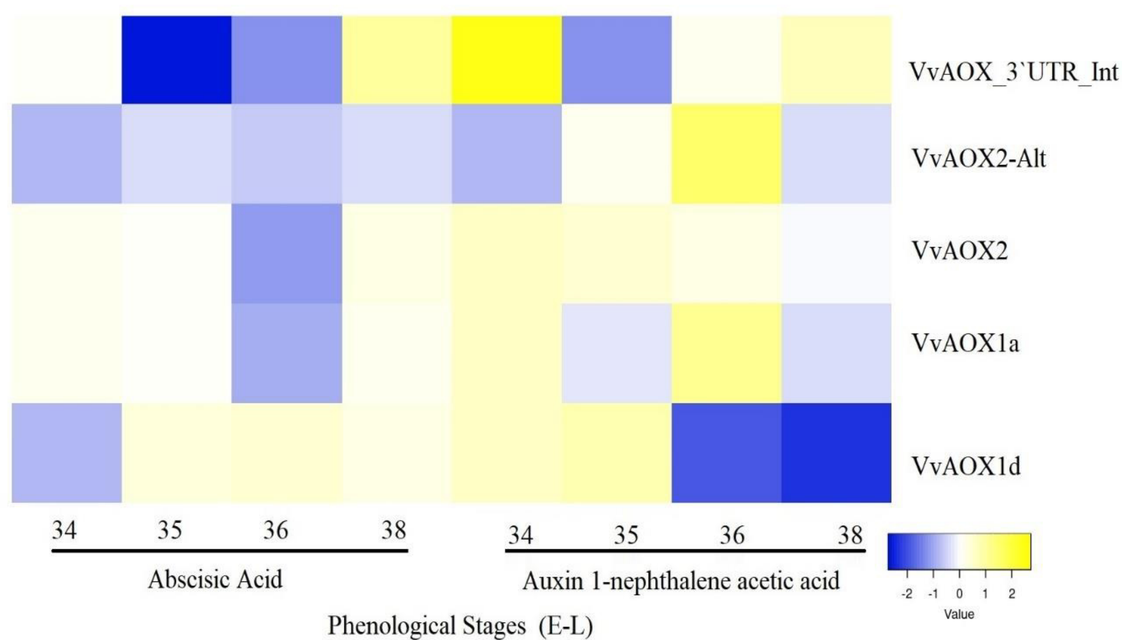

Figure 6. Heat map showing *AOX* family gene expression during development and ripening of abscisic acid- and auxin 1-naphthaleneacetic acid-treated berries. The data represent log2-fold changes in different phenological stages (34, 35, 36, and 38 E-L) in relation to the respective control conditions. In heat maps, yellow and blue represent up- and downregulated genes.

3.5 Transcriptome level expression profile of the selected genes in *V. vinifera* varieties under abiotic stress conditions

Figure 7 demonstrates the transcript level profile change of target *AOX* genes and alternative splicing in the berry transcriptome under water deficit (Bioproject PRJNA348619) conditions in five developmental stages and with high-temperature treatment (35 °C, 40 °C and 45 °C) (Bioproject PRJNA350310). Water deficiency significantly induced and suppressed the transcript levels of *AOX1a* and *AOX2-alt*, respectively, at the earliest stage of 26 days. *VvAOX1a-alt* responded to water deficit conditions with increasing transcription from 56 days. No significant changes were observed in *AOX2,* while *AOX1d* mRNA levels decreased at the final stage (106 days) of drought stress.

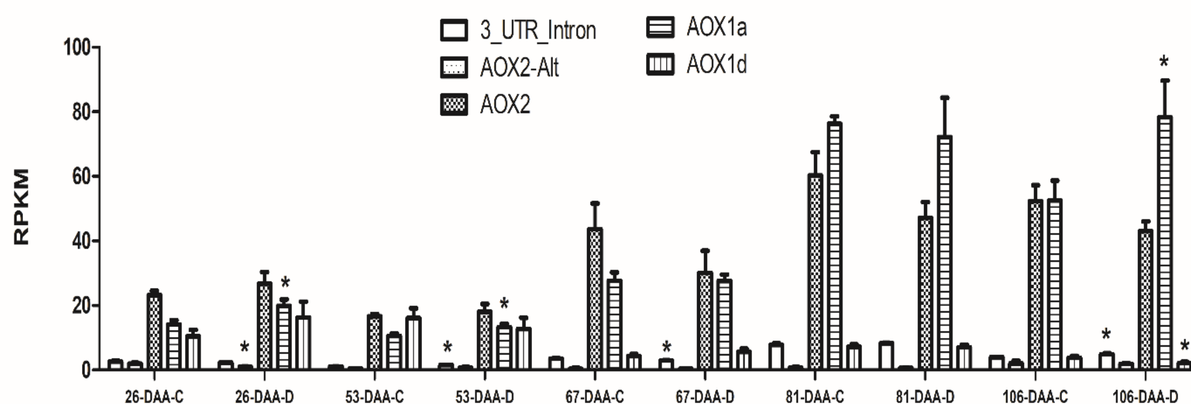

Figure 7. Gene expression of the *AOX* family during abiotic stress in grapes. Statistical analysis (t-test) was applied concerning the berry control of each developmental stage 26, 53, 67, 81 or 106 days after anthesis (DAA) under water deficit. Significant differences from the controls are indicated by * at $p < 0.05$.

AOX genes and alternative splicing were also evaluated in response to high temperatures (Figure 8; control, 35, 40, and 45 °C). Upon exposure to heat stress, a rapid reduction was observed in the *AOX1d* expression level from the first time point (35 °C) to the last point (45 °C). Conversely, the *AOX2* gene and the corresponding alternative splicing (*AOX2-alt*) were upregulated across the increasing intensity of temperature treatment. No remarkable dynamics were observed for the *AOX1a* transcript level, and *AOX1a-alt* was undetected.

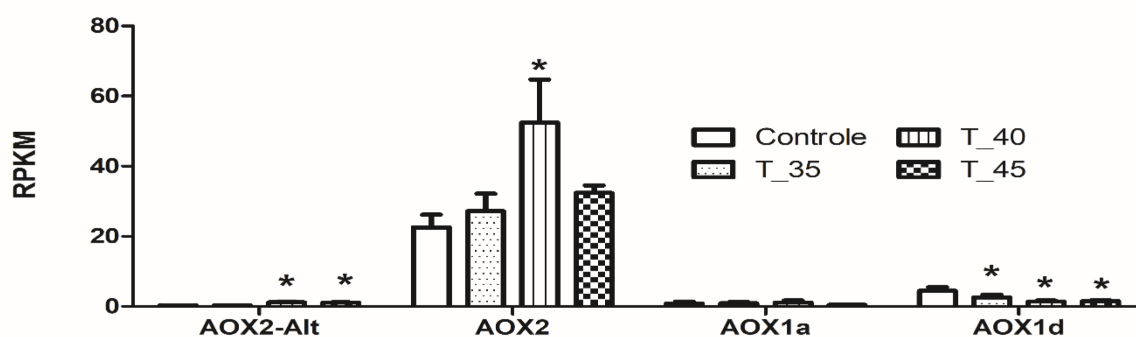

Figure 8. Gene expression of the *AOX* family during abiotic stress in grapes. One-way ANOVA, followed by the Bonferroni test, was applied concerning the leaf control of each temperature treatment (35, 40, or 45°C). Significant differences are indicated by * at $p < 0.05$.

3.6 Transcriptome level expression profile of the selected genes in *V. vinifera* varieties under biotic stress conditions.

Figure 9 depicts the transcript accumulation profile of *AOX* genes and alternative splicing in the powdery mildew (PM) (*Erysiphe necator*)-infected leaf transcriptome of *V. vinifera* genotypes Moscato and Nebbiolo. The infected cultivars were subjected to three resistance inducers (acibenzolar-S-methyl, potassium phosphonate, and laminarin) (Bioproject PRJNA508364). Only acibenzolar-S-methyl treatment exclusively stimulated transcript accumulation of *AOX*1a, 1d, and *AOX*2 in the Moscato cultivar. No effect on splicing alternative events was observed. The other resistance inducers (laminarin and potassium phosphonate) did not induce any remarkable change in the *AOX* mRNA level of Nebbiolo and Moscato cultivars.

The relative gene expression profiles of the selected genes in the transcriptome of the powdery mildew (*Erysiphe necator*) PM-infected Carignan cultivar at four different postinoculation times (12 and 24 h, 3 and 6 days) are presented under the section Bioproject PRJNA 254035 in Figure 9, and statistical analyses are shown in Supplementary Table 4. In Carignan leaves, a PM stress-induced late response with *AOX1a* and *AOX1a-alt* upregulation was observed at 3 and 6 days. On the other hand, *AOX2*-alt was downregulated as the postinfection duration increased.

We also evaluated the data from one PM-susceptible cultivar (Carignan) and seven PM-resistant cultivars (O34-16, Husseine, Karadzhandal, Khalchili, Late Vavilov, Sochal, and DVIT3351.27) at 1 and 5 days postinfection (DPI) in leaf transcriptome data (Bioproject PRJNA279229). Independent of PM susceptibility, most cultivars revealed *AOX1d* and *AOX1a* as early and late fungus infection-responsive genes, respectively. Additionally, *AOX2-alt* and *AOX2* were more highly expressed in Carignan, Husseine, Sochal, and DVIT3351.27 at 1 and 5 DPI, respectively. Regarding PM susceptibility, interestingly, higher expression of *AOX1d* and *AOX2-alt* was observed only in the PM-susceptible cultivar at 1 DPI. These data were significant at $p<0.05$, as shown in Supplementary Table 4.

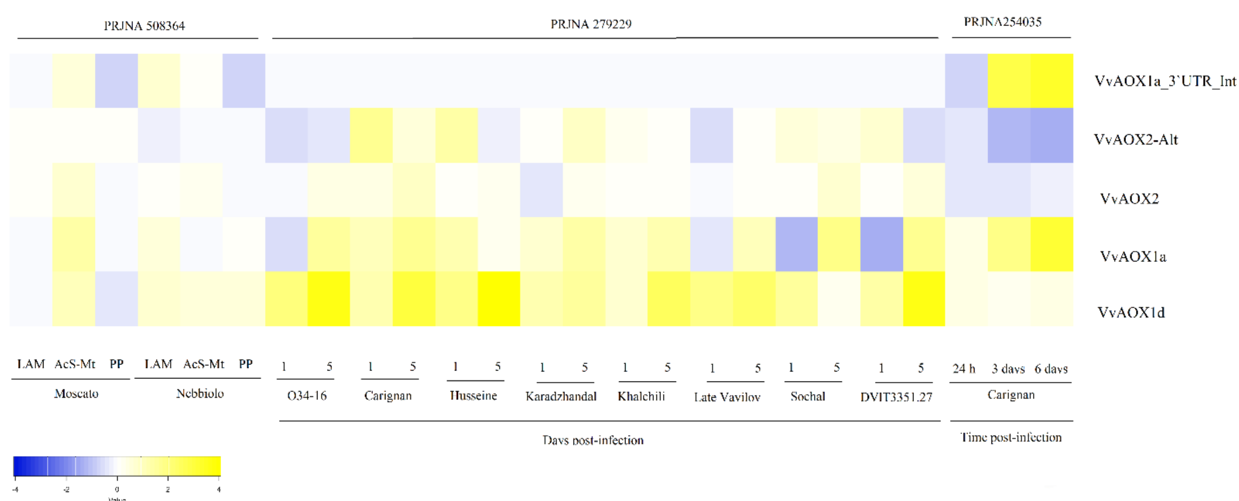

Figure 9. Heat map showing the gene expression of the *AOX* family in powdery mildew (*Erysiphe necator*)-infected leaves. The data represent log2-fold changes of two grape cultivars treated with resistance inducer concerning the control (Bioproject PRJNA508364); of different infection times in relation to the 12 hours post-infection (Bioproject PRJNA254035) and 1 and 5 days post-infection (DPI) of eight grape cultivars about respective control conditions (Bioproject PRJNA279229). In the heat map, yellow and blue represent up- and downregulated genes.

3.7 Identification and functional characterization of known and novel *AOX2* intron-derived miRNAs

We first searched for miRNA precursor sequences in the AOX gene introns to explore the intronic regulatory elements. The investigation revealed the identification of 16 stem-loop precursor sequences only in the introns of the *AOX2* gene, with an average length of 104-164 are shown in Supplementary Table 5. To identify miRNAs in the predicted precursors, we subjected the individual precursor sequences to high-throughput miRNA Seq-data of *V. vinifera* as a query through BLASTn. The search resulted in sequence similarity-based identification of sixteen homologous regions with 18 to 24 nucleotides as potential miRNA candidates. In further validation of the potential miRNA candidate sequences (18 to 24 nt), comparison with previously reported mature miRNA sequences of miRBase https://www.mirbase.org/ revealed the identification of nine previously reported miRNAs with a maximum of four nucleotide mismatches, as shown in Table 2.

Table 2. Computationally identified intronic miRNAs of the *V. vinifera AOX2* gene and their homologous miRNAs previously deposited in the miRBase database.

S. No	*V.vinifera* *AOX2* intronic miRNAs	Homologous miRNAs from miRBase	Attached article
1	vvi-mir536nr CAUAGAUGUGAAUCAUGUGA	gma-miR5671a CAUGGAAGUGAAUCGGGUGA	Song et al., 2011 Zhai et al., 2011
2	vvi-mir537nr AGGUCGGUGCAAUGGCGGCAACU	-	-
3	vvi-mir538nr UCGUGGUAACACAGAUUUUA	gma-miR10436 UCUUGGCAACACAACUUUUA	Zhao et al., 2015
4	vvi-mir539nr UCCGUAGGGCACCUUUUUUUUC	-	-
5	vvi-mir540nr CAGUACUUACCAAAUCCAAA	sbi-miR6233-3p CAUUAAUUACCAAAACCAAA	Thiebaut et al., 2012
6	vvi-mir541nr UGAAUAAGAGUAAUGUGGAGC	bra-miR5722 UGAAAUAGAGUCAUGUGGAAC	Yu et al., 2012
7	vvi-mir542nr UGUGGUCAUUUCCUCCUAUAUCA	-	-
8	vvi-mir443nr AAGAUGUAAUUCUUCUCUUG	-	-
9	vvi-mir544nr AAGUUUAGAUCUUUUGAACAUU	stu-miR7990b AAGUUACGAUCAUUUGAAAAUU	Zhang et al., 2013
10	vvi-mir545nr UCCUAGUAUGUUCUUUUAUUUUA	-	-
11	vvi-mir546nr AAUUUCUGGUUCUUCCUUCAUGU	-	-
12	vvi-mir547nr AGAGUUUAAUUUUCAUGCAC	lja-miR11097b-3p AGAGAUUAAUUUCUAUGCAC	Holt et al., 2015
13	vvi-mir548nr UUUCUGUGAAACUUAAGAAGCUUU	-	-
14	vvi-mir549nr CAUUCUUGCUGUUUUCAU	osa-miR5534b CAUUCUAGCUGUUGUCAU	Wei et al., 2011 Liu et al., 2017
15	vvi-mir550nr UUUGUAGCUAGGUCACCCCA	pta-miR398 UGUGUUCCAAGGUCACCCCA	Lu et al., 2007 Li et al., 2022
16	vv-mir551nr AAUGGAUACUGCAAAUGAA	stu-miR8015-5p AUUGGAUAUUGAAAAUGAA	Kuang et al., 2021

The remaining seven miRNAs did not show considerable similarity with the repositories of known miRNAs and could be potential novel miRNA candidates from the *V. vinifera* cultivar (Figure 10). We named the identified miRNAs according to the nomenclature rule described in the miRBase website https://www.mirbase.org/blog/category/nomenclature/.

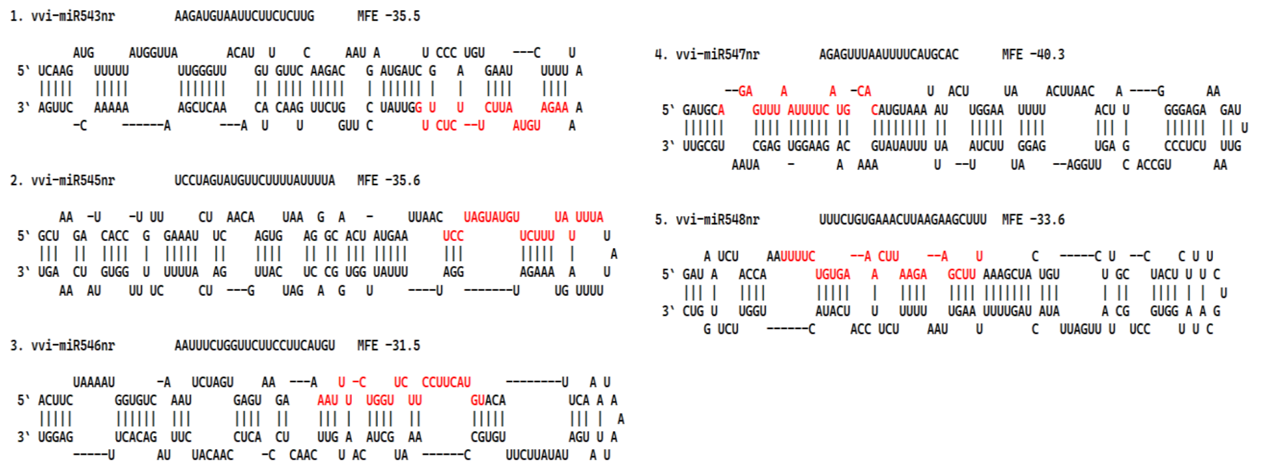

Figure 10. Folded hairpin structure of miRNA precursor sequences determined by the RNA-fold program. The sequences of mature miRNAs are highlighted in red letters. The abbreviation MFE stands for minimum free energy.

Target transcripts were predicted for all identified miRNAs except for vvi-miR538nr and vvi-miR549nr. Most target genes, 24.1%, 17.24%, and 10.34%, were among the targets of vvi-miR543nr, vvi-miR546nr and vvi-miR551nr, respectively. Rust resistance kinase Lr10, DNA repair protein, Elongation-factor_G-2, ABC-transporter, Disease_Resistance_RPP13, and trihelix transcription factor PTL were more frequently detected in target regions. Further characterization of the transcripts via the TF prediction tool PlantTFDB suggested three NF-YAs, two trihelixes and one GRAS family belonging to TF factors. The predicted transcripts/genes for each miRNA and other information originating from the prediction, including unpaired energy, aligned miRNA and transcript sequence, annotated gene function, and the regulation type: cleavage or translational inhibition, are shown in Supplementary File 1. While validating the miRNA's posttranscriptional regulatory function, an inverse relationship between the expression level of the miRNA hosting the *AOX2* gene and the target regions under biotic and abiotic stress conditions and berry developmental stages can be seen in Supplementary Figure 1-3. In addition, transcriptome-level expression of individual miRNA targets along the *AOX2* gene (regions under biotic and abiotic stress conditions and berry developmental stages) are presented in Supplementary File 2, 3, and 4 for biotic, abiotic, and developmental stages, respectively.

Gene Ontology (GO) enrichment analysis revealed the distribution of the target regions of miRNA in terms of biological processes, cellular components, and molecular functions. The miRNA targets were prominently involved in biological processes and molecular function. Most biological processes involve the regulation of protein stability, lipid localization, and cellular reproductive processes. While transcripts from the cellular component section were linked to molecule binding, the transmembrane moment of substances, ATPase activity, and receptor-binding proteins were prominent. The genes corresponding to molecular function categories were mainly associated with the microtubule complex, spindle, and extracellular matrix (Figure 11).

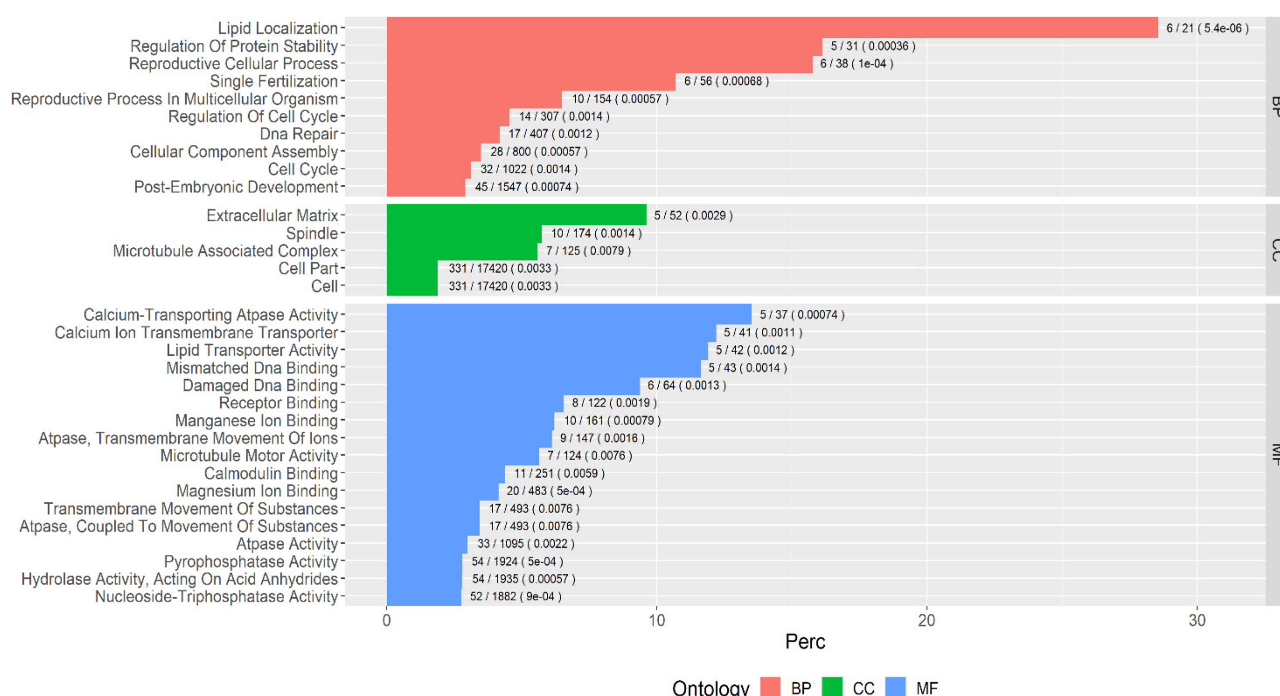

Figure 11. Gene ontology enrichment analyses of the predicted target regions of miRNAs. The AgriGO toolkit subgrouped the target genes into main GO vocabularies (1) biological process (B.P., red) (2) cellular component (CC, green), and (3) molecular function (MF, blue). The Y-axis is the percentage of genes mapped by the term, representing the GO term's abundance, and the X-axis is the definition of GO terms. The percentage of the input list was calculated by dividing the number of genes mapped to the GO terms by the number of all genes in the input list.

Following GO analysis, we used KEGG to construct a pathway enrichment of the predicted miRNA target genes. Many metabolic networks were found to be involved,

including metabolic pathways, mismatch repair, glycosylphosphatidylinositol, ABC transporters, plant–pathogen interaction, and beta-alanine metabolism, which were among the significant results ($P<0.05$) (Table 3).

Table 3. The miRNA target gene-enriched pathways are represented according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). The first three columns show the name, database, and I.D of pathways. The fourth column lists the number of input genes assigned to pathways, and the fifth P value of the statistical test

#Term	Database	ID	Input	Total	P Value
Metabolic pathways	KEGG	vvi01100	9	2390	9.29E-01
Mismatch repair	KEGG	vvi03430	6	42	2.88E-07
Glycosylphosphatidylinositol	KEGG	vvi00563	2	24	9.32E-03
ABC transporters	KEGG	vvi02010	3	32	9.96E-04
beta-Alanine metabolism	KEGG	vvi00410	1	57	2.79E-01
Glycine, serine and threonine	KEGG	vvi00260	1	67	3.18E-01
Protein processing in endoplasma	KEGG	vvi04141	2	216	3.47E-01
Di-carboxylate metabolism	KEGG	vvi00630	1	77	3.56E-01
Aminoacyl-tRNA biosynthesis	KEGG	vvi00970	1	132	5.28E-01
Phenylpropanoid biosynthesis	KEGG	vvi00940	1	202	6.82E-01
Biosynthesis of amino acids	KEGG	vvi01230	1	226	7.23E-01
Carbon metabolism	KEGG	vvi01200	1	263	7.75E-01
Biosynthesis of secondary meta...	KEGG	vvi01110	5	1271	8.48E-01
Glycosaminoglycan degradation	KEGG	vvi00531	3	15	1.33E-04
Arginine and proline metabolism	KEGG	vvi00330	2	62	5.04E-02
Nucleotide excision repair	KEGG	vvi03420	2	62	5.04E-02
Plant–pathogen interaction	KEGG	vvi04626	3	236	1.52E-01
Arginine biosynthesis	KEGG	vvi00220	1	34	1.79E-01
Fatty acid elongation	KEGG	vvi00062	1	39	2.02E-01
Ubiquinone and other terpenoids	KEGG	vvi00130	1	43	2.20E-01

3. Discussion

Transcriptomics is a useful approach to evaluate the transcriptome-level behavior of genes in an organism under a specific condition or developmental stage, thus enabling us to identify condition-specific marker genes (Arnholdt-Schmitt et al., 2006). Marker genes are more specific in expression for one or a few cells/genotypes over others in a particular condition and are important descriptors of cells and their behavior toward changing environments. As a genetic marker, the involvement of *AOX* in plant responses to stress mitigation, development, and growth processes by displaying altered expression levels has been established over the last decade (Arnholdt-Schmitt et al., 2006; Polidoros et al., 2009; Mohanapriya et al., 2021; Bharadwaj et al., 2021; Aziz et al., 2022). The alternative oxidase family of *Vitis vinifera* has been proposed as an attractive model to study the importance of genomic design (Costa et al., 2009). In the present work, considering the species-specific organization of *AOX*, we evaluated the transcriptome-level expression pattern of *AOX* gene family members across developmental stages and in response to several biotic and abiotic hostile conditions in *V. vinifera* genotypes. The *V. vinifera* Aox family revealed a retrotransposon element (T.E.) integrated with the ubiquitously expressed VvAOX2, which is 5028 bp in size (Costa et al., 2009). T.E.s are involved in gene regulation and can potentially induce alternative splicing, resulting in a relatively rapid change in a gene's function and contributing to its host's adaptive fitness (Arnholdt-Schmitt 2004; Xu et al., 2008). Depicting this information, we identified two alternative splicing events, one in the *AOX2* gene with significantly increased transcript accumulation of the *AOX2*-Alternative gene upon exposure to heat stress conditions. Another alternative splicing event was found in AOX1a, constituted by the retention of unusual intron 4 in the 3' UTR. AOX1a-Alt was upregulated under water deficit conditions.

In general, *AOX2* was a ubiquitous and highly expressed gene in all tissues and conditions (Supplementary Tables 2 to 4), confirming the previous observations in EST data by Costa et al. (2009). Concerning *AOX1a* and *1d*, these genes were also detected in all tissues/conditions. However, at lower levels than *AOX2* in most experiments evaluated. Perhaps this lower expression explains why these genes were detected only in specific tissue/conditions in EST data Costa et al. (2009).

For the first time, we identified two alternative splicing events (*AOX1a-alt* and *AOX2-*

alt) in *V. vinifera AOX,* which were detected at basal and low levels, but that expression fluctuated regarding tissues/conditions. Although *AOX* has been well studied in several angiosperm species, only *AOX1* alternative splicing (intron retention type) was previously documented in tomato (Fung et al., 2006) and wheat (Brew-Appiah et al., 2018). In tomato, *AOX1a-c* intron splicing was detected preferably at low temperature (Fung et al., 2006), which corroborates the *V. vinifera AOX1a-alt* (intron 4-splicing) increase under abiotic stress such as water deficit (Figure 7). Indeed, for the first time, *AOX2* alternative splicing associated with exon 1 change, instead of intron retention, was identified in an angiosperm species (Figure 1a).

The deduced *AOX*2-alt protein presents a serine instead of cysteine at the Cys I conserved position (Figure 1b), which might affect *AOX* activity in Arabidopsis (Selinski et al., 2017). Cysteine changes to serine at position Cys I rendered Arabidopsis *AOX1a* activated by succinate despite the insensibility of correspondingly substituted variants of *AOX1c* and *1d* (Selinski et al., 2017). In addition, Arabidopsis *AOX* isoforms can be differently activated by tricarboxylic acid cycle intermediates (Selinski et al., 2018); thus, it will be of great interest to investigate the activity regulation of the *AOX*2-alt protein to clarify the function of this alternative splicing in *V. vinifera*. Furthermore, *AOX1a* intron four retention (*AOX1a-alt*) may reflect specific regulation by miRNAs since the corresponding intron revealed eight miRNA targets (Table 1).

Fruit ripening analyses revealed that 3 *AOX* genes and two alternative splicing events could increase expression at the final stages. However, this full response occurred only in the Negroamaro and Refosco genotypes since in the other ten genotypes, at least one gene or alternative splicing event remained stable or decreased the expression (Figure 4). This finding provides evidence of the importance of *AOX* in grape berry ripening, and it will be crucial to extend this investigation by associating the genotypes with phenotypes to advance the understanding of the function of *AOX* isoforms in grape berry ripening.

In stress conditions, variable responses were observed according to biotic or abiotic stresses. *AOX1a* and *1a-alt* mRNA increased under drought, while high temperature stimulated *AOX2* and *2-alt* expression (Figures 7, 8). Under biotic stress, most genotypes revealed *AOX1d* and *AOX1a* as early and late fungus infection-responsive genes; however, *AOX2* and *2-alt* were also responsive depending on genotype (Figure 9; Supplementary Table

4).

Concerning the simultaneous *AOX1a* and *1d* stress response, similar behavior has been observed in monocot and eudicot species. In monocots, both *AOX1a* and *1d* from *Oryza sativa* were induced by stress conditions such as chilling, drought, and high salt (Feng et al., 2013; Li et al., 2013), while *AOX1a* and *1d2* from *Zea mays* were induced by specific inhibitors of respiratory complexes (Karpova et al., 2002). In eudicots, *AOX1a* from Arabidopsis is a strong stress-responsive gene (Saisho et al., 1997; Clifton et al., 2005; 2006; Giraud et al., 2008; Ho et al., 2008). However, *AOX1d* also seems to be among the most stress-responsive genes (Clifton et al., 2006). In *Solanum lycopersicum*, *AOX1a* and *1d* are the most responsive genes to tobacco mosaic virus infection (Fu et al., 2010; Liao et al., 2012) and cold treatment of fruit (Holtzapffel et al., 2003). With regard to *AOX2,* the same stress response was observed in our analyses (Supplementary Tables 4). In fact, in other species, this gene is more related to development. However, some plants, such as *Vigna unguiculata* (Costa et al., 2010), *Glycine max* (Matos et al., 2009) and two species of the *Medicago* genus (*M. sativa* and *M. trucatula*) (Cavalcanti et al., 2013), revealed an *AOX2* (*AOX2d type*) stress response together with *AOX1*. However, *V. vinifera AOX2* is classified as the *AOX2abc* type (Costa et al., 2014), and this stress behavior is uncommon. We observed that *V. vinifera AOX2* seems critical to different developmental and environmental conditions with regulatory elements (miRNAs) or alternative splicing hosted in large introns.

The popular notion that genes exhibiting high constitutive expression have short introns and are small in number is not justified for plant *AOXs*, where the constitutive and highly expressed *V. vinifera AOX2* has an intron whose length is more than 60% of the gene length (up to 62% in *V. vinifera*) (Costa et al., 2009). The ability of some introns to surprisingly stimulate mRNA accumulation even when the promoter has been deleted reveals that our understanding of gene expression remains incomplete (Rose et al., 2019). Most reported plant miRNAs emerged from intergenic regions or nonannotated genes (Zhang et al., 2013; Voinnet. 2009). By taking advantage of previous studies and following the strict rules of plant miRNA prediction and identification (with a maximum of 4 mismatches) suggested by Meyers et al. (2008), shedding light on the mysterious large intron of the *V. vinifera AOX*2 gene revealed the identification of seven novels and nine known miRNAs reported to be involved in the regulation of various biological pathways. We found more potential miRNAs when more relaxed filtering conditions were applied. Recent findings indicate that plants assign miRNAs as critical gene-expression regulators in a sequence-specific manner to

attenuate plant growth and development under numerous adverse conditions they face during their growth cycle (Dong et al., 2020; Yadav et al., 2020; Begum et al., 2022; Asefpour et al., 2020). Previous studies have found that miRNAs function in plants mainly by cleaving or inhibiting mRNA at the posttranscriptional level during developmental stages or to grow out of stress conditions (Dong et al., 20122).

The predominant sugars that are present in grapes are glucose and fructose. Only a few high-sucrose content cultivars have been detected in *Vitis labrusca* and *V. vinifera* (Liu et al., 2006). In our analyses, *AOX* mRNA accumulation increased with increasing sugar level (Figure 5). As a consequence of the obtained knowledge, our present study explores the homologous sequence of miRNA of sbi-miR6233-3p, as vvi-mir540nr is known to be a keen regulator of sucrose metabolism in sugarcane (*Saccharum* species hybrid) (Banerjee et al., 2006).

Furthermore, Giannuzzi et al. (2013) performed segmental duplication (SD) analyses in *V. vinifera* and demonstrated that 17% of the total genome was duplicated, and the duplicated genes were involved in the biosynthesis of compounds mediating stress adaptation. We identified vvi-mir538nr homologous to gma-miR10436 associated with evolutionary gene duplication events with primary roles in plant–pathogen interactions (Zhao et al., 2015). Furthermore, in confirmation of the pathogen-interacting role of *AOX,* in *Solanum lycopersicum*, *AOX1a* and *1d* are the most responsive genes to tobacco mosaic virus infection (Fu et al., 2010; Liao et al., 2012). In our analyses, we observed that *AOX2* and *AOX1a* and *1d* responded to powdery mildew (*Erysiphe necator*) infected leaves (Figure 9) (Supplementary Table 7).

AOX2 coordinates growth and diverse responses to environmental factors, and miR398 has been reported as a master regulator that coordinates growth and diverse responses to a series of stress conditions (Li et al., 2022). Thus, identifying the miR398 homologous miRNA "vvi-miR550nr" in the intronic region of *AOX*2 nailed the hidden regulatory and stress mitigation potential of the *AOX2* gene. Moreover, including the master growth regulator miRNA, we identified gma-miR4366 (as, vvi-mir542nr) involved in cotyledon development, homologous to bra-miR5722 (vvi-mir541nr) associated with lipid metabolism, and importantly, the target gene of bra-miR5722 encodes chlorophyll a-b binding protein 1 (BrLHCB1.2), principally regulating photosynthesis (Goettel et al., 2014; Wang et al., 2017).

Furthermore, reflecting the vital role of the exceptionally large intron of the AOX2 gene and reflecting the constitutive expression of the *AOX2* gene, our newly explored vvi-miR551nr shows homology with the model microRNA stu-miR8015 family known to be involved in the regulation of TOR-dependent (target of rapamycin) posttranscriptional gene regulatory networks in diverse metabolic pathways (Deng et al., 2021). Our study provides a base level of knowledge concerning the hidden potential regulatory role of the large intron, and the exhibiting miRNAs need to be further studied.

4. Concluding remarks

V. vinifera is an attractive model species for studying alternative respiratory pathways, including alternative splicing events and the regulatory role of large introns in plants. This study aimed to provide theoretical baseline information for further elucidation of *AOX* genes as functional markers and the hidden regulatory potential of mysteriously large introns involved in host gene functional diversification. Further experimental validation of the predicted novel miRNAs and their posttranscriptional regulatory roles is needed. The targets of the conserved and novel miRNAs have diverse functions, and their regulatory roles in *V. vinifera* require further study. We hope this discovery encourages experimental investigation into this novel mode of action, which could advance *AOX* research and the current knowledge of intron-mediated regulatory mechanisms associated with plant development, adaptation and stress alleviation.

Author's contribution.

This manuscript is part of the PhD dissertation of S.A. He conceptualized and designed the study and finalized the manuscript. TAG contributed to the statistical analyses and helped with manuscript preparation. AERO helped with the computational analyses. KLLT and LMS for Basic Data Preparation. BA-S helped with theoretical development and conceptualization. JHC directed the study, reviewed the data, interpreted the results, coordinated the discussions, and advanced the final manuscript. All the authors have agreed to the final manuscript submission.

Conflicts of Interest.

The authors have no conflicts of interest to declare.

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3. ARTICLE 2

TRANSCRIPTOME ANALYSES IN A SELECTED GENE SET INDICATE ALTERNATIVE OXIDASE (AOX) AND EARLY ENHANCED FERMENTATION AS CRITICAL FOR SALINITY TOLERANCE IN RICE

Transcriptome analyses in a selected gene set indicate alternative oxidase (AOX) and early enhanced fermentation as critical for salinity tolerance in rice

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Abstract

Plants subjected to stress need to respond rapidly and efficiently to acclimatize and survive. In this paper, we investigated a selected gene set potentially involved in early cell reprogramming in two rice genotypes with contrasting salinity tolerance (Pokkali tolerant and IR29 susceptible) in order to advance in early molecular mechanisms of rice dealing with salt stress. Selected genes were evaluated in available transcriptomic data over a short period of 24 hours and involved enzymes that avoid ROS formation (AOX, UCP and PTOX), impact ATP production (PFK, ADH and COX), or relate to the antioxidant system. Higher transcript accumulation of AOX (ROS balancing), PFK and ADH (alcohol fermentation) was detected in the tolerant genotype, while the sensitive genotype revealed higher UCP and PTOX transcript levels, indicating a predominant role for early transcription of AOX and fermentation to confer salt stress tolerance in rice. Antioxidant gene analyses supported higher oxidative stress in IR29 with transcript increases of cytosolic CAT and SOD from all cell compartments (cytoplasm, peroxisome, chloroplast, mitochondria). In contrast, Pokkali increased mRNA levels from AsA-GSH cycle as cytosolic/mitochondrial DHAR involved in ascorbate recovery. In addition, these responses occurred from 2h in IR29 and 10h in Pokkali, indicating an early but ineffective antioxidant activity in the susceptible genotype. Overall, our data suggest that AOX and ADH can play a critical role during early cell reprogramming for improving salt stress tolerance by efficiently controlling ROS formation in mitochondria. We discuss our results in view of gene engineering and editing approaches to develop salinity-tolerant crops.

Keywords. Cell reprogramming; ROS formation control; Pokkali; IR29; Crop development

1. Introduction

Salinity is the major impediment shattering the productivity of cultivated land areas. Excessive salt accumulation causes severe ionic toxicity, increases soil compactness, reduces plants' ability to acquire water, and clog efficient transportation of nutrients, thus interfering with crop production and yield worldwide [1]. Rice (*Oryza sativa*) model cereal crop is a premier staple food ensuring a large proportion of the human population's food and income for billions across the globe. Rice is categorized as a typical glycophyte and vulnerable to climate changes, thus alarming food security [2, 3]. However, abundant natural variability and various cultivated rice genotypes demonstrate contrasting responses to salt stress. Pokkali can withstand salinity, which is used as a positive control in screening salt-tolerant rice cultivars, while IR29 is considerably salt-sensitive and used as negative control [4]. Understanding genes and mechanisms that regulate environmental stress in crops is critical for boosting agricultural yield and quality, safeguarding food security and even protecting important crops from extinction. Comparative analysis of stress responding genes and their interconnected networks in rice genotypes with contrasting responses to salinity stress may help better comprehend salinity tolerating mechanisms and identify relevant genes for molecular breeding.

Tolerance against stresses is a complex phenomenon involving several particular gene loci with distinct regulation, molecular aspects, and an array of interconnected mechanisms that maintain plant homeostasis on exposure to hostile conditions [5]. In general, stress tolerance is linked to the maintenance of cellular redox homeostasis, regulating the levels of reactive oxygen species (ROS) required to initiate biological processes and function as signaling molecules to trigger plant defense responses [6]. Plants have different systems that act to regulate ROS formation by using alternative oxidase (AOX), uncoupling protein (UCP) and plastid terminal oxidase (PTOX) or ROS scavenging by enzymatic and non-enzymatic antioxidants.

The inner-facial mitochondrial membrane of plant cells harbors energy dissipating alternative respiratory systems mediated by AOX. The AOX gene family in angiosperms is a nucleus-encoded, composed of one to six members in two subfamilies (AOX1 and AOX2) linked to stress and housekeeping functions [7,8,9, 10]. However, in monocots, the AOX2 is restricted only to some species of Alismatales order [10], while most studies show that

monocots have 4 or 5 AOX1 genes [9]. In rice, 4 AOX1 genes (*AOX1a*, *1c*, *1d, 1e*) have been found [11,9] which *AOX1a and/or AOX1b (renamed to AOX1d) in [9]* were induced by different stress conditions as chilling, drought, and high salt while *AOX1c* was stably detected and *AOX1e* was barely expressed in germinating seeds[12, 13,14, 15, 16,17, 9, 18] In stress conditions, AOX relaxes the highly coupled and tensed electron transport process by driving them from quinol to oxygen, thereby alleviating tensed conditions and reducing ROS production [19]. These characteristics may allow them to flexibly deal with the challenge of changing scenarios and induce plasticity, facilitating plant persistence.

Besides AOX, the plant mitochondrial inner membrane possesses UCPs. The UCPs belong to the superfamily of mitochondrial carrier proteins dissipating the proton electrochemical gradient generated by the respiratory chain complexes [20]. In plants, these proteins are involved in mitochondrial energy flow regulation. They have been suggested to play a critical role in mitigating ROS production by the mitochondrial electron transport chain [21]. Moreover, another terminal oxidase is the PTOX, which is located in chloroplasts. PTOX is a key factor for maintaining the plastoquinone (PQ) pool redox balance and functions as a "safety valve" to protect photosynthesis [22]. It is a stress-responsive protein and could protect plants from various harmful stresses [23].

With changing environment AOX, PTOX, and UCP genes show differential expression patterns and are induced by multiple signaling pathways [24, 25, 26]. Together, AOX, UCP and PTOX are considered primary defense lines mitigating ROS production as their excess, causing progressive oxidative damage and ultimately cell death. Thus, these protein systems allow flexibly dealing with the challenge of several stressors, restoring respiratory activities and correcting metabolism.

Cellular damage manifests when the delicate balance between ROS production and elimination is disturbed on exposure to severe stress. To minimize the damaging effect of ROS, plant has developed an efficient antioxidant system with two components: enzymatic and non-enzymatic antioxidants. In plants, the non-enzymatic antioxidants ROS-scavenging pathway involves ascorbate and glutathione metabolites mediated by ascorbate–glutathione cycle (AsA-GSH) in chloroplasts, cytosol, mitochondria, and peroxisomes [27, 28]. Enzymatic components of the antioxidant defense system comprise several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX),

which catalyze ROS degradation, and enzymes of the ascorbate-glutathione (AsA-GSH) cycle, such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) that regenerate soluble antioxidants [29, 30].

To mitigate and recover from the damaging effects of adverse environmental conditions understanding and developing mechanisms such as effective reprogramming of the damaged cell is among the primordial needs. Induced cell reprogramming permanently facilitates plants immediate persistence with environmental factors variability throughout the lifetime. Consequently, it promotes individual cell growth and organism survival. Thus, early reprogramming in response to multiple individual and combined stressors can be a unique positive response across plant species and even across diverse taxonomic classes [31, 32, 33]. AOX demonstrated a significant role in plant homeostasis, reprogramming and plant growth adaptation in response to diverse abiotic and biotic stresses [34, 35, 36, 37, 38]. AOX has positive physiological roles in certain developmental processes and adaptation to environmental stresses. In doing so, AOX improved the ability of cells to rapidly recover its energy status [19]. Short and long-term fine-tuning of AOX at the transcriptional level was essential for positive performance effects [7, 39]. Finally, adaptive plant robustness in the field was shown to connect to the capacity of efficient cell reprogramming, which could be measured already at the level of seeds [34, 38]. Prediction of plant holobiont robustness could be linked in a technically simple way to AOX by inhibiting its activity. These tests promoted its use in seed screening for diverse species also for low-cost *on-farm* seed selection and are awaiting broader validation [34, 38, 40, 41, 42].

Recently, our group demonstrated that transcript accumulation of genes linked to early cell reprogramming under stress-related primarily to ROS/RNS balancing and energy status connected to cell restructuration and cell cycle regulation [33, 43, 44, 45, 46]. Our approach is in conformity with the view that "optimization of adaptive potential requires reconfiguration of developmental attributes to allow growth adjustment and stress avoidance" [47]. Thus, in the current study, we explored a selected gene set in public transcriptomic data of two rice cultivars with contrasting responses to salt stress (Pokkali tolerant and IR29 susceptible) during 24 hours post salt stress treatment to advance knowledge on the relevance of early cell reprogramming for general plant plasticity and robustness. Pokkali is most famous for salt tolerance but is appropriate for our approach since this traditional cultivar has a broader

spectrum for resilience [48, 49]. Here, we focused on gene expression involved in ROS formation (AOX, UCP and PTOX), impact ATP production (PFK, ADH and COX), and associated with the antioxidant systems (APX, MDHAR, DHAR, GR, CAT, SOD, GPX) in different cell compartments in order to gain insight in early cell reprogramming of salinity tolerance in rice.. The results are discussed in a connected view on redox homeostasis and energy supply as critical traits for salinity tolerance.

2. Material and Methods

2.1 Gene expression analyses of RNA-seq data.

This study used publicly available RNA-seq data of two rice genotypes with contrasting responses to salt stress (Pokkali tolerant and IR29 susceptible) [50]. Both genotypes were grown in growth chambers to the 3 leaf stage. The salt stress treatment was applied by watering 2-week old seedlings with 300 mM NaCl solution or by normal watering in control plants. Shoots (stem and leaves) were harvested at 1, 2, 5, 10, and 24 hours post-treatment to obtain transcriptomic data [50]. The transcriptomic data are available in SRA database from Genbank (NCBI) under the following Bioproject numbers: PRJEB4671 (Pokkali) and PRJEB4672 (IR29).

The expression analysis of target genes in transcriptomic data, with three replicates for each sample, was performed in three steps such as: 1) mapping of reads by Magic-Blast software [51], 2) quantification of mapped reads using HTseq program [52] and 3) normalization of reads amount in all samples. Thus, in the mapping of the reads, the target cDNAs were aligned against RNA-seq data. After quantification of mapped reads, the normalization of reads among different samples was carried out using the RPKM (Reads Per Kilobase of transcript per Million of mapped reads) method [53] according to the following equation: RPKM = (number of mapped reads × 109)/(number of sequences in each database X number of nucleotides of each gene).

The target genes were associated with glycolysis, fermentation, aerobic respiration, anti-ROS formation, and antioxidant. Thus, these genes included seven gene members encoding cytosolic PFK (Phosphofructokinase) [54] to represent total PFK in glycolysis. Four ADH (Alcohol Dehydrogenase) genes [55] to denote total ADH in alcohol fermentation while mitochondrial aerobic respiration was represented by total COX (cytochrome c oxidase) with eleven gene members [56]. In addition, total AOX (alternative oxidase) with 4 genes [11,9] and UCP (UCP 1 and 2, [57] were the systems involved in mitochondrial ROS formation control, while PTOX single gene, Tamiru et al. [58] represented the system regulating ROS formation in the chloroplast (Supplementary Table 1). Regarding antioxidant enzymes, multiple gene families of APX, MDHAR, DHAR, GR, SOD, GPX, CAT encode proteins with different subcellular destinations such as cytosol, peroxisome, mitochondria and chloroplast (Supplementary Tables 1 and 2) were evaluated. In general, all antioxidant genes presented at

least one member associated with each compartment, except GPX (without members to cytosol) and CAT (without members to chloroplast and mitochondria).

2.2 Prediction of subcellular localization of antioxidant proteins

For the prediction of the subcellular localization from corresponding deduced antioxidant proteins (listed in Supplementary Table 2), the following tools were used: TargetP-2.0 (http://www.cbs.dtu.dk/services/TargetP/), MitoProtII (https://ihg.gsf.de/ihg/mitoprot.html), DeepLoc-1.0 (http://www.cbs.dtu.dk/services/DeepLoc/) and Plant-mSubP (http://bioinfo.usu.edu/Plant-mSubP/) [59, 60, 61]. In addition, experimental confirmation of subcellular localization was available for proteins of some gene members of APX [62, 63] MDHAR [64, 65, 66] CAT [67], SOD [68].

2.3 Statistical analysis

The gene expression data statistical analyses were performed using the GraphPad Prism 9.0 software. The results were expressed as means (RPKM values) ± standard deviation (SD) from three biological replicates. The data obtained were subjected to analysis of variance (ANOVA) using GraphPad Prism 9.0 software, and Bonferroni's test compared averages at 5 % probability.

3. Results

3.1. Tolerant genotype shows elevated transcript levels of PFK (Glycolysis) and ADH (fermentation)

In figure 1, total transcript levels of PFK, ADH and COX are shown to infer the status of glycolysis, fermentation and aerobic respiration in two rice genotypes differing in salt stress tolerance [Pokkali (tolerant) and ir29 (susceptible)].

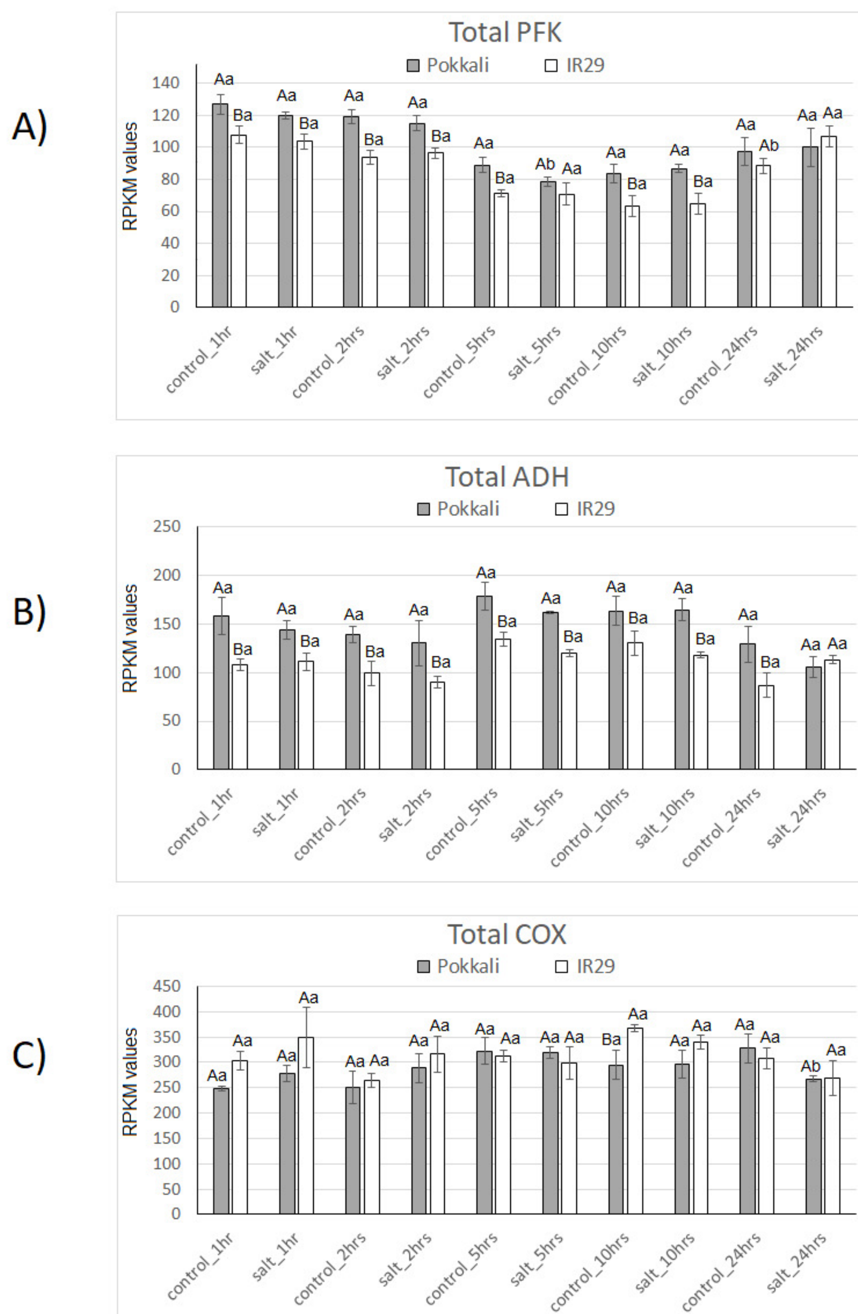

Figure 1. Expression profiles of Total *PFK*, *ADH* and *COX* genes in Pokkali and ir29 genotypes of *Oryza sativa* under salt stress. Data represent RPKM means with standard deviations from 3 biological replicates. For each gene, different capital letters indicate

significant differences (at $p < 0.05$) between genotypes (Pokkali and ir29) while lowercase letters designate significant differences between treatments (control and stress) at the same time point and genotype, according to Bonferroni's test.

In general, higher mRNA levels of total PFK and ADH were observed in the tolerant genotype across the time points, with a significant difference in most cases. The only exception was the point salt stress at 24 h, in which similar levels of both transcripts were detected for Pokkali and ir29 genotypes (Figures 1A and 1B). Regarding the salt stress effect, a significant difference in controls was found in PFK expression, with a decrease at 5h in Pokkali and an increase at 24 h in ir29 (Figures 1A and 1B).

Interestingly, the differential gene expression pattern observed for total PFK and ADH among both rice genotypes differed from the pattern of total COX transcripts (Figure 1C). Overall, similar COX transcript levels were observed in both genotypes across all time points except in the time point control 10h. Significant higher COX mRNA levels were observed in the ir29 genotype. A single significant COX mRNA decrease occurred at 24 h in the Pokkali genotype under salt stress.

3.2 Alternative oxidase expression is preponderant in tolerant genotype among different ROS formation control systems.

The total transcript levels of AOX, UCP and PTOX, are shown in Figure 2 to gain insight into energy dissipating systems in mitochondria (AOX and UCP) and plastid (PTOX) in rice genotypes under salt stress.

Curiously, higher total AOX transcripts (Figure 2A) were observed in the tolerant (Pokkali) genotype compared to the susceptible (ir29) one, with significant data in the majority of cases. This difference can be associated with a higher expression of *AOX1a* (Figure 2D). Concerning salt stress effect, total AOX mRNA decrease (significant) was observed at times 1, 5 and 10 h in Pokkali, while some AOX mRNA increase was observed at 24 h in ir29 (not significant) (Figure 2A).

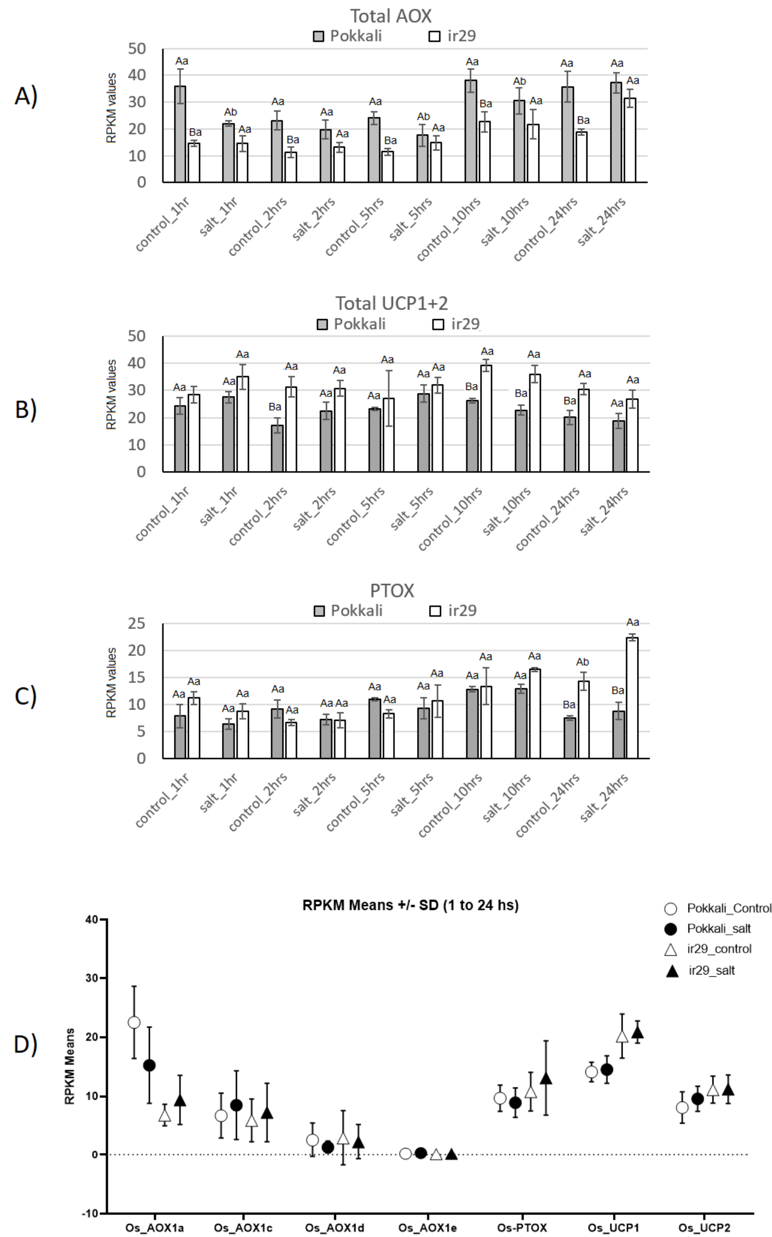

Figure 2. Expression profiles of Total *AOX*, *UCP* and *PTOX* genes in Pokkali and ir29 genotypes of *Oryza sativa* under salt stress. Data represent RPKM means with standard deviations from 3 biological replicates. For each gene, different capital letters indicate significant differences (at $p < 0.05$) between genotypes (Pokkali and ir29) while lowercase letters designate significant differences between treatments (control and stress) at the same time point and genotype, according to Bonferroni's test.

For UCP, higher mRNA levels were detected in the susceptible ir29 genotype than the tolerant in both control and salt conditions, although the values were not significant in most time points. No significant salt stress effect was observed (Figure 2B). This difference between genotypes can be connected with higher UCP1 expression in ir29 (Figure 2D).

Concerning to PTOX, differences between two genotypes and treatments were found mainly at 24 h. At this time point, significantly higher PTOX mRNA levels were observed in ir29, which increased significantly in response to salt stress (Figure 2C).

3.3. Antioxidant gene expression indicates redox status compartmentalization in rice genotypes under salinity.

The expression analyses of different gene members of APX, MDHAR, DHAR, GR, SOD, GPX, and CAT indicated the redox status of different subcellular compartments such as cytosol, peroxisome, mitochondria and chloroplast. In general, the main increase responses to salt stress differed between genotypes, with ir29 responding from 2 h while Pokkali from 10 h (Figure 3).

For genes encoding proteins to the cytosol, three transcripts (APX, DHAR, and SOD) presented significant changes in response to salt stress compared to the control in both genotypes. APX transcripts significantly increased in Pokkali at 10 and 24h and in ir29 at 2 and 10 h. On the other hand, DHAR increased in Pokkali (1 a 24h) and decreased in ir29 (1h), while SOD decreased in Pokkali (1 and 5 h) and increased in ir29 (2 to 24 h). In addition, cytosolic CAT mRNA decreased in Pokkali and increased in ir29 (1h). Also, cytosolic GPX mRNA increased in Pokkali (non-significant) and remained stable in ir29.

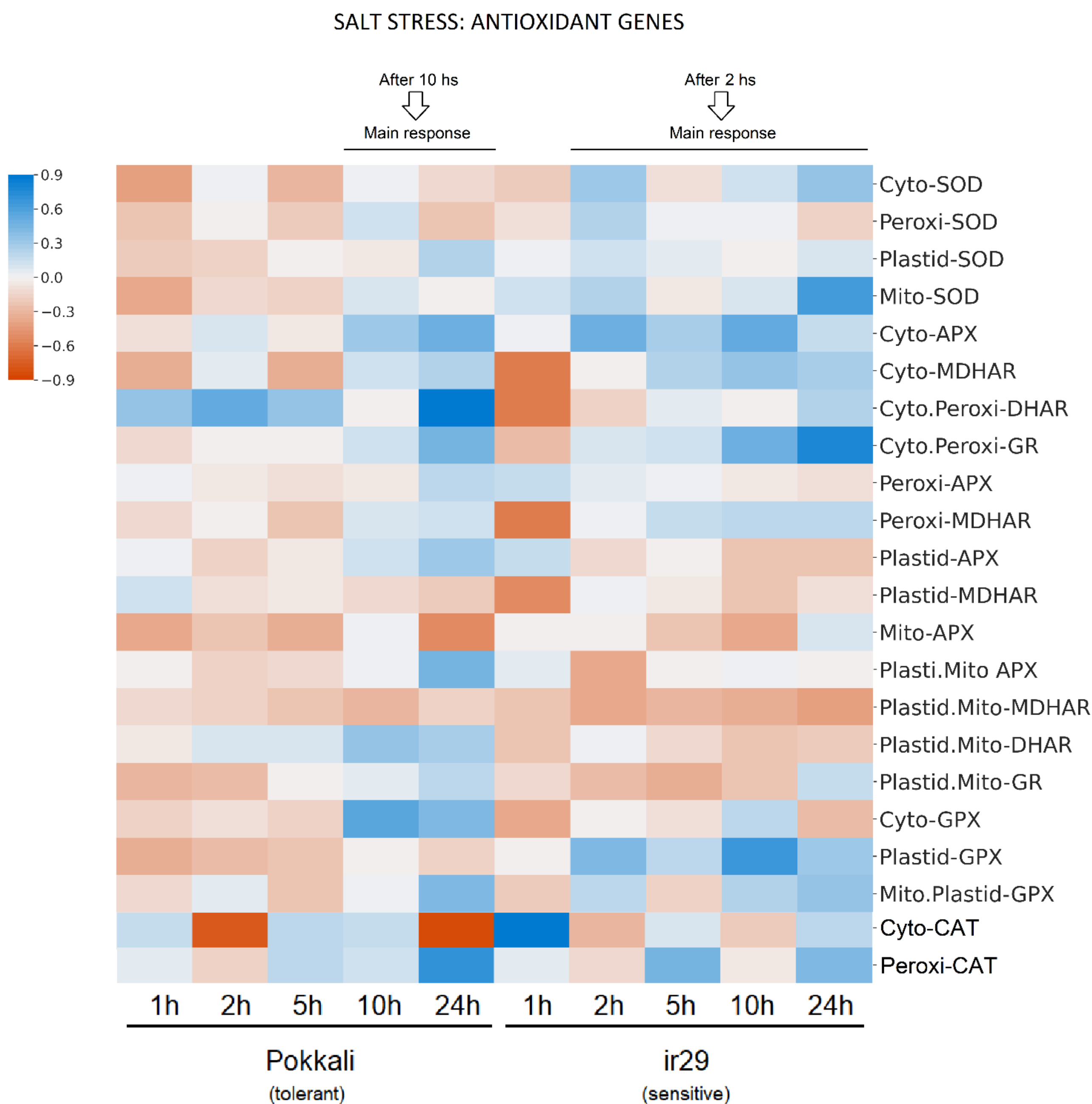

Figure 3. Heat map showing the gene expression of antioxidant enzymes in different cellular compartments of *Oryza sativa* genotypes under salt stress. The analyzed genes were APX, MDHAR, DHAR, GR, SOD, GPX, and CAT in Pokkali and ir29 genotypes. The data represent log2 fold change of salt treatment values at 1, 2, 5, 10 and 24h concerning the respective control conditions. In heat maps, the colours blue and orange represent up and down-regulated genes, respectively. Statistical analyses of the RPKM means with standard deviations from 3 biological replicates are shown in Supplementary Tables 3 and 4.

In peroxisome, both MDHAR and DHAR transcripts significantly decreased in IR29 at 1h, while DHAR transcripts increased in Pokkali at times 1 to 24 h. In response to salt stress, CAT transcripts increased significantly in Pokkali (24 h) and ir29 (5 and 24h). Also,

peroxisomal SOD decreased in Pokkali, and an increase in ir29 was observed (insignificant).

In Chloroplast, GPX revealed significant changes in response to salinity in both genotypes. GPX transcripts increased in ir29 at 2 to 24h and decreased in Pokkali at 1 to 24 h. In addition, plastid.mito APX transcripts increased significantly in Pokkali (24h) and decreased in ir29 (2h). Likewise, SOD increases were observed in both genotypes (not significant). GPX mRNA increases were observed in both genotypes (not significant). In addition, SOD increased in ir29 and decreased in Pokkali (insignificant). Plastid.mito DHAR increases in Pokkali (10 and 24 h), while a slight decrease was observed in ir29.

4. Discussion

In this research, we support the hypothesis that salt tolerance in rice involves AOX linked to rapidly induce alternative energy production via glycolysis-driven aerobic fermentation. Following the current insight that transcript level changes during early cell reprogramming can be critically relevant [33, 43, 44, 45] for predicting later performance, we explored salt-induced transcript accumulation changes for the selected gene sets up to 24h. Thus, we selected transcriptomic data from two rice genotypes with known contrasting salinity stress tolerance in the field (Pokkali and IR29) treated at the seedling stage with 300 mM NaCl. Recently, our group confirmed that AOX might play a relevant role under mild stress (e.g. at watering for seed germination) and also under severe stress conditions (e.g. induction of somatic embryogenesis) and demonstrated that this was connected to temporarily enhanced fermentation [34, 38]. Here, we advanced our knowledge on redox homeostasis (AOX/Antioxidant enzymes) and energy supply (glycolysis/fermentation) under severe salt stress in rice. Singh et al. [69] pointed the importance of mild salt stress as critical for reproductive stage. However, these authors pointed also to the danger of higher salt concentrations under higher temperatures and low relative humidity when transpiration increases [70].

In fact, the higher transcript levels of AOX, PFK and ADH in the tolerant (Pokkali) compared to susceptible (ir29) genotype (Figures 1 and 2) support a critical involvement of AOX and glycolysis/fermentation in salt stress tolerance. Corroborating these findings, AOX expression variation was also observed in *Vigna unguiculata* cultivars, contrasting in salt/drought stress tolerance [8]. In rice, generally, *AOX1a* and *AOX1d* are the stress-responsive genes (9, 12, 13, 14, 15, 16, 17, 18]. However, regarding the present experiment (seedling stage under 300 mM NaCl during 24 h) the higher AOX expression in the tolerant genotype was due to *AOX1a* (Figure 2D). In this regard, very recently, Challabathula et al. [71] also observed this peculiarity of higher *AOX1a* expression in rice cultivars tolerant to drought and salinity. Among other metabolic pathways, glycolysis transcripts increased under salinity in stress-tolerant rice cultivars [72]. More recently, Bharadwaj et al. [38] showed that adaptive reprogramming during early seed germination requires enhanced fermentation and it involves a critical role of AOX to maintain the metabolism homeostasis. Also, Costa et al. [43, 44, 45] showed that variable ROS/RNS rebalancing and temporarily increased aerobic fermentation appear to generally combine stress defense mechanisms in humans. These traits are connected to cell restructuration and can discriminate stress factors and distinguish

genotypes respectively cell origins.

Furthermore, Zheng et al. [73] identified AOX pathway involvement with waterlogging tolerance in watermelon, which was associated with increased fermentation instead of aerobic respiration. They compared two contrasting genotypes, YL (tolerant) and Zaojia8424 (sensitive) and observed a strong increase of *AOX* and *ADH* transcripts in the tolerant genotype until 24 h. Also, higher *AOX* and *ADH* mRNA levels were always detected in the tolerant genotype during all analysis times (until 72h). Considering the relevance of aerobic respiration during early hours after stress perception these authors observed a strong decrease of *COX* transcripts in both genotypes up to 24 h of stress. In rice, we detected similar *COX* mRNA levels in both genotypes in the majority of the evaluated time points up to 24 h (Figure 1). Overall, these data support our findings in rice genotypes indicating a critical role of AOX in stress tolerance followed by efficient respiration and mitigating oxidative impairment in the tolerant genotype (Pokkali).

Since AOX function avoids ROS formation, it is also important to investigate the antioxidant systems among genotypes with contrasting tolerance. According to Lakra et al. [74] Pokkali genotype has a more efficient antioxidant system than other salt-susceptible genotypes. In our data, the susceptible ir29 genotype, overall, revealed early antioxidant response from 1 or 2 h compared to Pokkali that responded from 10h (Figure 3). This early response could be due to the higher oxidative stress in ir29 than Pokkali. In fact, SOD transcripts in ir29 increased in response to salt stress in all cell compartments, while in Pokkali *SOD* expression increased only in the chloroplast (Figures 3 and 4), supporting a disseminated O_2^- overproduction in ir29. In this context, it is of interest that Costa et al. [46] observed an early and higher transcript increase of ASC-GSH cycle genes in susceptible soybean genotypes compared to the tolerant ones in response to different biotic and abiotic stresses.

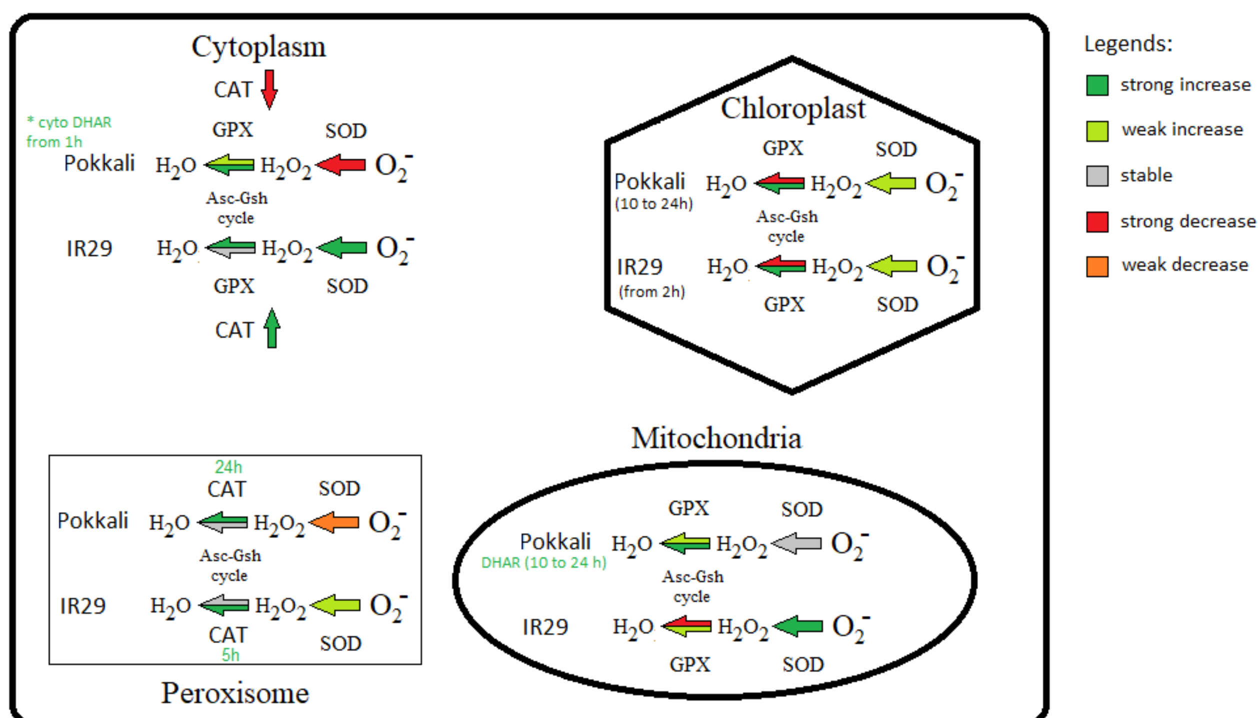

Figure 4. Schematic representation of antioxidant enzymes in different cellular compartments of *Oryza sativa* genotypes under salt stress. Significant values denoted substantial transcript increases or decreases while non-significant values indicated weak increases or decrease according to Supplementary Tables 3 and 4.

In addition, given that APX, GPX and CAT are the main H_2O_2 scavenging enzymes in plants [75] our data indicated that rice genotypes under salinity used different enzymatic pathways to control H_2O_2 concentration in cell compartments (Figure 4). Apparently, Pokkali activates preferably ASC-GSH cycle genes to scavenger H_2O_2 via APX from cytosol and organelles (mitochondria and chloroplast) while ir29 seems to rather activate ASC-GSH cycle in cytosol and GPX in organelles (Figures 3 and 4). Also, the ascorbate recycling via ASC-GSH cycle appeared more active in Pokkali since cytosolic / organelles *DHAR* mRNA levels increased only in this genotype (Figure 3). In this regard, stress-tolerant genotypes in *Glycine max* also differed from susceptible genotypes increasing transcripts involved in ascorbate regeneration [46]. Some works showed that *DHAR* overexpression successfully conducted transgenic plants to abiotic stress tolerance as aluminum and cold [76]. Among these enzymatic systems, APX appears to be the pivotal antioxidant enzyme to maintain the H_2O_2 balance since it has higher H_2O_2 affinity, acting the same in very slow protein concentration [77, 78] whereas CAT is more associated to H_2O_2 detoxification [79]. Thus, while higher CAT could act in peroxisome scavenging toxic H_2O_2 in both genotypes (Figures 3 and 4), the cytosolic CAT transcript decrease observed only in Pokkali suggests that cytosolic H_2O_2

concentration is regulated mainly by the ASC-GSH cycle via APX. However, the higher antioxidant efficiency observed in Pokkali (tolerant) can start much earlier by the alternative pathway activity to avoid ROS formation, denoted by the higher AOX mRNA levels observed in this genotype than ir29 (susceptible). Supporting our findings, very recently, Challabathula et al. [71] observed that increased *AOX1a* mRNA levels with an efficient antioxidant system were essential for tolerant rice cultivars to maintain lower ROS, higher photosynthesis rates, and stress tolerance.

Interestingly, higher *AOX*, *PFK* and *ADH* transcript levels already occur at Pokkali control plants, suggesting that this genotype could also be more resistant to other stress conditions. In this context, some studies show Pokkali as more resistant to lead (Pb) accumulation [48], or have a similar expression profile of abiotic inducible genes in response to multiple stress as NaCl, ABA, polyethylene glycol (PEG) or cold (4°C) [80]. Thus, Pokkali could have a resourceful genetic background [81] or an intrinsic environmental feature involved in stress tolerance. In this sense, recently, Sampangi-Ramaiah et al. [82] identified a salt-tolerant endophyte (Fusarium sp.,) in Pokkali, which could confer salt tolerance when colonizing the salt-sensitive rice variety IR-64. Nevertheless, it was possible to detect this endophyte in Pokkali genotype in our transcriptomic data, but in a small amount (data not shown). However, we identified Fusarium also in ir29 at a similarly low amount (data not shown). Fungal endophyte diversity in plants depends not exclusively on genotypes and their effects on the surrounding environment. Natural environmental contexts importantly influence plant endophyte diversity mainly in the rhizosphere and, thus, depends on agricultural management, respectively experimental conditions [83]. Bharadwaj et al. [38] suggested that microbiota can provide a sink for stress-induced higher levels of sucrose and, in this way, might help to alleviate oxidative stress through overloaded mitochondria as a consequence of enhanced glycolysis. In this sense, genotype-compatible endophytes can complement fermentation and alternative respiration in their effect on maintaining host metabolic and energetic homeostasis. Furthermore, it was suggested that endophyte-born AOX genes could complement plant AOX capacities as an added value that evolved under plants' holobiont natures [38, 84, 85, 86]. However, preliminary observations of Bharadwaj et al. [38] indicated also that more robust plant genotypes can act more independently on microbiota assistance.

Our studies suggest that developing functional marker-assisted rice breeding or genetically engineered respectively edited rice plants by targeting AOX and

glycolysis/fermentation-related genes can be among the promising strategies to confer salt stress and sustain rice productivity. However, such strategies require considering carefully the following general and specific state-of-the-art insights: 1) AOX genes have shown to be highly polymorphic in exon and even more pronounced in non-exon regions [86, 87, 88, 89, 90, 91, 92, 93] In general, causative polymorphic sites within a gene were found to have low degree of conservation and phenotypic variation in a target trait can be linked to diverse sequence polymorphisms [94] the relevance of AOX comes by its link to coordinating early plasticity provoked by continuously acting, ever-changing diverse environmental conditions, where salinity is only one among many stressors. This flexibility can be expected to rely on allelic polymorphisms [93] and flexible switching between polymorphic sites [95] depending on environmental and metabolic conditions; thus, diversity in AOX genes might be a desired trait per se; 3) as also shown in the present research, AOX is embedded in complex networking contexts [96] that evolved in unique complex systems/organisms. This also includes the level of cells and their unique context in the plant body- shaped tissue- and organ landscapes and concerning cell-free spaces (apoplast). Thus, the species-specific role of target cells for defined agronomic traits needs to be considered [97] (4) gene technology and gene editing have technical obstacles (reviewed, e.g [98] because they require *in vitro* culture as a first step. However, this means applying strong stress [86, 91, 99] and requires tissue and cell disruption from established networks typically. In contrast to functional marker-assisted selection at seed and plant level, this bares at least the risk of undetected somaclonal variations by epigenetic and genetic side effects, which might change intrinsic deeper phenotype characteristics of the original plant that can escape breeders' awareness through the focus on restricted agronomic or quality selection criteria.

In conclusion, our data support relevant involvement of alternative pathways and glycolysis/fermentation in the more efficient stress response observed in a salt stress-tolerant rice genotype. This response is primarily associated with adaptive ROS balancing by AOX (Via *AOX1a* expression), effective tuning of the antioxidant system, and, secondarily, rapid energy production (via fermentation). Both contribute to sustaining and optimizing respiration. We cannot exclude that this intrinsic feature observed for stress-tolerance performance could have been modified by host-endophyte interaction since we confirmed the holobiont nature of both genotypes.

Author's contribution.

JHC & SA conceived the basic idea and planned the study. SA performed data analyses, helped write the basic draft and prepared the final manuscript for submission. TAG contributed to statistical analyses. RSM, KLLT and MCB helped SA in basic data preparation. JHC revised the data analysis and results interpretation and wrote and advanced the final manuscript. BA-S coordinated the final manuscript discussion and revision. All authors agreed on the final manuscript submission.

Conflicts of Interest.
The authors have no conflicts of interest to declare.

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4. ARTICLE 3

MAJOR COMPLEX TRAIT FOR EARLY DE NOVO PROGRAMMING ‘COV-MAC-TED’ DETECTED IN HUMAN NASAL EPITHELIAL CELLS INFECTED BY TWO SARS-COV-2 VARIANTS IS PROMISING FOR DESIGNING THERAPEUTIC STRATEGIES

Major complex trait for early de novo programming 'CoV-MAC-TED' detected in human nasal epithelial cells infected by two SARS-CoV-2 variants is promising for designing therapeutic strategies

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Abstract

Background: Early metabolic reorganization was only recently recognized as essentially integrated part of immunology. In this context, unbalanced ROS/RNS levels that connected to increased aerobic fermentation, which linked to alpha-tubulin-based cell restructuration and control of cell cycle progression, was identified as *ma*jor *c*omplex *t*rait for *e*arly *d*e novo programming ('CoV-MAC-TED') during SARS-CoV-2 infection. This trait was highlighted as critical target for developing early anti-viral/anti-SARS-CoV-2 strategies. To obtain this result, analyses had been performed on transcriptome data from diverse experimental cell systems. A call was released for wide data collection of the defined set of genes for transcriptome analyses, named 'ReprogVirus', which should be based on strictly standardized protocols and data entry from diverse virus types and variants into the 'ReprogVirus Platform'. This platform is currently under development. However, so far an *in vitro* cell system from primary target cells for virus attacks that could ideally serve for standardizing data collection of early SARS-CoV-2 infection responses was not defined. *Results*: Here, we demonstrate transcriptome level profiles of the most critical 'ReprogVirus' gene sets for identifying 'CoV-MAC-TED' in cultured human nasal epithelial cells. Our results (a) validate 'Cov-MAC-TED' as crucial trait for early SARS-CoV-2 reprogramming for both tested virus variants and (b) demonstrate its relevance in cultured human nasal epithelial cells. *Conclusion*: *In vitro*-cultured human nasal epithelial cells proved to be appropriate for standardized transcriptome data collection in the 'ReprogVirus Platform'. Thus, this cell system is highly promising to advance integrative data analyses by help of Artificial Intelligence methodologies for designing anti-SARS-CoV-2 strategies.

Keywords: immunology paradigm shift, melatonin, ADH5, E2F1, cell cycle, SARS-CoV-2 Δ382, SARS-CoV-2 helicase, SARS-CoV-2 RdRp, anti-viral diagnosis and strategies, microbiota, repurposing drugs

1. Background

Is there a paradigm shift in understanding immunology?

It is increasingly understood that plants and animals have similar responses and cell memory mechanisms to manage immunology (1, 2). Effective immunologic protection requires a diversity of innate and adaptive cell responses and cell memory tools (1-4). However, immunologic responses are energy consuming and require efficient metabolic reprogramming, but metabolic reorganization is not fully recognized as an integrated part of immunology (5-8). Viruses have comparatively low Gibbs energy due to their chemical compositions (9, 10). This makes their replication highly competitive and is the basis for their 'structural violence' against the host cell metabolism [see discussion on this term in Costa et al., 2021 (11)]. Virus-induced host cell reprogramming that is intended to support host defense is turned ad absurdum for the host and favors virus replication as the driving force. As a consequence of this conflict, any viral infection provokes struggling for commanding coordination of host cell program and this starts in the initially infected cells. Competing for bioenergy and for 'territories' is decisive for the success of virus reproduction and evolution.

These insights stimulated our group to take profit of mixed plant and human cell-based experimental systems for gaining general, relevant knowledge on early reprogramming during SARS-CoV-2 infection. Our complex approach was explained on the basis of extensive literature reviews in a recent paper entitled 'From plant survival under severe stress to anti-viral human defense – a perspective that calls for common efforts' (11, 12). Having applied our theoretical concept by comparing transcriptome data from a resilient plant system with coronavirus-infected human cells of diverse origins, a *ma*jor *c*omplex *t*rait for *e*arly *de* novo programming upon SARS-CoV-2, named 'CoV-MAC-TED', was identified that should help tracing critical virus footprints. This trait covers unbalanced levels of ROS/RNS (i.e. reactive oxygen species in relation to reactive nitrogen species), which connects to temporarily increased aerobic fermentation that links to α-tubulin-based cell restructuration and cell cycle regulation (11).

Resilience can depend on the capacity for efficient early reprogramming – learning from plants

Plants as settled organisms are especially challenged to rapidly confront highly diverse and complex abiotic and biotic environmental constraints, including virus threats. Marker development is essentially required to permanently advance breeding strategies that can timely cope with ever changing environmental conditions, such as climate changes. Consequently, prediction of adaptive robustness that provides resilience is best explored in plants (13). In this context, early reprogramming in target cells and tissues that impacts final agronomic or quality characteristics (such as yield stability or richness in secondary metabolites) was raised by our group as a trait *per se* (14-20). We developed unique concepts and tool kits that allow predicting plant robustness in the field from respiration traits as early as several hours during seed germination (21-26). This innovative approach was tested and preliminary validated by using diverse plant species (13, 25-26). This observation suggests common mechanisms for resilient life performance across plant species. As a hypothesis, we expect similar performance across oxygen-dependent, respiring eukaryotic organisms. Applying a higher degree of abstraction, we argued that these results might also be promising to identify critical mechanisms in human cells under virus stress including SARS-CoV-2, which might help to pave the way for designing early combating strategies (11, 12).

Early reprogramming can link to ROS/RNS equilibration and sugar-dependent fermentation

We found that adaptive early reprogramming in plants can be critically connected to temporarily enhanced, sugar-dependent fermentation (11, 26). This process was essentially regulated by an enzyme named alternative oxidase (AOX), which maintains ROS and RNS levels equilibrated, regulates metabolic and energetic homeostasis and adjusts respiration overload and fermentation, which at the same time associates to induction and regulation of cell division growth (11, 26). These characteristics make AOX a highly promising functional marker resource for improving general plant resilience. Its role in early reprogramming was indicated in several applied systems cited in Arnholdt-Schmitt et al. (12). However, humans do not possess this enzyme (see discussions in 11, 12). Nevertheless, understanding the functional importance of AOX in plants can guide research strategies to identify marker gene candidates for resilience in human target cells against virus attacks appropriate to characterize resilient traits early at reprogramming. Costa et al. (11) suggested that melatonin could at least in part substitute the early role of AOX during reprogramming. Melatonin is a natural

hormone in humans, which has also been recognized as a phytohormone (27). It is produced in most organs and cells (27-31), including human salivary gland cells (32). Costa et al. (11) observed that *ASMT* transcript levels were increasing dependent on MOI level and infection time in MERS-CoV-infected MRC5 cells, which encouraged studying markers for melatonin metabolism in nose cells. Melatonin is known to possess anti-oxidant and pathogen defense-related properties and shows high fluctuation in its cellular concentration (27, 33-37). Furthermore, melatonin is since long proposed as anti-viral agent (38) promising as repurposing drug to treat SARS-CoV-2 infections (39, 40).

Driving a standardized collection of data on virus-induced early reprogramming

Arnholdt-Schmitt et al. (12) initiated common efforts for a standardized collection of transcriptome data during early reprogramming after virus infection from a defined set of genes, named 'ReprogVirus'. The principle intention of this approach was to identify common early target traits that could help in designing therapeutic strategies, which could be applied for a high diversity of virus types and variants. In the present communication, we reduced the number of tested genes from ReprogVirus to promising core markers for CoV-MAC-TED components (11). Thus, genes were selected to identify a shift in ROS/RNS (ASMTL, SOD1, SOD2, ADH5, and NOS2), represent glycolysis (PFK, GAPDH, Eno) and lactic acid fermentation (LDH) as well as structural cell organization (α-Tub). We assumed that ASMTL could indicate oxidative stress equilibration, while SOD1 and SOD2 genes mark anti-oxidative activities and were selected to indicate oxidative stress. ASMTL is a paralog of ASMT*,* which is involved in melatonin synthesis in human cells (28). However, we could not find ASMT gene transcripts in collected human nasal epithelial cells. ADH5 is known to be involved in ROS/RNS equilibration through NO homeostasis regulation (40-42) and the inducible NOS gene, NOS2, relates to the induction of NO production (11, 43). NOS1 and NOS3 had not been encountered in sufficient quantities in the collected epithelial nose cell data. Further, we selected SNRK and mTOR to highlight cell energy-status signaling (44-47). The mTOR is activated when there is excess of energy in contrast to SNRK, where higher expression indicates energy depletion. Genes for E2F1 together with mTOR were included to indicate changes in cell cycle regulation, namely cell cycle progression (G1/S and G2/M transitions) (47). E2F1 belongs to the transcription factor family E2F and is known as cell cycle activator. The interferon regulator factor, IRF9, demonstrated early transcription in

SARS-CoV-2 infected human lung adenocarcinoma cells and was therefore proposed as functional marker candidate that could signal initiation of the classical immune system (11). Here, we studied validity of our approach for SARS-CoV-2 in human nasal epithelial cells (NECs) that was originally identified to cause the Coronavirus Disease 2019 (COVID-19). Additionally, we tested whether the same approach could be applied to a SARS-CoV-2 variant (SARS-CoV-2 Δ382). This mutant was detected in Singapore and other countries and had been associated with less severe infection (48, 49).

2. Material and Methods

2.1 Gene expression analyses of RNA-seq data from SARS-CoV2 infected human nasal epithelial cells

In this work we analyzed the expression of the main ReprogVirus genes (11, 12, for gene abbreviations see in **supplementary table S1**) in transcriptomic data from human nasal epithelial cells infected with two SARS-CoV2 variants (wild type and mutant Δ382) at 0, 8, 24 and 72 hpi (hours post infection). Transcriptomic data (RNA-seq data) are available in SRA database at GenBank (NCBI) under the Bioproject PRJNA680711 previously published by Gamage et al. (48). The ReprogVirus genes analyzed were involved in shift in ROS/RNS (ASMTL, SOD1, SOD2, ADH5, NOS2), glycolysis [Total PFK (PFKM, PFKL and PFKP), GAPDH, Total Eno (Eno1, Eno2 and Eno3)], lactic acid fermentation [Total LDH (LDH-A, LDH-B, LDH-C, LDH-AL6A, LDH-AL6B)] structural cell organization [Total α-Tub (TUB-A1B, TUB-A1C, TUB-A4A)], cell cycle activator (E2F1), cell energy-status (SNRK, mTOR) and immune response (IRF9). Accession numbers of these genes are available in Costa et al. (11). SARS-CoV-2 proliferation was monitored evaluating transcript levels of virus helicase (YP_009725308.1) and virus RNA-dependent RNA-polymerase (RdRp) (YP_009725307.1) genes.

Gene expression was evaluated by mapping, quantifying and normalizing the reads of each ReprogVirus gene in the RNA-seq data with three biological replicates. For this, ReprogVirus cDNAs were aligned against RNA-seq data using the Magic-Blast software (50). Specific parameters in Magic-Blast as word size of 64 was included to certificate specific read detection for each gene. The number of mapped reads was obtained using the HTSeq (51) program exploring an alignment file (in SAM format) derived from Magic-Blast. Normalization of reads was performed using the RPKM (Reads Per Kilobase of transcript per Million of mapped reads) method (52) according to the following equation: RPKM = (number of mapped reads X 10^9) / (number of sequences in each database X number of nucleotides of each gene).

2.2. Statistical analyses

Normality and homogeneity of variances from the analyzed variables (in RPKM) were tested with Shapiro-Wilk test and Levene tests, respectively, using InfoStat 2018I. Then,

ANOVA tests for single measures were performed for each donor and virus variant along different time points by using Excel datasheet. Significance levels were set at $\alpha=0.05$.

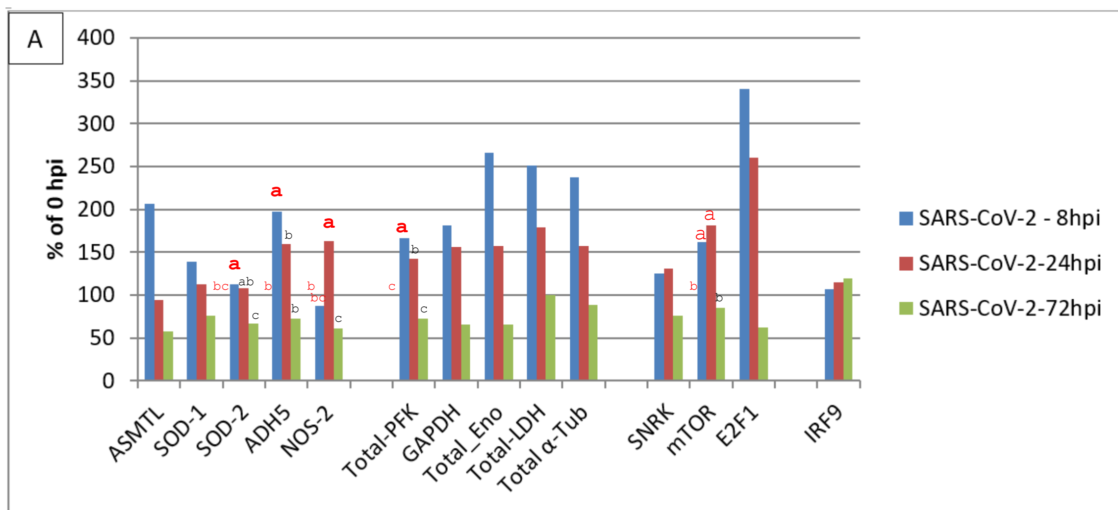
We highlight that we interpret our data as 'real' observations under the employed conditions involving only small samples, which certainly provide insights that cannot get relevance or not relevance by using significance calculation. Nevertheless, we applied significance calculations at usual p-values for biological research as an aid to focus our insights. Readers are encouraged to making themselves familiar with the current paradigm change related to the usage of statistical significance (53-57).

3. Results

Figure 1 shows relative transcript level changes of selected ReprogVirus genes in epithelial nasal cells infected with SARS-CoV-2 or SARS-CoV-2 mutant Δ382 at 8 hpi, 24 hpi and 72 hpi. Transcript level changes are expressed as % of 0 hpi. Standard errors (SE) are listed for all genes in **supplementary table S1**.

Transcript profile level changes for SARS-CoV-2 (original virus) infection are given in Fig.1A and demonstrate similar increases for ASMTL and ADH5 (207% and 197%) at 8 hpi and indicate unbalanced ROS/RNS at 24 hpi (94% ASMTL and 159% ADH5), which is supported by near-basal SOD1 and SOD2 transcript levels (112% and 108%) in relation to an increased transcript level of NOS2 (163%). At 72 hpi, transcript levels of all ROS/RNS related genes were simultaneously reduced to below basal values observed at 0 hpi (57% ASMTL, 76% SOD1, 68% SOD2, 73% ADH5 and 61% NOS2). Transcription of glycolysis-related enzymes was rapidly increased at 8 hpi with highest transcript accumulation for enolase (267%). This high increase at 8 hpi was associated to a similar increase in LDH transcription (251%) linked to highly enhanced transcript levels also for α-tubulin (238%). SARS-CoV-2 Δ382 (Fig. 1B) shows a very similar profile for ROS/RNS signaling and metabolism-and structural cell organization-related traits. They also display almost identical signaling for a change in cell cycle regulation and the lack of strong stimulation of the classical immune system represented by the missing of IRF9 transcript accumulation level changes. Both genotypes demonstrate high increase in E2F1 transcript stimulation at 8 hpi, which signals rapid cell cycle activation. E2F1 transcript levels decreased slightly at 24 hpi, but fall rapidly below the basal values observed at 0 hpi to 62% respectively 72% (at 72 hpi). These observations together with increased transcript levels of SNRK and mTOR at 8 hpi (126% and 162%) and 24 hpi (131% and 182%) for the original SARS-CoV-2, and for SARS-CoV-2 Δ382 at 8 hpi (111% and 150%) and at 24 hpi (149% and 205%) point for both virus variants to early energy depletion and rapidly driven cell cycle progression plus cell cycle arrest at 72 hpi. At 72 hpi, no energy depletion is signaled anymore for both virus variants (SNRK: 76%, 83%). However, SARS-CoV-2 Δ382 shows at 72 hpi a slower decrease of mTOR transcript accumulation (109% vs 86% for original virus), and this together with the slower decrease also for E2F1 transcription (72% vs. 62% for original virus) might indicate slightly delayed cell cycle progression and arrest for the mutant. These last observations link to the postponed increase in transcript levels of LDH (at 8 hpi 247% mutant vs 251% original

virus and at 24 hpi 205% mutant vs 179% original virus) and α-Tub (at 8 hpi 220% mutant vs 238% original virus and at 24 hpi 189% mutant vs 157% original virus). Further, considering delayed cell cycle progression for SARS-CoV-2 Δ382 is supported by 2-ways ANOVA analysis. This analysis identified significant transcript level increases of SOD2, ADH5 and PFK early at 8 hpi and highlighted a significant increase for NOS2 from 8 hpi to 24 hpi only for the original virus. On the other hand, it demonstrated a significant increase for mTOR from 8 hpi to 24 hpi only for the mutant. However, the effect of such differences between original virus and the mutant did not substantially influence the initiation kinetics of IRF9 transcription. Transcript level increase for IRF9 was marginally seen at 72 hpi for both virus variants (>110%, both non-significant) and only a slightly different extent is indicated between original virus and mutant (120% vs 111%). The infection trials had been performed in unsynchronized nasal cell cultures (48, 58). Thus, it cannot be excluded that the observed differences in transcript levels between both virus variants may be due to experiment-dependent, differentially non-synchronized cell cycle phases.



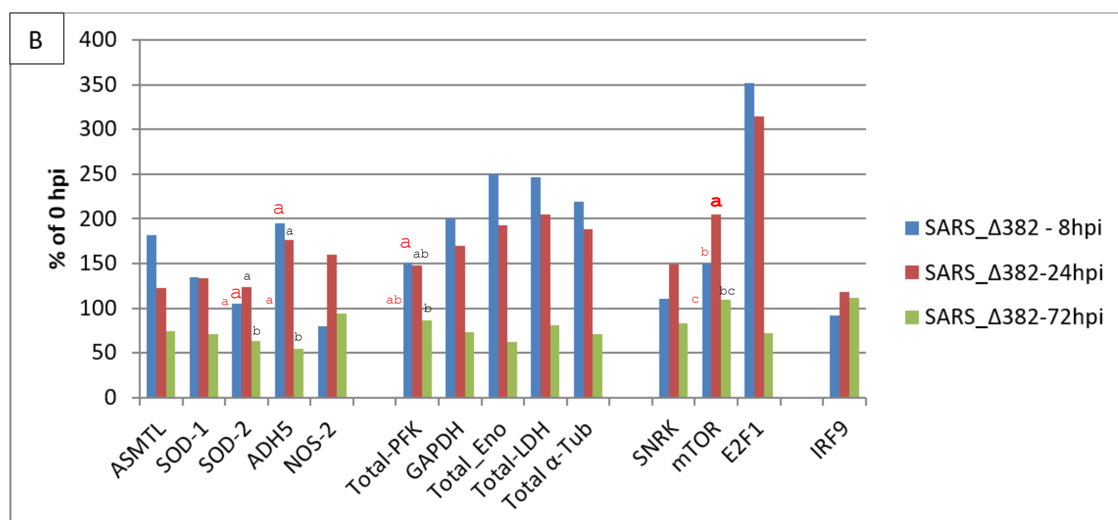


Figure 1: Transcript accumulation of selected *ReprogVirus* marker genes in human nasal epithelial cells infected with two SARS-CoV-2 variants at 8 hours post infection (hpi), 24 hpi and 72 hpi. Transcript levels are averages from three cell origins (donators/cell cultures) given in % of 0 hpi. A – SARS-CoV-2 (virus originally discovered); B – SARS-CoV-2 Δ382. 2-way ANOVA analysis identified differential transcript level changes along early times between the original virus and the mutant (marked by enlarged, fat letters). Different letters indicate significant differences between net RPKM for $\alpha = 0.05$. Letters on the 100% horizontal line correspond to 0 hpi. The result of 2-way ANOVA analysis for all genes can be consulted in **supplementary Figure S1**).

Figure 2 shows transcript accumulation in RPKM for both virus variants from nasal epithelial cells separated for the three cell origins, resulting in the averages shown in Figure 1. This figure also integrates transcript accumulation of virus helicase and RNA-dependent RNA-polymerase (RdRp) as markers for virus proliferation. In **Figure 2A**, it can be seen that cells from origin 1 demonstrate lower transcript levels for enolase and LDH at 24 hpi. This was observed after infection by both SARS-CoV-2 variants. Also for both virus variants, virus helicase and RdRp indicate the start of virus replication at around 24 hpi though to a slightly lower degree for the SARS-CoV-2 Δ382. In **Figure 2B**, transcript accumulation of virus helicase and virus RdRp point to a burst of virus replication at 72 hpi. The level of virus transcripts is lower for the mutant than for the original SARS-CoV-2 virus. However, cells that originated from donator 1 that showed lower transcript accumulation for enolase and LDH at 24 hpi in comparison to cells from donators 2 and 3 in response to both virus variants did not indicate a burst of virus replication at 72 hpi, but only a slow increase from 24 hpi. Nevertheless, cells of donator 1 also demonstrated lower virus RdRp transcript levels at 72 hpi (**Fig. 3**). This differential performance of cells from donator 1 connects to higher transcription levels of SNRK at 72 hpi in cells from donator 1 after infection with both, the original SARS-CoV-2 and SARS-CoV-2 Δ382 (**Fig. 2 and 3**). It signals higher depletion of energy for donator 1 cells at 72 hpi and confirms critical energy-dependency of SARS-CoV-2 replication for both variants. However, the higher values for enolase and LDH observed at 24 hpi for cells infected by the mutant, the differential development of SNRK over time and higher values at 72 hpi observed for mTOR from donator 1 in the mutant (**Fig. 3**) argues for prolonged cell cycle progression respectively a delay in cell cycle arrest related to both, the origin of cells from donator 1 and infection by the mutant. Nevertheless, since longer observation times and technical repetitions in synchronized cultures are missing, it cannot be concluded whether individual origin or differentially unsynchronized cell cultures caused these differences in the emergence of a burst in virus replication.

A

RPKM

500 450 400 350 300 250 200 150 100 50 0

Eno-24hpi LDH-24hpi Helicase-24hpi Polymerase-24hpi

SARS-CoV-2-1
SARS-CoV-2-2
SARS-CoV-2-3
Δ382-1
Δ382-2
Δ382-3

B

RPKM

4000 3500 3000 2500 2000 1500 1000 500 0

Eno-24hpi LDH-24hpi Helicase-24hpi Polymerase-24hpi Helicase-72hpi Polymerase-72hpi SNRK-72hpi (x100) IRF9-72hpi

SARS-CoV-2-1
SARS-CoV-2-2
SARS-CoV-2-3
Δ382-1
Δ382-2
Δ382-3

Figure 2: Burst of SARS-CoV-2 virus proliferation at 72 hpi in nasal epithelial cells from three origins infected by two SARS-CoV-2 variants indicates an energy-dependent link to aerobic glycolysis and fermentation at 24 hpi.

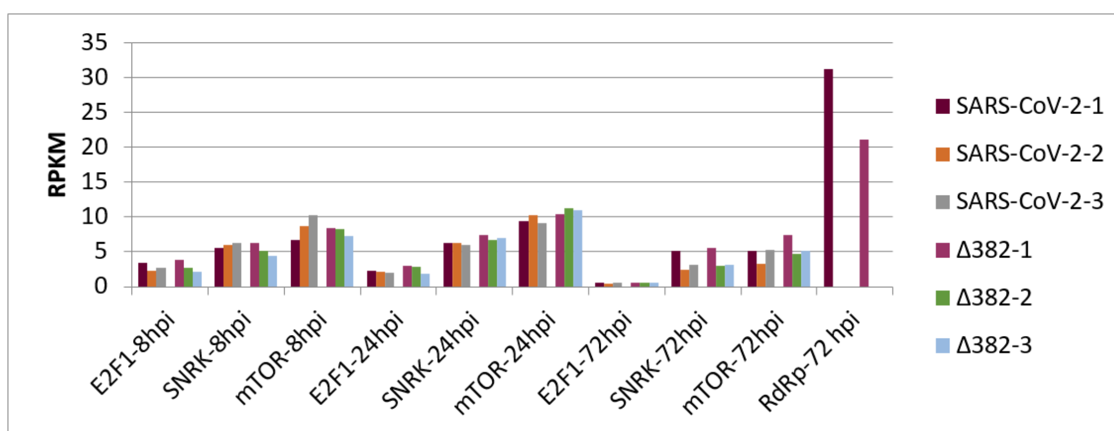


Figure 3: Transcript accumulation of E2F1, SNRK, and mTOR in human nasal epithelial cells

infected by two SARS-CoV-2 variants at 8 hpi, 24 hpi and 72 hpi and transcript levels of RdRp (x 0,5) at 72 hpi for cell origin 1

4. Discussion

This is the first time that relevance of the marker system ‘CoV-MAC-TED’, which is based on a relatively small set of key genes, was validated in primary human target nose cells for respiratory SARS-CoV-2 infections. It is also the first time that this approach was tested for two SARS-CoV-2 variants, which associate to differences in clinical effects (48, 49).
Our analyses have been performed in public RNA sequencing data provided from experiments with human NECs described in Tan et al. (58) and Gamage et al. (48). Thus, results and conclusions of these authors helped us additionally to validate relevance our key marker candidate approach: Transcriptome accumulation of the selected set of ‘ReprogVirus’ genes showed similar performance of ‘CoV-MAC-TED’ in human NECs infected by the originally discovered SARS-CoV-2 virus compared to infection by SARS-CoV-2 Δ382. In this aspect, our results are in conformity with the conclusions drawn by Gamage et al. (48). In addition, our observation that both virus variants induced cell cycle activator E2F1 to highest transcript levels at 8 hpi among all tested genes and times is in good agreement with results of Gamage et al. (48). These authors highlighted enhanced numbers of transcription factor E2F targets during early virus infections and a decrease during time combined with high activity related to cell cycle checkpoint G2M.

However, applying CoV-MAC-TED indicated also differences between both SARS-CoV-2 variants. The complex marker components revealed virus-induced ROS/RNS de-balancing, differential glycolysis, fermentation and cell cycle regulation that pointed to delayed cell response and cell cycle arrest for the mutant, which connected to delayed virus propagation. Interestingly, a delay in cell cycle arrest was also indicated for one of three cell origins (cells from donator 1), which pointed to the relevance of lower levels of glycolysis and aerobic fermentation for postponing virus replication (Figure 2).

In the same RNAseq data that we used for the presented research, the providing authors Gamage et al. (48) had intensively studied the complex immune response of SARS-CoV-2 infected NECs compared to cells infected by influenza H3N2. Influenza infection showed an earlier burst of virus replication at 48 hpi related to a pronounced early initiated immune response. This gave us the opportunity to further validate, advance and standardize our approach in a parallel study with a focus on influenza H3N2 infected NECs (59, preprint). In this way, we could validate our choice of IRF9 (12) as an appropriate general marker for

the classical immune system. The usefulness of CoV-MAC-TED to identify similar and differential early cell responses was strengthened. Both virus types showed early unbalanced ROS/RNS and temporarily increased aerobic fermentation linked to α-tubulin-marked cell restructuration. However, CoV-MAC-TED indicated the absence of initial cell cycle progression for influenza A H3N2 infections that connected to rapid energy-dependent IRF9-marked immunization and this contrasted our present findings during infections with both SARS-CoV-2 variants.

In **Figure 4**, we present a simplified scheme from the results here obtained in human NECs infected by SARS-CoV-2 for the five main components of CoV-MAC-TED (1) ROS/RNS balance shift, represented by ASMTL, SOD1, SOD2, ADH5, and NOS2, (2) glycolysis and fermentation, represented by enolase and LDH, (3) structural reorganization and cell cycle progression and arrest, represented by E2F1, mTOR and α-tubulin, (4) energy status signaling, represented by SNRK and mTOR, and (5) initiation of the classical immune system, represented by IRF9. For our conclusions and hypothetical inferences, we consider the dynamic interplay between virus infection, ROS/RNS signaling, carbohydrate stress metabolism, aerobic fermentation and cyt respiration based on knowledge related to our recently published insights (11, 26) and state-of-the-art knowledge on SARS-CoV-2 infection and general stress biology. Thus, we expect that the indicated increase of oxidative stress signaled at 8 hpi by ASMTL and ADH5 upon SARS-CoV-2 infection associates with rapidly increased sucrose/glucose cell levels that stimulate the Cyt pathway via enhanced glycolysis, pyruvate production and increased TCA cycling in a way that the respiration chain gets overloaded by electrons. Consequently, ROS and RNS concentrations might increase. On the other hand, the Cyt pathway will temporarily be restricted due to rapidly consumed oxygen. In turn, aerobic lactic acid fermentation will be activated. Depending on stress level and the amount of sugar and duration of the situation of high-sugar level, anaerobic glycolysis can reach high turnover during cell reprogramming and a level of ATP production corresponding to the Warburg effect. Warburg effects are increasingly recognized as being part of normal physiology (60, 61) that enables host cells to rapidly mobilize energy for host maintenance and stress escape. Results of Bharadwaj et al. (26) suggests that microbiota can help stress alleviation through forming a sink for sucrose that supports their own growth, and, depending on their nature, this could support symbiotic or parasitic performance. Viruses are parasitic structures. These structures are supposed to profit from the mobilized high energy favored by the thermodynamic conditions of their replication. However, in case of SARS-CoV-2 our

results indicate that during the first hours post infection (8 hpi to 24 hpi) rapidly available energy is consumed first for cell cycle progression (E2F1, mTOR and α-tubulin signaling) and only from around 24 hpi energy is increasingly used for virus replication (Figure 2). This might signal during early hours after infection stress (observed at 8 hpi by highly increased ASMTL and ADH5 transcription) relieve to the host cell (low oxidative stress indicated by ASMTL, SOD1 and SOD2), lower sugar concentration (less glycolysis combined with lower degree of fermentation observed from 24 hpi) and, thus, enable normalization of TCA cycling and decrease of energy depletion as it is SNRK-signaled after 24 hpi at 72 hpi before the expected burst of the classical immune system. However, the change for ROS/RNS in favor of NO production at 24 hpi, when virus replication starts, points to a ‘non-normal’ unbalanced situation.

5. Conclusion

The high relevance of metabolism-driven, early cell re-organization that we observed by studying CoV-MAC-TED in infected primary target cells for two respiratory viruses, SARS-CoV-2 and influenza A H3N2, stimulate re-thinking of current understanding of the immunological system and its determinants. Future anti-viral effective therapeutic strategies should, to our view, explore more sensitive diagnostic virus tools for rapid virus identification combined with effective individual treatment through early targeting CoV-MAC-TED components. Lower transcript levels observed for enolase and LDH observed at 24 hpi for cells/cell cultures from one origin that was linked to delayed virus replication might point to a strategic target for combating early initiation of SARS-CoV-2 replication by blocking the Warburg effect and its link to cell cycle progression.

In vitro-cultured human nasal epithelial cells proved to be appropriate for standardized transcriptome data-collection in the 'ReprogVirus Platform'. Thus, this cell system is highly promising to advance integrative data analyses by help of Artificial Intelligence methodologies for designing anti-SARS-CoV-2/anti-viral strategies.

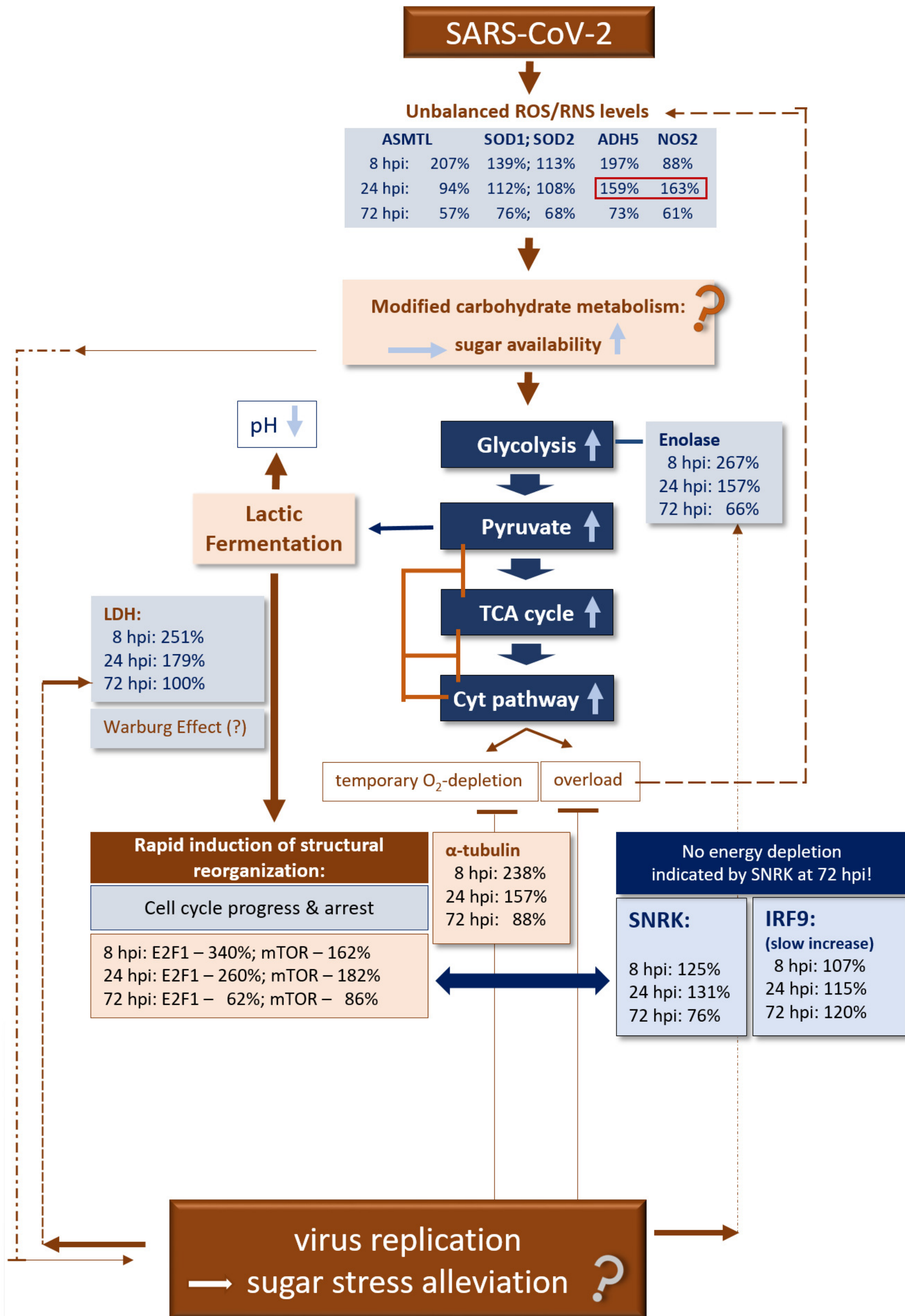


Figure 4: Validating CoV-MAC-TED as crucial trait for early SARS-CoV-2-induced reprogramming in human nasal epithelial cells – A simplified scheme for hypothetical metabolic principles and validation of markers.

Author Contribution

JHC & BA-S conceived the basic idea and plan of the study. SA performed BLAST and helped JHC in transcriptome analyses and manuscript preparation. CN performed statistical analyses. BA-S reviewed data analyses, interpretation, wrote the manuscript and coordinated revision of the manuscript. All co-authors commented and approved the final manuscript.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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4 CONCLUSION

Since the emergence of *V. vinifera* as a model species for *AOX* research, this study characterized the species-specific profile of the *AOX* family gene related to differential tolerance performance in several *V. vinifera* cultivars and alternative splicing events regulating the environmental fitness of the plant by creating multiple variants of *AOX2* mRNA. In addition, we identified sixteen miRNAs in the large introns of the *AOX2* genes associated with stress-coping mechanisms and growth regulation.

Furthermore, we validated the recently raised hypothesis of *AOX* and its complemented genes from the ReprogVirus dataset, which equilibrates ROS formation during the first hours of stress perception as an important target for individual resilience prediction and therapeutic strategies, by evaluating the transcriptome data of two rice genotypes with contrasting salinity tolerance and human nasal epithelial cells (NECs) infected with SARS-CoV-2 variants.

Earlier experimental validation of the works presented here, annotating *AOX* introns/exons in major *V. vinifera* genotypes is necessary to ascertain the same length and exhibit potential miRNA sequences. Second, before conducting clinical trials, wider studies are required to confirm the functionality of the AOX-complemented gene response to viral infection. Furthermore, we suggest that SNP genotyping of the coding and noncoding sequences of crucial functional marker genes can help define the risk of an individual’s resilience capacity.

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