Grapes for the Desert: Salt Stress Signaling in *Vitis*

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Karlsruhe, den 19. Juli 2013

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Abbreviations

$[Ca^{2+}]_{cyt}$, cytoplasmic calcium

$^1O_2$, singlet oxygen

2, 4-D, 2, 4-dichlorophenoxy-acetic acid

ABA, abscisic acid

ABI1/2/5, ABA insensitive 1/2/5

ACN, acetonitril

AIDS, Acquired Immunodeficiency Syndrome

AOC, allene oxide cyclase

AOS, allene oxide synthase

APX, ascorbate peroxidase

ASH, ascorbic acid

BRs, brassinosteroids

cADPR, cyclic ADP-ribose

CaM, calmodulin

CAMTAs, camodulin binding transcription activators

CAT, catalase

CaVs, The voltage-gated Ca^{2+}-selective channels

CAXs, High-capacity vacuolar calcium exchangers

CCaMKs, Ca^{2+}/CaM dependent protein kinases

CDPKs, Ca^{2+}-dependent protein kinases

CHS, chalcone synthase

CK, cytokinin

CRD, completely randomized design

DEPC, diethylepyrocarbonate

DHAR, dehydroascorbate reductase

DHE, dihydroethidium

DMSO, dimethylsulfoxide

ds.m^{-1}, decisiemens per metre

ER, endoplasmic reticulum

ET, ethylene

FAO, Food and Agriculture Organization of the United Nations

GB, glycine betaine

$GdCl_3$, gadolinium chloride

GFP, green fluorescent protein

GOPX, guaicol peroxidase

G-protein, GTP-binding protein

GPX, glutathione peroxidase

***gpx1*,** glutathione peroxidase 1

GR, glutathione reductase

GSH, glutathione

GST, glutathione-S- transferase

H^+, Hydrogen ion

H_2O_2, hydrogen peroxide

H_3O, hydronium ion

HAB1/2, homology to ABA ½

$HO_2^{\bullet}$, perhydroxy radical

HPLC, high performance liquid chromatograph

HR, hypersensitive response

IAA, natural auxin or indole-3-acetic acid

I_{Ca}, non-selective cation current

iGluRs, ionotropic glutamate receptors

IP3, inositol 1,4,5-trisphosphate

IP6, inositol hexakisphosphate

JA, jasmonic acid (or jasmonate)

JA-Il, (+)-7-iso-Jasmonoyl-l-isoleucine

JAR1, jasmonate-Resistant 1

JAZ/TIFY, jasmonate ZIM/tify-domain

JIPs, jasmonate-induced proteins

KAT1, K^+-inward rectifier 1

LEA, late embryogenesis abundant proteins

LOX, lipoxygenase

MAPK, mitogen-activated protein kinase

MDHAR, monodehydroascorbate reductase

MeJA, methyl jasmonate

mM, millimolar

NaCl, sodium chloride

NHX1, Na^+/H^+ antiporter

NINJA, Novel Interactor of JAZ/TIFY

nM, nanomolar

NO, nitric oxide

NSCCs, non-selective cation channels

$O_2^{\bullet-}$, superoxide radicals

OAT, ornithine δ-aminotransferase

OECD, the Organization for Economic Co-operation and Development

OH^-, hydroxyl ion

$OH^\bullet$, hydroxyl radical

OPC-8:0, 3-oxo-2(2'[Z]-pentenyl)cyclopentane-1-octanoic acid

OPDA, 12-oxophytodienoic acid

OPR3, OPDA reductase 3

OST1, an ABA-activated protein kinase, homologue of SnRK2.2/SnRK2.3, that are positive regulators of ABA responses. The name is due to the physiological phenotype of the mutant *open stomata* that is caused by the suppression of OST1 expression.

P5CS, Δ^1-pyrroline-5-carboxylate synthetase

PAs, polyamines

PCD, programmed cell death

PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α

Phenidone, 1-phenylpyrazolidinone

PLC, phospholipiase C

PP2Cs, protein phosphatases which fall under the category of type 2C; some members are negative regulators of ABA-induced responses.

PR genes/proteins, pathogenesis-related genes/proteins

Pro, Proline

PUSFs, polyunsaturated fatty acids

Pyrabactin, common name for 4-bromo-N-[pyridin-2-yl methyl]naphthalene-1-sulfonamide. This growth regulator partially mimicks the inhibitory effects of ABA on seed germination, seedling growth, and its impact on gene expression.

QACs, quaternary ammonium compounds

R genes/proteins, resistance genes/proteins

RCARs/PYR1/PYLs, regulatory components of ABA receptor/pyrabactin resistance protein1/PYR-like proteins; a family of START domain proteins; demonstrated to inhibit clade A PP2Cs.

RNS, reactive nitrogen species

RO$^{\bullet}$, alkoxy radicals

ROS, reactive oxygen species

RsGluR, plasma membrane-targeting glutamate receptor from small radish

RT-PCR, reversible transcription PCR

SA, salicylic acid

SHM1, serine hydroxymethyl transferase 1

SLAC1, slow anion channel-associated 1

SLs, strigolactones

SnRK2, sucrose non-fermenting 1-related protein kinase 2

SOD, superoxide dismutase

SOS1, salt overly sensitive 1

SOS2, salt overly sensitive 2

SOS3, salt overly sensitive 3

START, steroidogenic acute regulatory-related lipid transfer proteins.

STS, stilbene synthase

STZ, salt tolerance zinc finger protein

SV, slow vacuolar

TPL, Groucho/Tup1-type corepressor TOPLESS

TPRs, TPL-related proteins

TSCs, tertiary sulfonium compounds

ub, ubiquitination

UBP16, ubiquitin-specific protease

UPS, ubiquitin–26S proteasome system

wfp, world food program

ZIM/tify, zinc-finger protein expressed in inflorescence meristem

α-GDH, α-glutamate dehydrogenase

α-LA, α-linolenic acid

Zusammenfassung

Salzbelastung setzt nicht nur Pflanzen unter Stress, sondern auch die Biologen, die mühsam versuchen, die verschlungenen Elemente der pflanzlichen Antwort auf Salzstress zu entwirren. Es ist von großer Bedeutung für die Pflanzenwissenschaften, die Mechanismen zu verstehen, mit denen Pflanzen Salzstress wahrnehmen, verarbeiten und darauf antworten. In Stress-Signalwegen sind häufig dieselben Moleküle an Stress-Schäden und an der Stress-Anpassung beteiligt. Ausserdem wird die Anpassung an Salzstress nicht durch einen „Generalschalter“ aktiviert, sondern hat sich aus der Vernetzung verschiedener Regelkreise entwickelt.

Um diese Regelkreise ansprechen und diese Komplexitäten entschlüsseln zu können, nutze ich in meiner Arbeit die natürliche Biodiversität in einem experimentellen System, wo osmotische Anpassung in Abgrenzung von pflanzlicher Abwehr untersucht werden kann. Ich setzte hierzu zwei Zell-Linien aus eng verwandten Rebarten ein, die sich in ihrer osmotischen Anpassungsfähigkeit unterscheiden: die Felsenrebe *Vitis rupestris* besiedelt felsige, sonnige Abhänge und hat daher evolutiv eine sehr gute osmotische Toleranz ausgebildet. Im Gegensatz dazu siedelt die Uferrebe *Vitis riparia* in feuchten Auwäldern und ist weniger gut an osmotischen Stress angepasst. Ich verfolgte schnelle Änderungen im zellulären Gehalt von Natrium und Calcium, reaktiven Sauerstoffspezies, apoplastischer Alkalinisierung und der Transkription spezifischer Gene, aber auch langsamere Antworten im Pegel von Jasmonsäure und ihrem aktiven Isoleucinkonjugat, ebenso wie von Abscisinsäure und Stilbenen. Unterschiede in Zeitmuster und der Empfindlichkeit gegenüber dem Lanthanoid Gd oder exogenem Calcium legen eine adaptive Funktion der frühen Natriumaufnahme durch nicht-selektive Kationenkanäle nahe. Diese Ionenkanäle wirken stromauf von Calcium- und Protonenströmen. Die apoplastische Alkalinisierung wurde durch das Lanthanoid $GdCl_3$ entweder völlig (*V. riparia*) oder zumindest teilweise (*V. rupestris*) gehemmt. Analog den durch osmotischen Stress

induzierten Ionenflüssen werden die durch Salzstress ausgelösten Calcium- und Protoneneinströme durch Lanthanoid-Ionen gehemmt. Die frühesten zellulären Antworten auf Salzstress scheinen also mit denen von mechanischem Stress übereinzustimmen.

Ich kann außerdem eine Korrelation zwischen Salzempfindlichkeit und unkontrollierter Jasmonatbildung zeigen. Hingegen korreliert erfolgreiche Anpassung an Salzstress mit einer strikten Kontrolle der Akkumulation von Jasmonsäure und seinem Isoleucin-Konjugat., was von einer Akkumulation von Abscisinsäure begleitet wird. Ausserdem wird infolge der Stress-Adaptation die Bildung von Stilbenen unterdrückt, wichtigen Schaltern für den programmierten Zelltod bei der pflanzlichen Abwehr. Ausserdem werden früh während der Adaptation JAZ/TIFY Proteine induziert, die mit zahlreichen Transkriptionsfaktoren der hormonellen Signalverarbeitung interagieren und deren Aktivität modulieren können.

Aus diesen Daten ergibt sich ein Modell, wonach die rechtzeitige Dämpfung des Jasmonat-Signalwegs darüber entscheidet, ob die Zellen der Weinrebe die frühen Stress-Signale als salzstress-bezogen erkennen und daher mit einer Aktivierung der Salzadaptation reagieren, oder ob diese frühen Signale als abwehrbezogene Stress-Faktoren gedeutet werden, worauf die Zelle mit programmiertem Zelltod antwortet.

Summary

Salinity does not only stress plants but also the biologists who sincerely try to discriminate its tangled elements. Understanding the mechanism by which plants sense, signal, and respond to salinity stress is of great interest to plant biologists. In stress signalling often the same molecules are involved in both, damage-related and adaptive, events. In addition, adaptation to salinity is not provided by one single “master-switch”, but must have evolved from tuning the cross-talk of different regulatory circuits. To address these circuits and dissect these complexities, I decided to make use of natural biodiversity in a

system, where osmotic adaptation can be studied in contrast to defense-related signaling. I employed two cell lines from two closely related grapevine species differing in osmotic tolerance: *Vitis rupestris* inhabits rocky, sunny slopes, and therefore has evolved a considerable osmotic tolerance. In contrast, *Vitis riparia* occurs in alluvial woods and performs poorly under osmotic stress. I followed rapid changes in the cellular content of sodium and calcium, ROS, apoplastic alkalinisation, gene transcripts, and slower responses in the levels of jasmonic acid, its active isoleucine conjugate, and abscisic acid, as well as of stilbenes. Differences in timing and sensitivity to either the lanthanoid Gd or exogenous calcium provide evidence for an adaptive role of early sodium uptake through non-selective cation channels acting upstream of Ca^{2+} and H^{+} fluxes. The lanthanoid $GdCl_3$ completely blocked (*V. riparia*), or at least impaired (*V. rupestris*), apoplastic alkalinisation. Similar to osmotically induced ion fluxes, pretreatment with lanthanoid ions can block salinity induced calcium and proton fluxes. Thus, the earliest events of the cellular salinity response are shared with those triggered by mechanic challenge. I find a correlation of salt sensitivity with unconstrained JAs signalling, whereas salt adaptation correlates with tight control of jasmonic acid and its isoleucine conjugate, accompanied by accumulation of abscisic acid and suppression of stilbenes that trigger defense-related cell death. Furthermore, JAZ/TIFY proteins, that interact with multiple transcription factors central for plant hormone signaling, are more actively induced in context with salt adaptation. From these data a model for salt adaptation is developed, where efficient quenching of JA-signaling determines whether grapevine cells recognise the early stress signals as salinity related and therefore respond by salinity adaptation, or whether they interpret these signals as defense-related and therefore respond by programmed cell death.

1 Introduction

1.1 What does stress mean for a plant?

Cormophytic plants are terrestrial organisms, and firstly appeared about 480 million years ago according to the fossil records (Gehrig *et al*., 1996). Since that time, the battle with drought and salt stress has shaped plant evolution, as land plants are frequently exposed to this type of stress during their life cycles.

The concept of stress has been originally derived from physics, where it is exactly defined as relation between inputs and output of a system and can be exactly determined. Stress is referred to as the force per unit area of a material that causes, when sufficient, deformation of this material. The term strain is derived from the change in length caused by the material deformation (Lubliner, 2008). In biology, however, the term stress is usually used improperly and can have a wide range of meanings, although several definitions have been suggested and none of them being universally accepted. For example, in humanities and psychological medicine, stress is considered as a consequence of proximal life events ranging from extreme situations such as natural catastrophes to more usual events like marriage, birth, death, and unemployment (Dohrenwend, 2000). Zoologists view stress as a psychological/biological response of the individual to different environmental conditions and correlate it with an adaptive response called fight-or-flight (Monroe, 2008). Based on his experiments on rats, the biomedical scientist Hans Selye (1936) suggested the term “general adaptation syndrome” as stress concept. This syndrome develops in three stages; (1) a general alarm reaction, (2) cellular reactions on different levels that prioritize the defense-related metabolites over growth, resulting in adaptation and recovery, and (3) exhaustion that hugely depends on the severity of the damaging agent. In botany, the stress concept must differ as

plants as sessile organisms are confined to the place where they grow, and therefore must constantly monitor their ever-changing environment, adapting their architecture and lifestyle (Jaillais and Chory, 2010). An environmental condition that is optimal for one species could be stressful for another. Moreover, within the same species, cultivars vary in their growth behavior during the same biological event. Depending on the factors, stress elicitors (inducer) can be distinguished from stressors that cause disease or deviations of physiology or growth. Such factors that are maybe either favorable or unfavorable, could be either originate from the physical environment (abiotic stress), but they could be as well biotic, for instance, pathogenic (Cassells and Curry, 2001). Any situation is considered as stressful when it limits the rate of dry-matter production of the plant below its genetic potential (Jones *et al*., 1993).

The plasma membrane is considered as the first target of the stressor or elicitor (Mahajan and Tuteja, 2005). Stress primarily alters the physiological state and impairs the performance of a vital function (Gaspar *et al*., 2002). In the same line, Munns and Tester 2008 defined stress as an adverse circumstance that disturbs or is likely to disturb the normal physiological functioning of an individual. It is possible to transfer the triphasic biomedical stress concept of Selye (1936) to plant stress as well, as has been worked out for seed stress (Kranner *et al*., 2010). In this example, as an “alarm” response, post-translational modification and stress signaling through the cross-talk between reactive oxygen species (ROS), reactive nitrogen species (RNS), and seed hormones (ABA and GA) are utilized by challenged seed to induce transcriptome modifications. The second phase, “adaptation”, is achieved when the “building blocks” of the resistance response have been successively assembled. Finally, depending on dose and exposure time, the exhaustion phase can occur, and the result is seed death.

A widely accepted definition considers plant stress as any external factor that negatively affects the plant (Taiz and Zeiger, 2010). These stress factors fall in two groups; biotic or abiotic stress. Biotic stress refers to biological factors

such as insects, weeds, and pathogens (bacteria, fungi, viruses, nematodes). On the other hand, abiotic stress includes nutrient deficiency, oxidative stress (such as ozone), drought (water deficit), flooding (water excess), salinity, high temperature, low oxygen, mechanical damages (such as wind), chilling, freezing, pollution, and radiations (such as high intensity of UV).

1.2 Environmental stress affects the world population

Growth and productivity of stressed plants are highly decreased causing a shortage of food supplies. According to the World Food Program 2011, there are about one billion undernourished people in the world today. This means that one in seven people does not get enough food to be healthy and lead an active life. The risk of hunger and malnutrition is seen as the number one risk to global health. Its negative impact is considered much higher than that of AIDS, malaria and tuberculosis combined. East, central and southern Africa harbor the highest percentage of hungry people (**Fig. 1**) (FAO, 2011). In addition, the recently-appeared annual report of the OECD and FAO 2012 discussing the production, consumption, stocks, trade and prices for different agricultural products for the period 2012 to 2021 are alarming for the future of the world population. According to this annual report, the World population, approximately 7 billion people in 2011, is expected to increase by about 680 million people in 2021, and by 2050 the world's population is expected to be 9.1 billion. Those additional people will need additional food, fiber and fuel for their life. In other word, the agricultural production has to increase by 60% over the next 40 years to meet the rising demand for food. Moreover, additional production will also be necessary to provide feedstock for expanding biofuel production (OECD and FAO, 2012).

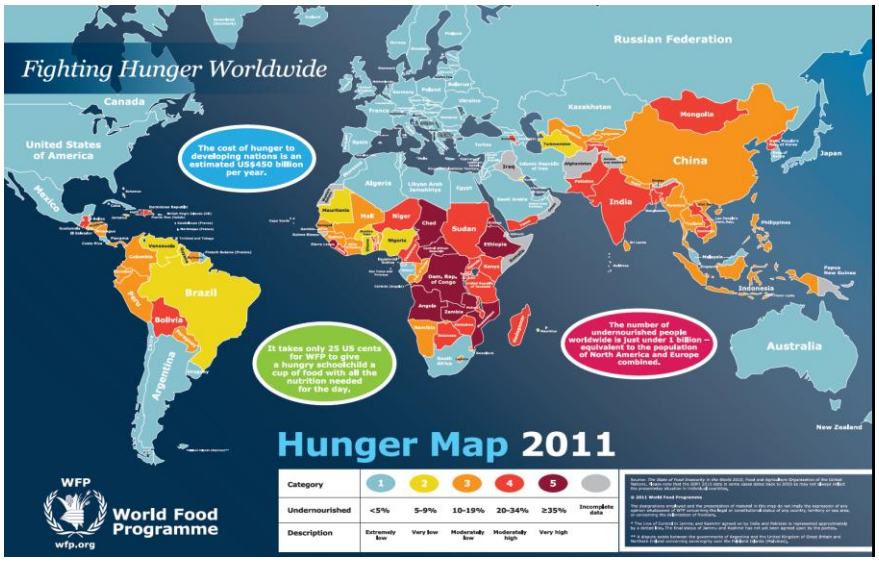


Fig. 1: The world hunger map according to wfp.org 2011. The categary ranges from 1, as the best situation (malnutrition < 5%) to 5, as the severest situation (malnutrition ≥ 35%).

This information means that the world population needs to find ways to fill the gap between the available and demanded food. Unfortunately, several reasons make this task not easy at all. Firstly, water limitation is considered as one of the major restricting factors in many countries and as overlooked problem for many others (Cominelli *et al*., 2009). Secondly, non-sustainable farming and soil erosion irreversibly degrade significant portions of agricultural lands - estimates range by 25% (OECD and FAO, 2012). Thirdly, the growing impact of the global climate changes seriously affect crop growth and threaten the conservation of cultivated land (Takeda and Matsuoka, 2008). All of these elements, in fact, are abiotic stress factors which, together with biotic stress factors, result in crop failures worldwide estimated by 65 – 87 % (Buchanan *et al*., 2002).

The world total arable land according to this OECD and FAO 2012 annual reports will increase by only 69 million hectares (less than 5%) by 2050. This means that the world needs to increase the food production almost exclusively by increased productivity in the same way as it has for the past 50 years. At the same time, the improvement of the sustainable use of available land, water, marine ecosystems, fish stocks, forests, and biodiversity should be considered (OECD and FAO, 2012).

1.3 The salinity stress syndrome

High salinity is commonly caused by high sodium (Na^+) and chloride (Cl^-) in the soluble fraction of the soil with electric conductivity values higher than 4 dS m^{-1}. Salinity stress generates both hyperionic and hyperosmotic imbalance. In addition, the presence of high NaCl alters water potential, inhibits growth, and promotes cell death (Hasegawa *et al*., 2000). For more than 3000 years, salinity has been a threat to agriculture in some parts of the world (Flowers, 2006). More than 80 million hectares of arable land worldwide are estimated to be affected by salt (Munns and Tester, 2008) resulting in estimated annual global costs equivalent to 11,000 million US$ in 2011 (http://www.fao.org/ag/agl/agll/spush/). Saline soils negatively affect both the ability of crops to take up water and the availability of micronutrients, while they increase the concentration of ions that are toxic to plants, and may degrade soil structure. The FAO (2007) has estimated soil properties worldwide and mapped the actual soil profile data as a soil production index (**Fig. 2**) (http://www.fao.org/nr/land/soils/en/). This production index of the soil tells a clear message: the agricultural horizontal expansion is limited and vertical expansion (i.e. by increase of productivity) is the only way to meet the world demand.

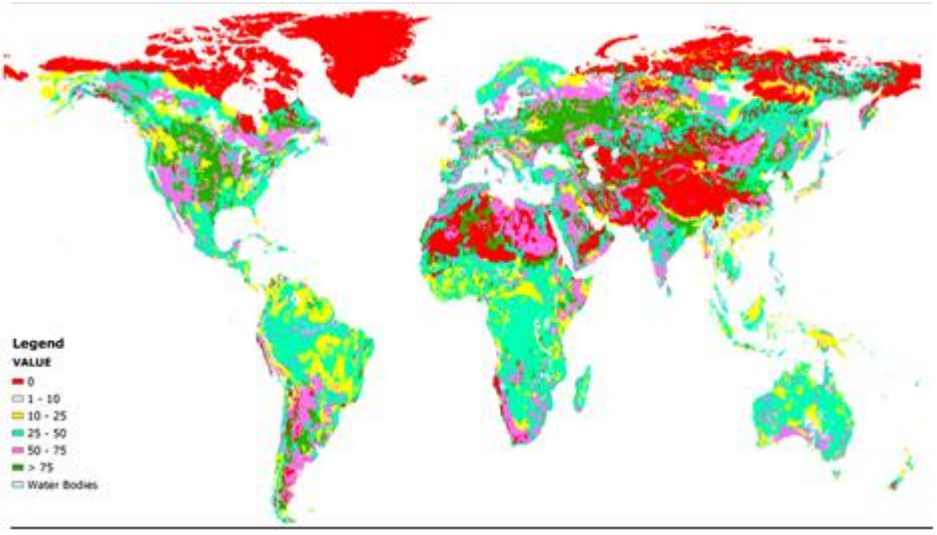


Fig. 2: The soil production index map ranging from zero as no productivity (red) to more than 75% as the highest production (dark green) (from FAO 2007).

Plants are divided into two groups according to their behavior under salinity stress. Halophytes (naturally inhabiting saline environments) require electrolyte concentrations higher than those found in nonsaline soils (approximately from 20 to 500 mM). On the other hand, glycophytes, which represent the most economically crops, are stressed under salinity condition ranging from 10 to 100 mM (Flowers *et al*., 1977). Some of the glycophytes have evolved different adaptive mechanisms to control the negative events caused by salt stress and therefore are in the focus of research interest. Adaptation usually refers to a genetically determined level of resistance acquired by a process of selection over many generations (Taiz and Zeiger, 2010). Therefore, plants are classified to three groups depending on their tolerance or sensitivity. The tolerant crops such as date palm and barley can withstand a salt concentration up to 10 g/l (~170 mM NaCl). The moderately tolerant crops such grapevine and olive can manage concentrations of salt up to 5 g/l (~85 mM NaCl). Finally, the sensitive group including peach and apple is able to survive only below 2.5 g/l (~40 mM NaCl) (FAO Manual 1985). Based on this classification, 40 mM NaCl is considered as the threshold of salinity stress (equaling approximately $1ds.m^{-1}$). The newest FAO classification (2011) divides plants to four groups as shown in table 1.

Sensitive (0-4 dS m^{-1})	Moderately Tolerant (4-6 dS m^{-1})	Tolerant (6-8 dS m^{-1})	Highly Tolerant (8-12 dS m^{-1})
Almond	Corn	Fig	Barley
Bean	Grain Sorghum	Oats	Cotton
Clover	Lettuce	Pomegranate	Olive
Onion	Soybean	Sunflower	Rye
Potato	Tomato	Wheat	Wheatgrass

Table 1: Salinity tolerance of some economic crops. Salinity expressed as electrical conductivity (FAO 2011).

Salinity-challenged plants show some damage events which could lead to death. At the cellular and molecular levels, salt stress causes membrane disorganisation, metabolic toxicity, formation of reactive oxygen species (ROS), inhibition of photosynthesis, and reduced nutrient acquisition (Hasegawa *et al.*, 2000; Tuteja, 2007; Zhu, 2002). Such factors can initiate more catastrophic events by which plants enter programmed cell death.

The temporary effects of salinity are clearly due to rapid and often transient changes in plant-water relations. However, the subsequent changes in growth rate, and the underlying molecular or metabolic events, are not so easily ascribed to water stress or salt-specific effects alone (Munns, 2002). Instantly after salinization, cells dehydrate and shrink, but they are able to regain their original volume hours later (Läuchli and Grattan, 2007). The concept of a “two-phase growth response to salinity” has been developed by Munns 1993 as an attempt to understand the differences in response to osmotic versus ionic salt stress (**Fig. 3**). The first phase of growth reduction takes place quickly, within minutes of exposure to salinity. This response is due to the osmotic effect caused by salt ions in the rhizosphere. Hormonal signals coming from the roots are assumed to regulate the growth reduction during this phase. The second phase of growth reduction is a resultant of a salt-specific effect, and needs some times (days, weeks or months) to develop. Cellular damage is due to salt accumulation in transpiring leaves leading to excessive levels which exceed the ability of the cells to compartmentalize salts into the vacuole (Läuchli and Grattan, 2007; Munns, 1993; Munns, 2002; Munns, 2005).

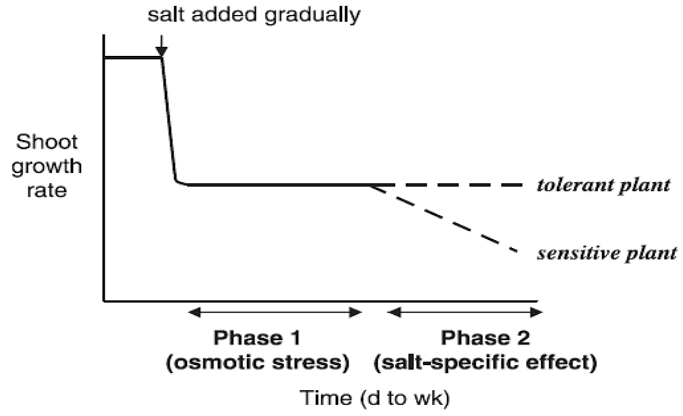


Fig. 3: Schematic illustration of the two-phase growth response to salinity for genotypes that differ in the rate at which salt reaches toxic levels in leaves (Munns, 2005).

1.4 Salinity perception and signal transduction

As salinity poses ionic (Na^+) and osmotic stresses, its perception could be achieved by both signals. Osmotic stress is suggested to be sensed partly by

cytoskeleton-associated stretch-activated ion (Ca^{2+}) channels, accompanied by redox-sensing, and transmembrane protein kinases, including two component histidine kinases. On the other hand, ionic stress is proposed to be perceived by a membrane receptor for sensing the extracellular Na^+, whereas intracellular Na^+ could be sensed by a membrane protein or Na^+-sensitive enzymes in the cytoplasm (Türkan and Demiral, 2009). The plasma membrane Na^+/H^+ antiporter (SOS1; salt overly sensitive 1), which is also responsible for exporting Na^+ from the cell, is proposed to be one of the Na^+ sensors (Zhu, 2002). After perception, a set of signal transduction pathways has been proposed to mediate salinity responses in plant cells. The detailed pathways and their elements will be discussed below.

1.4.1 Calcium signaling

Calcium ions (Ca^{2+}), which are essential for all life forms, are considered as the most prominent ubiquitous second messenger in cells ranging from bacteria, and plants, up to specialized neurons. However, prolonged high levels of calcium can activate degradative processes or apoptosis. Therefore, the intracellular levels of Ca^{2+} as well as the spatial distribution of this signal are tightly regulated by complex mechanisms such as chelation, export, and compartmentalization in different intracellular organelles including ER, chloroplast, mitochondria, and the vacuole (Clapham, 1995; Bouché *et al*., 2005). The normal cytoplasmic Ca^{2+} level is ~100-200 nM while in the cell wall and organelles it is ~1-2 mM. In plant cells, many Ca^{2+}-permeable channels have been identified by patch-clamp studies and assigned to plasma membrane, tonoplast, endoplasmic reticulum, chloroplast and nuclear membranes (White, 2000). The voltage-gated Ca^{2+}-selective channels (CaVs), which are plasma membrane ion channels, represent the fastest Ca^{2+} signalling proteins. When open, each channel conducts approximately a million Ca^{2+} ions/sec driven by the 20,000-fold gradient between apoplast and cytoplasm (Clapham, 2007). Cytoplasmic Ca^{2+} (Ca^{2+}_{cyt}) signals can be generated by influx or efflux of ion from the extracellular space (cell wall or apoplast in plants) or intracellular compartments (such as vacuole,

chloroplast or mitochondria), respectively. It is known that Ca^{2+}_{cyt} signals are often produced by Ca^{2+} release from internal stores (Peiter, 2011). In particular, a release of vacuolar Ca^{2+} into the cytosol has been demonstrated in response to a wide range of signalling transduction pathways, including biotic and abiotic stresses and hormone signals (Hirschi, 2001). Several Ca^{2+} channels on the tonoplast have been identified on the molecular level and classified into ligand-gated and voltage-gated channels (Peiter, 2011). Ligand gated channels activated by inositol 1,4,5-trisphosphate (IP3), inositol hexakisphosphate (IP6) or cyclic ADP-ribose (cADPR), along with the depolarization-activated slow vacuolar (SV) channel act in concert to shape Ca^{2+} signals (Peiter, 2011; Hetherington and Brownlee, 2004). The spatial pattern of Ca^{2+} signals (e.g. cytosol, nucleus, organelles, or localized region within the cell), temporal propagation of Ca^{2+} levels, the amplitude of the signal, and the frequency of Ca^{+2} oscillations are all informative signalling elements conveyed by Ca^{2+} ions (Reddy *et al*., 2011).

These changes in Ca^{2+} levels are perceived by adaptor proteins or Ca^{2+}-modulated proteins which function as sensors for the Ca^{2+} signals and regulate down-stream signalling events. The majority of Ca^{2+}-modulated proteins contain one or more highly conserved motif, the EF hand, which is a helix-loop-helix structure (Bouché *et al*., 2005). In plants, the superfamily of EF-hand proteins is categorized into two major classes, termed sensor relays and sensor responders (Kudla *et al*., 2010; Reddy *et al*., 2011). The class of sensor relay proteins includes calmodulins (CaMs) (with exception of CaM7), CaM-like proteins (CMLs), and calcineurin B-like proteins (CBLs). Sensor responders are the Ca^{2+}-dependent protein kinases (CDPKs), Ca^{2+}- and Ca^{2+}/CaM dependent protein kinases (CCaMKs), some DNA or lipid binding proteins, and a few enzymes. Additionally, other proteins lacking an EF-hand motif can bind Ca^{2+} such as annexins and C2 domain-containing proteins (Bouché *et al*., 2005; Cheng *et al*., 2002; Kudla *et al*., 2010; Reddy *et al*., 2011).

Ca^{2+} signals are highly interconnected to signalling pathways involved in the

response to nearly all developmental, hormonal, and stress cues. For example, the camodulin binding transcription activators (CAMTAs), transcription factors contain CaM binding domain, are induced upon cold (CAMTA1), heat (CAMTA3-6), ABA and SA (CAMTA2, 4, and 6), MeJA and ET (CAMTA1, 3, and 4), H_2O_2 (CAMTA2-6), and auxin IAA (CAMTA1), and all of them are induced by wounding (Reddy *et al*., 2011). Additionally, a plasma membrane-targeted glutamate receptor first identified in small Radish (RsGluR), homologous of the neural ionotropic glutamate receptors (iGluRs) that are ligand gated nonselective cation channels (NSCCs) mediating fast excitatory neurotransmission, has been shown to mediate, upon overexpression in Arabidopsis, glutamate-mediated Ca^{2+} Influx resulting in enhancing pathogen resistance by upregulation of JA-biosynthetic gene(s) (Kang *et al*., 2006).

As to be expected from this general role in signalling, Ca^{2+} plays also a crucial role in salinity tolerance involving different signalling elements. Upon salinity, CAMTA1 to 6, except 5, are induced (Yang and Poovaiah, 2002). Ca^{2+}-activated salt overly sensitive 3 (SOS3), a member of the CBLs family known as CBL4, is triggered to interact with SOS2 (a CBL-interacting protein kinase, CIPK24). The SOS3/SOS2 complex, in turn, activates SOS1 (a plasma membrane Na^+/H^+ antiporter) through its phosphorylation. The activated SOS1 extrudes Na^+ from the cell thus reducing its harmful effects on cellular metabolism (Harper *et al.,* 2004; Munns and Tester, 2008; Zhu, 2002). Additionally, Ca^{2+} can act directly without the need for a protein adaptor. The Arabidopsis salt stress-responsive gene 1 (*AtNIG1*), a basic/helix-loop-helix-type transcription factor, is the first known calcium-binding transcription factor involved in salt-stressed plant (Kim and Kim, 2006). In addition, Ca^{2+} ions interplay also with H_2O_2, ABA, and JA in the activation of the potassium outward channels causing stomatal closure (Pei *et al*., 2000; Suhita *et al*., 2004). Interestingly, auxin-induced Ca^{2+} elevations produced the opposite response leading to stomatal opening illustrating the point that it is not calcium per se, but its spatiotemporal pattern that confers response specificity (Ng and McAinsh, 2003). Fig. 4 represents a schematic

presentation of calcium signalling pathways mediating the specific responses to different environmental stresses (Bouché *et al*., 2005).

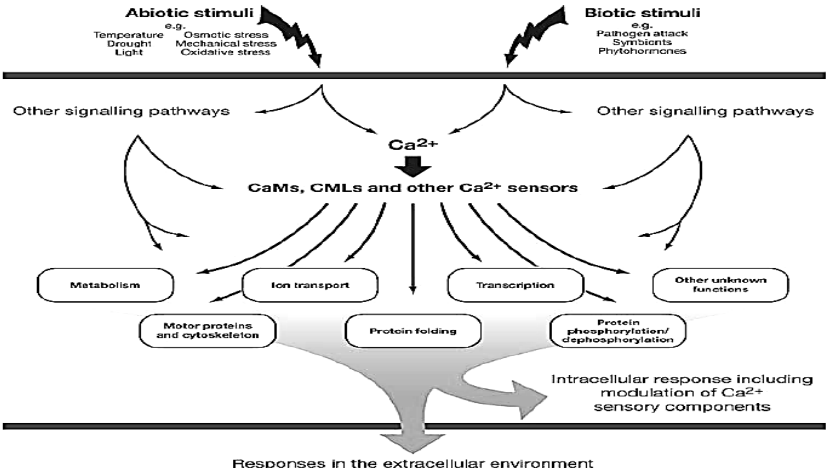


Fig. 4: Model for different signal responses mediated by Ca^{2+} and Ca^{2+}-modulated proteins (Bouché *et al*., 2005).

1.4.2 Extracellular and intracellular pH

Water molecules spontaneously ionize forming a hydronium ion (H_3O) and a hydroxyl ion (OH^-). The reverse reaction also takes place very readily establishing an equilibrium. By tradition, the H_3O^+ concentration is usually referred to as "H^+" concentration, even though nearly all H^+ in an aqueous solution is present as H_3O^+ (Alberts *et al*., 2008). The hydrogen ion (H^+) concentration is expressed using a logarithmic scale known as the pH scale. Pure water represents a neutral solution with pH 7. Also the interior pH of the cell is kept close to neutrality by powerful proton transport mechanisms at the plasmalamma, and by the high buffering capacity of the cytosol (Kurkdjian and Guern, 1989). In biology, the proton activity (H^+) plays crucial roles in cell signaling either directly or in cross-talk with phytohormones or Ca^{2+} (Gao *et al*., 2004). It might be also important for the regulation of various aspects of metabolism. For example, proteins are sensitive to changes of H^+ concentration in their environment and pH regulates the activity of key enzymes and metabolic steps, such as the enzyme activities responsible for malate synthesis, during cation accumulation in polar transport of the plant hormone (auxin), and in the intracellular response to substances such as

auxin mimicked by the mycotoxin fusicoccin, which both cause proton extrusion (Kurkdjian and Guern, 1989; Roberts *et al*., 1980). Due to its biochemical impact, proton concentration is tightly controlled by all cells. Additionally, the intracellular pH is considered as a second messenger for several signaling pathways. For example, cytoplasmic alkalinization conveys MeJA and ABA signalling during stomatal closure of *Arabidopsis thaliana* (Suhita *et al*., 2004), and is also involved in plant responses to salinity, auxin, and gravity (Gao *et al*., 2004; Fasano *et al* 2001; Kurkdjian and Guern, 1989).

A plant specific topic is the apoplast, considered as the first plant domain challenged by environmental signals. The apoplast is probably not only involved in the response to numerous environmental signals, but also in their perception and transduction and herefore cooperates with the plasma membrane (Hoson, 1998). Changes in apoplastic pH have been intensively studied in different plant species, and under different conditions. For example, tomato cells elicited by chitin or bacterial flagellin (Felix *et al*., 1993, Felix *et al*., 1999), root hair cells of *Sinapis alba* elicited by fusicoccin (Bertl and Felle, 1985), alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals, *Vicia faba* leaves responding to different stress factors (Felle and Hanstein, 2002), and *Vitis* cells elicited by the bacterial elicitor Harpin (Qiao *et al*., 2010) have been reported to produce rapid, and transient increases of apoplastic pH. Although still under debate, these pH responses have been proposed to act as signals for changes of light intensity, drought, oxygen deficiency, and microbial attack (Felle, 2001).

1.4.3 ROS induction

Reactive oxygen species (ROS) are continuously generated in plant compartments such as mitochondria, chloroplasts, and peroxisomes as unavoidable byproducts of metabolic processes like photosynthesis and respiration (Apel and Hirt, 2004). The term ROS comprises both free radical ($O_2^{\bullet -}$, superoxide radicals; $OH^{\bullet}$, hydroxyl radical; $HO_2^{\bullet}$, perhydroxy radical and $RO^{\bullet}$, alkoxy radicals), and non-radical (molecular) forms (H_2O_2, hydrogen

peroxide and $^1O_2$, singlet oxygen) (Gill and Tuteja, 2010). The different ROS species vary not only in their chemical nature, but also in their toxicity. The Superoxide $O_2^{\bullet-}$ is considered as earliest ROS, while $OH^{\bullet}$ is among the most highly reactive ROS known. The accumulation of ROS causes oxidative damage to DNA, proteins, carbohydrates, and lipids. However, they also could function as signalling molecules regulating responses of development and various aspects of stress. Therefore, they must be closely regulated by orchestrated mechanisms (Miller *et al*., 2010).

For different stimuli, the elevated levels of ROS are sensed at the plasma membrane, for instance by two-component signalling systems (membrane-localized histidine kinases) that, in turn, activate the MAPK signalling cascades (**Fig. 5**). However, ROS can also change the expression of genes by modifying their transcription factors (Apel and Hirt, 2004). Additionally, the elevation of H_2O_2 is considered as a modifier of key signalling enzymes through reversible oxidation of critical thiols (Forman and Torres, 2002). For example, salt-stressed tobacco plants increased H_2O_2 levels in the intracellular space, which in turn leads to the activation of the glutathione peroxidase 1 (*gpx1*) promoter (Avsian-Kretchmer *et al*., 2004). A second example for this type of mechanism is the induction of α-glutamate dehydrogenase (α -GDH) subunit by salinity-stimulated ROS. The anionic iso-GDHs assimilate ammonia, thus relieving stress damage by ammonia detoxification and production of glutamate glutamate for proline synthesis (Skopelitis *et al*., 2006). The elicitor-induced ROS production activates ion channels, defense gene, stomatal closure, and phytoalaxin synthesis (Jabs *et al*., 1997; Pei *et al*., 2000). Moreover, ROS are a hallmark of plant-specific forms PCD, so called necroptosis (Coll *et al*., 2011).

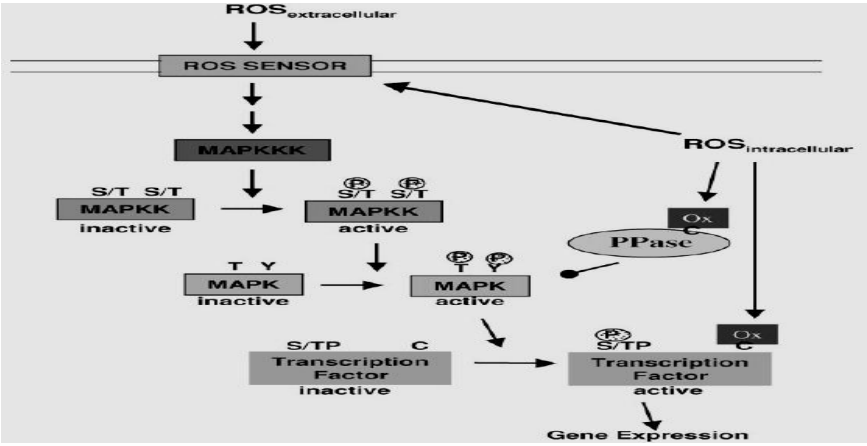


Fig. 5: Schematic depiction of cellular ROS sensing and signaling mechanisms adopted from Apel and Hirt, 2004.

1.4.4 Nitric oxide signals

Nitric oxide (NO) is a small, uncharged, short-lived, water and lipid soluble, highly diffusible, and ubiquitous, volatile, highly reactive free radical that functions as a biological mediator during key physiological processes in both animals and plants (Neill *et al*., 2003; Siddiqui *et a*l., 2011). It acts as second messenger during vasorelaxation, neurotransmission, immunity, and cytotoxicity processes (Neill *et al*., 2001). In plants, NO plays crucial roles in a broad spectrum of stress responses (biotic and abiotic) and developmental processes (e.g. germination, root growth, stomatal closure). It acts as a Ca^{2+}-mobilizing messenger and modulates the activity of proteins through nitrosylation and probably tyrosine nitration (Besson-Bard *et al*., 2008). Due to this general signalling role, NO is meanwhile considered as a phytohormone (Santner and Estelle, 2009). The cross-talk between NO and Ca^{2+}-CaM, H_2O_2, ABA, and IAA signalling has been established (Sang *et al*., 2008; Terrile *et al*., 2012; Wang *et al*., 2012). Interestingly, NO can be either cytotoxic or cytoprotective. The cytoprotective effect of NO is linked with its ability to neutralize Fenton-type oxidative damage by scavenging superoxide, and therefore preventing the formation of oxidants (such as $O_2^{\bullet-}$, H_2O_2, and alkyl peroxides) which makes it easier to recover a redox homeostasis. However, when $O_2^{\bullet-}$ reduces NO giving rise to the strong oxidant peroxinitrite ($ONOO^-$), NO functions as a cytotoxic agent (Lamattina *et al*., 2003). In

addition, NO is considered as a redox regulator of the NPR1/TGA1 system (both of them are key redox-controlled regulators of systemic acquired resistance in plants) (Lindermayr *et al*., 2010).

1.4.5 Mitogen-activated protein kinases

Mitogen-activated protein kinase (MAPK) cascades, that are basic signalling tools conserved in all eukaryotes, transduce environmental and developmental signals into intracellular responses. MAPKs cascades are composed of MAPKKKs, MAPKKs, and MAPKs that are activated in a sequential process. MAPKKKs (also known as MAP3Ks or MEKKs) that function upstream of MAPKs signalling, are activated via plasma membrane receptors. The resulting phosphorylation cascades activate, in turn, MAPKKs (also known as MAP2Ks, MEKs or MKKs) that eventually activate MAPKs (also known as MPKs) (Rodrigues *et al*., 2010; Taj *et al*., 2010; Wrzaczek and Hirt, 2001).

In planta, MAPKs signals regulate numerous events, including stress responses, innate immunity, and developmental processes. ROS signalling mediates the activation of MAPK signalling pathways under various abiotic stresses (Fig. 5) (Apel and Hirt, 2004). For salinity stress, different MAPK elements are activated such as MAPK4, MAPK6, and MAPKK1 (Taj *et al*., 2010). Arabidopsis overexpressors for AtMAPKK2 exhibited constitutive MAPK4 and MAPK6 activity, constitutively upregulated expression of stress-induced marker genes, and increased freezing and salt tolerance. Full genome transcriptome analysis of this mutant showed altered expression of 152 genes involved in transcriptional regulation (such as, *STZ* (Salt tolerance zinc finger protein), *WRKY*, *MYB*), signal transduction (such as a MAPKK5-related protein and a putative calmodulin), cellular defense (such as lipoxygenase and the ACC synthase *AtACS-6*), and stress metabolism (including a flavonol synthase and *P5CS*, a gene encoding a key enzyme of proline biosynthesis) (Teige *et al*., 2004). However, MAPK signalling can also act as antagonist – for instance, AtMAPK1 negatively regulates a putative Na^+/H^+ antiporter, leading to salinity sensitivity (Chinnusamy *et al*., 2006).

1.4.6 Cytoskeletal reorganization

Cytoskeletal polymers are unique, because, here, macromolecular subunits are linked by non-covalent bonds. As a consequence, the elements of the cytoskeleton, microtubules and actin filaments, are highly dynamic structures and assemble or disassemble in response to factors that control protein-protein interactions like ionic strength or temperature (Buchanan *et al*., 2002). The dynamicity of cytoskeletal networks affects almost every intracellular activity, from division to movement, morphogenesis and signal transduction. The plant cytoskeleton alters SOS signaling, Ca^{2+} influx, and the proteolysis of cytoskeleton associated proteins in response to salinity stress (Wang *et al*., 2011). Cytoskeleton-associated mechanosensors participate in the perception of osmotic stress signals in plants (Türkan and Demiral, 2009).

1.4.7 Roles of plant hormones

Phytohormones are small molecules that are transported to regulate plant growth, development, and stress responses. Auxin, ABA, cytokinin (CK), gibberellin (GA) and ethylene (ET) are identified during the first half of the twentieth century. In addition, several additional compounds are meanwhile considered as hormones, including jasmonate (JAs), brassinosteroids (BRs), salicylic acid (SA), nitric oxide (NO), polyamines (PAs) and strigolactones (SLs) (Santner and Estelle, 2009). Among those plant hormones, ABA and methyl jasmonate (MeJA) play central roles in plant adaptation to stress. They channel plant metabolism towards defense causing inhibition of root growth and cell cycle, reduction of photosynthesis, transpiration, and seed germination, and induction of stomatal closure (Suhita *et al*., 2004). Recent advances in the signalling of these two plant hormones will be discussed in the following pages.

1.4.7.1 JA biosynthesis and mode of action

JA and related compounds, collectively named jasmonates (JAs), are ubiquitously occurring lipid-derived compounds, and function as a master switch in plant responses to several abiotic and biotic stresses such as wounding (mechanical stress), drought and salt stress, ozone and pathogen infection, and insect attack (Wasternack, 2007). Methyle jasmonate (MeJA) was firstly isolated and identified in 1962 as a new odoriferous component from jasmine oil of *Jasminum grandiflorum* (Demole *et al*., 1962). JAs have become accepted as a new class of plant hormone, mainly involved in defense (Browse, 2009). Genes coding for plant defense proteins, so-called jasmonate-induced proteins (JIPs), such as proteinase inhibitors or enzymes of phytoalexin synthesis (in grapevine mainly the stilbene resveratrol) are upregulated by JA, while those coding for housekeeping proteins such as Rubisco are downregulated (Wasternack, 2007). In addition, JAs regulate many aspects of plant development and growth such as seed germination, fruit ripening, production of viable pollen, root growth, tendril coiling, photomorphogenesis, leaf abscission, and senescence (Creelman and Mullet, 1995, 1997a, 1997b; Conconi *et al.*, 1996; Rao *et al.*, 2000; Riemann *et al.,* 2003; Riemann *et al.*, 2013; Haga and Iino, 2004; Ma *et al.*, 2006; Robson *et al.,* 2010; Wang *et al.,* 2011).

JAs and octadecanoids (including 12-oxophytodienoic acid (OPDA) and OPDA-containing derivatives) are members of the oxylipin family, which includes metabolites formed by oxidation of polyunsaturated fatty acids (PUSFs). JA, a cyclopentanone compound carrying two side chains (a pentenyl and a carboxylic acid), is derived from α-linolenic acid (α-LA), 18:3, via the octadicanoid pathway (Wasternack, 2002). Several of key enzymes, including, lipoxygenase (LOX*)*, allene oxide synthase (AOS), and allene oxide cyclase (AOC), which are located in the chloroplasts, catalyze the biosynthetic pathway from the conversion of α-LA to OPDA. OPDA reductase 3 (OPR3), located in the peroxisome, reduces OPDA to 3-oxo-2(2'[*Z*]-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0), which in turn

undergoes three cycles of β-oxidation and finally is converted to (3R,7S)-JA (Browse, 2009; Wasternack, 2007).

JAs signals (Avanci *et al.*, 2010; Browse, 2009) have been extensively studied, using biochemical and molecular genetical approaches, in *Arabidopsis thaliana* and other species (Chini *et al*., 2009; Fonseca *et al.*, 2009; Memelink, 2009; Reinbothe, 2009; Schaller and Stintzi, 2009). (+)-7-iso-Jasmonoyl-l-isoleucine (JA-Il) generated by the enzyme JAR1 (Jasmonate-Resistant 1) represents the endogenous bioactive form of JAs (Staswick and Tiryaki, 2004, Staswick, 2008, Fonseca *et al.*, 2009). Similar to GA and IAA signaling, jasmonate-dependent gene activation involves hormone-induced degradation of a transcriptional repressor, the jasmonate ZIM/tify-domain (JAZ/TIFY) proteins (Vanholme *et al*., 2007). To date, 12 *JAZ/TIFY* genes have been identified in *Arabidopsis thaliana* (Chini *et al.*, 2007). These *JAZ/TIFY* genes share two conserved domains, a ZIM/TIFY (Zinc-finger protein expressed in Inflorescence Meristem), and a Jas (Jasmonate-associated) domain. The ZIM/TIFY domain mediates homo- and heteromeric hormone-independent interactions between individual JAZ/TIFY proteins (Chini *et al.*, 2009). In contrast, the Jas domain is required for hormone-dependent interactions of JAZ/TIFY with a bHlH-type transcription factor AtMYC2 and the F-box protein COI1 (Chini *et al.*, 2007; Thines *et al.*, 2007) and for nuclear localisation (Grunewald *et al.,* 2009). In the absence of stress, JAs in plant cells are maintained at low levels. However, in response to environmental or developmental signals that stimulate the biosynthesis of JAs, the elevated levels of JA-Ile promote the interaction of JAZ/TIFY repressors with SCF^{COI1}-mediated ubiquitination (ub), followed by degradation of JAZ/TIFY proteins via the 26S proteasome, which in turn frees MYC2, MYC3, MYC4, and other transcription factors (Wager and Browse, 2012). Activation of MYC2 induces transcription of early JA-responsive genes including the *JAZ/TIFY* genes themselves. The newly synthesised JAZ/TIFY proteins dimerise with MYC2 thus restoring the repression of MYC2 and deactivation of the pathway (Chico *et al*., 2008; Chung *et al.*, 2009). They also recruit the Groucho/Tup1-type corepressor TOPLESS (TPL) and

TPL-related proteins (TPRs) through a Novel Interactor of JAZ/TIFY (NINJA). NINJA acts as a transcriptional repressor, whose activity is mediated by a functional TPL-binding EAR repression motif and both NINJA and TPL proteins function as negative regulators of jasmonate responses (Pauwels *et al.,* 2010) (**Fig. 6**).

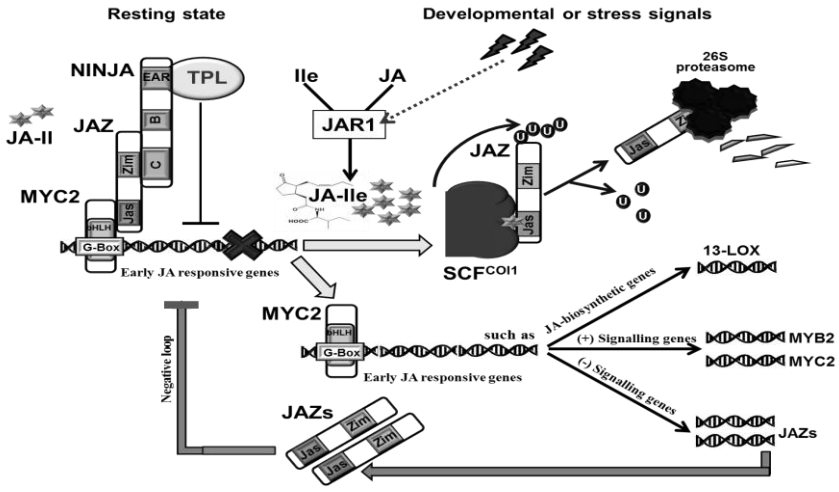


Fig. 6: Molecular mechanisms for activation and deactivation of JA signalling summarizing the current state of knowledge from the references described in the text.

1.4.7.2 ABA biosynthesis and mode of action

ABA, a growth inhibiting substance, was originally isolated and identified in the 1960s by several groups, and called *dormin* and *abscising II*. Later, it was renamed ABA to reflect its supposed role in the abscission process, although in the meantime, this name appears somewhat inappropriate, because abscission is not triggered directly by ABA, but via the induction of ET (Cutler *et al*., 2010). ABA regulates numerous developmental processes in plants including, the inhibition of growth and germination, bud dormancy, and seed development. Moreover, during seed development, ABA initiates embryo maturation, synthesis of storage reserves and late embryogenesis abundant (LEA) proteins, as well as the induction of seed dormancy (Xiong and Zhu, 2003). Additionally, ABA has vital roles in plant adaptive responses to stresses such as drought, salt, ozone, and pathogen infection, and therefore is also seen as a stress hormone. In salt- or drought-stressed plants, ABA

promotes the closure of stomata, in order to reduce the transpirational water loss (as discussed later). ABA also increases the plant stress tolerance by activating many stress-responsive genes that encode enzymes for the biosynthesis of compatible osmolytes (as shown for water-stress-induced betaine in pear leaves (Gao *et al*., 2004)), dehydrins, and LEA-like proteins that play important roles in osmotic adjustment. In addition, ABA can induce proteins that protecting membranes under stress (Hasegawa *et al*., 2000; Zhu, 2002). In several respects the role of ABA is due to its antagonistic interactions with other plant hormones such as IAA, GA, BRs, ET, CK, and JAs (Taiz and Zeiger, 2010).

ABA is synthesized via the terpenoid pathway and using condensation of isopentenyl diphosphate (IPP) as basic building block. The biosynthetic pathway is primarily launched in chloroplasts and other plastids by a zeaxanthin epoxidase (ZEP) which can catalyze the epoxidation of zeaxanthin (C40), and antheraxanthin (C40) to violaxanthin (C40) (Nambara and Marion-Poll, 2005; Taiz and Zeiger, 2010; Taylor *et al*., 2000). Violaxanthin is converted to 9'-cis-Neoxanthin (C40) which in turn undergoes oxidative cleavage to Xanthoxal (C15) catalysed by the 9-cis-epoxycarotenoid dioxygenase (NCED). NCED is considered as a key enzyme in the ABA biosynthesis pathway (Eckardt, 2002; Milborrow, 2001). Finally, Xanthoxal is then exported to the cytoplasm, where it is converted to ABA through oxidative steps involving the intermediate(s) ABA-aldehyde and/or possibly xanthoxic acid (Taiz and Zeiger, 2010).

ABA perception and signalling pathways (Cutler *et al*., 2010; Raghavendra *et al.,* 2010) have been extensively studied in *Arabidopsis thaliana* and other species using biochemical and molecular genetical approaches (Hasegawa *et al*., 2000; Ishibashi *et al*., 2012; Nishimura *et al*., 2010; Xiong and Zhu, 2003; Zhu, 2002). The long search for the ABA receptor led in 2009 to the identification of pyrabactin resistance 1 (PYR1), a member of the PYR/PYR1-like (PYL)/regulatory component of ABA receptor (RCAR) group of proteins. This novel ABA-binding protein was demonstrated as soluble

ABA receptor by two independent research groups, considered as breakthrough for the understanding of ABA signalling (Ma *et al*., 2009; Park *et al*., 2009). These receptors termed now PYR/PYL/RCAR, represent a family of soluble proteins of about 150–200 amino acids that share a conserved START domain. Crystallographic structure studies revealed that PYR1 is a direct receptor for ABA receptor. The PYR1 undergoes conformational changes when ABA binds to its interior cavity. The ABA-free “open-lid” conformation of PYR1 is converted to a more compact and symmetric closed-lid dimer upon binding to ABA (Nishimura *et al*., 2009).

In the absence of stress, the ABA signal is switched off by the clade A protein phosphatases 2C (PP2C) (mainly HAB1, ABI1, and ABI2), that negatively regulate downstream kinases. However, when in response to environmental or developmental signals, ABA is synthesized and bound to PYR1, this receptor, in turn, binds to PP2Cs inducing there a conformational change resulting in its inhibition, and thus terminating the inhibition of the downstream ABA-activated kinases (OST1/SnRK2.6/SRK2E, SnRK2.2, and SnRK2.3). The released SnRK2 is able to phosphorylate downstream factors, such as the AREB/ABF bZIP-type transcription factors, ion channels (SLAC1, KAT1), and a NADPH oxidase (AtrbohF) (**Fig. 7**) (Hubbard *et al*., 2010; Joshi-Saha *et al*., 2011; Umezawa *et al*., 2010). Till now, more than 100 genes and several secondary messengers such as Ca^{2+}, ROS, NO, cyclic nucleotides, and phospholipids have been identified as downstream targets of these early events in ABA signalling (Cutler *et al*., 2010; Pei *et al*., 2000; Suhita *et al*., 2004).

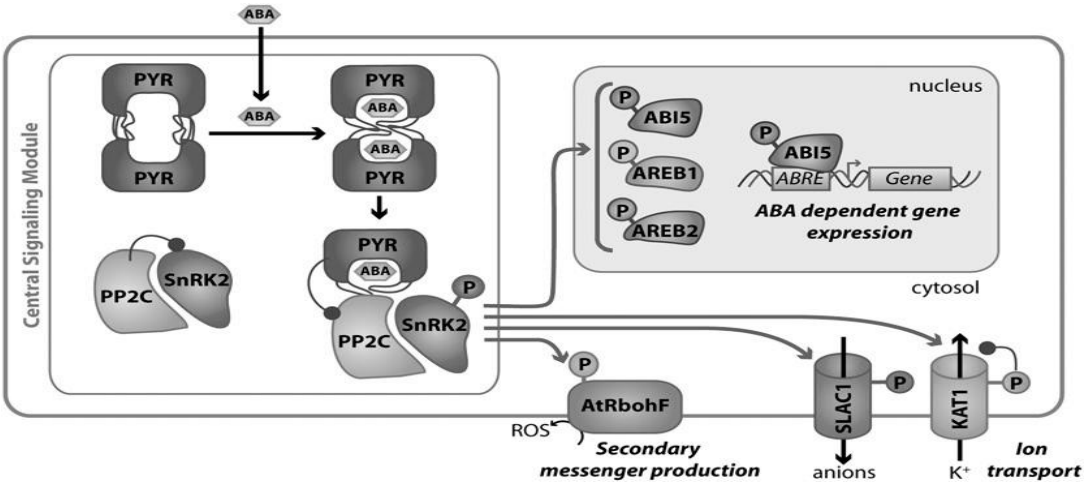


Fig. 7: Mechanisms of early abscisic acid signal transduction, adapted from Hubbard *et al*., 2010.

1.4.8 Ubiquitin–26S proteasome system (UPS)

In eukaryotyes, such as plants, the ubiquitin–26S proteasome system (UPS) is a tightly regulated and highly specific machinery that is devoted to specific proteolysis (Sullivan *et al*., 2003). Plants utilize the UPS to modulate almost all aspects of their biology including growth, development, and stress response (Santner and Estelle, 2010). The crucial roles of UPS are reflected in the number of genes encoding UPS components. A genomic analysis of *Arabidopsis thaliana* showed that, over than 1400 genes (or >5% of the proteome) code for UPS components (Smalle and Vierstra, 2004). Interestingly, several enzymes in the UPS are hormonal receptors. Moreover, the UPS controls the levels of essential downstream signalling proteins in hormonal signal transduction (Santner *et al*., 2009). Thus, the plant UPS not only removes abnormal proteins that arise due to biosynthetic errors or normal proteins with wrong configuration (Vierstra, 2009), but, in addition to this canonical function, controls signal specificity by removal of specific repressors.

As discussed previously, salt-stressed plants increase the levels of different hormones such as JA and ABA activating specific branches of the UPS. The elevated levels of JA-Il promote the proteolytic degradation of JAZ proteins

via UPS releasing MYC2 and probably other transcription factors (Chini *et al.*, 2007; Thines *et al.*, 2007). Conversely, in ABA signalling, the synthesis of the RING E3 ABI3-INTERACTING PROTEIN 2 (AIP2) is increased, which in turn suppresses the ABA transcriptional regulator ABI3, however, the degradation of ABI5, a positive regulator of ABA signalling, is blocked (Vierstra, 2009). As to be expected, it should be possible to modulate stress tolerance through the UPS. In fact, the ubiquitin-specific proteases UBP16, which is a functional ubiquitin-specific protease, was found to increase salt tolerance by stabilization of SERINE HYDROXYMETHYLTRANSFERASE1 (SHM1) that can reduce ROS burst and therefore repress cell death and at the same time positively regulates plasma membrane Na^+/H^+ antiport activity (Zhou *et al*., 2012).

1.5 Adaptive mechanisms to salinity

Life requires that an internal homeostasis is continuously defended against fluctuations of the environment. When this homeostasis is lost in consequence of external challenges, the organism experiences stress. Stress conditions will induce adaptive responses aimed to reestablish homeostasis. Since plants cannot run away, stress adaptation is their only remedy to cope with the adversities of life. The signals culminating in stress adaptation have therefore been of central scientific interest and great agronomical impact (Ingram and Bartels, 1996; Hasegawa *et al*., 2000; Zhu, 2002; Huang *et al*., 2012). A very simple mechanism to respond appropriately to stress would be to use stress-induced imbalance or its cellular consequences (in the following designated by the term *stress damage*) as signal to activate *stress adaptation*. In fact, this is the case for reactive oxygen species (ROS) that play a dual role as toxic byproducts of stress-evoked metabolic imbalance, and as central signals for the adaptation to osmotic stress (Miller *et al*., 2010). It is therefore far from trivial to discriminate events linked with *stress damage* from the events that confer *stress adaptation*. To assign a given event to either *stress damage* or *stress adaptation*, it is necessary to define timing as well as the phenotype observed

upon inactivation or activation of this event.

Salinity-dependent stress damage includes loss of turgescence (leading to growth arrest), membrane disorganisation, metabolic imbalance, formation of ROS, inhibition of photosynthesis, and reduced nutrient acquisition (Hasegawa *et al*., 2000; Zhu, 2002). The cellular response depends also on the timing: it differs for a situation, where salt concentration is increased instantaneously from a situation, where the concentration increases slowly over a longer period (which is the natural situation). This distinction has been termed “salt shock” versus “salt stress” (Shavrukov, 2013) and adds a further level of complexity. However, as in most experiments, in the current study we used the design of a “salt shock” to get a clearer temporal sequence of responses. To achieve stress adaptation, the cell has to restore turgescence against the inverted gradient of water potential, and it has to quell damage-related signaling to escape programmed cell death (PCD). The velocity and amplitude of these adaptive responses will define the degree of salinity tolerance of the respective plant.

Plants have evolved different adaptive strategies in order to survive under hostile conditions. They can respond to salinity challenge in an individual way as cells and in synergistically way as a whole organism. The coping events vary in their strength and level according to the tolerance ability of the plant. At the cellular level, changing in cell wall, cell wall architecture, cell division, endomembrane system, and vacuolization of cells all lead to salt stress tolerance. At the biochemical level, plants alter their metabolism and direct it in order to accommodate salinity. Finally on the molecular level, they could link the perception of a stress signal with their genomic responses by which they fastly respond stress (Parida and Das, 2005; Taiz and Zeiger, 2010). The detailed adaptive mechanisms are discussed below.

1.5.1 Stomatal closure

Stomata (*mouth* in Greek; singular: stoma), are minute aperture structures on

the epidermis of plants, each delineated by two guard cells (Evert and Eichhorn, 2006). They are responsible for the regulation of the exchange of water vapour and CO_2 between the internal plant tissues and the atmosphere. As an immediate response to salt stress, stomata close in order to mitigate ion flux to the shoot (Hasegawa *et al.,* 2000). Meanwhile, considerable efforts have been devoted to identify signalling elements regulating and controlling stomatal responses to different kind of stresses. ABA and MeJA play pivotal roles for the control of stomatal closure under stress conditions. Interestingly, their transduction pathways leading to stomatal closure share overlapping signalling elements (Suhita *et al*., 2004). Under drought condition, several crucial events take place. Firstly, both JAR1 and OST1 function upstream of cytoplasmic alkalinization in the MeJA and ABA-signalling pathway, respectively. Secondly, protein phosphorylation is essential for cytoplasmic alkalinization which leads to ROS production by NAD(P)H oxidase. The plasma membrane receptor kinase, GHR1, that is a critical early component in ABA signalling, mediates ABA- and H_2O_2-regulated stomatal movement through the phosphorylation and activation of S-type anion currents SLAC1 in guard cells (Hua *et al*., 2012). The induction of H_2O_2 activates non-selective cation currents (I_{Ca}), leading to Ca^{2+} influx and elevations of cytoplasmic calcium $[Ca^{2+}]_{cyt}$ causing stomatal closure by increasing the K^+ efflux from the guard cells. Methyl jasmonate (MeJA) requires calcium for the induction of stomatal closure, while ABA functions independently of calcium through the IP3 pathway (Evans, 2003; Pei *et al*., 2000; Suhita *et al*., 2004). Although the stomatal closure is a fast response to drought or salt stress, this closure cannot be maintained for a long period as plants need to fix CO_2 for survival.

1.5.2 Ionic homeostasis

High salinity disrupts the extra- and intracellular ions homeostasis of plant cells. The accumulation of Na^+ in the cytoplasm impairs K^+ acquisition and negatively affects the entire metabolism. The concomitant increase of Ca^{2+}_{cyt} can induce pathological processes, although it might also be used as signal to initiate adaptation. Salt-stressed plants defend ionic homeostasis by

various pumps, ions, and Ca^{2+} sensors (Tuteja, 2007). In the following, the involvement of SOS pathway, NHX1, and other factors in controlling ion homeostasis will be discussed.

1.5.2.1 Na^+ homeostasis

1.5.2.1.1 Na^+ transport

Plants absorb water from the soil, simultaneously translocating salt ions through the endodermal membrane barrier into roots to shoots with the transpirational flux that is required to maintain their water status. Those ions are selectively conducted through plasma membrane channels according to the difference in electrochemical gradient. In addition, ion movement is constrained by gating (the proportion of the time during which the chanells are open or shut) (Yeo, 1998). However, the initial influx of Na^+ from the soil solution into the cortical cytoplasm of plant roots takes place in a passive fashion (Tester and Davenport, 2003). Nonselective cation channels (NSCCs) passively catalyse fluxes of cations through plant membranes including the primary Na^+ influx (Essah *et al*., 2003). *In planta*, NSCCs have been subdivided according to their voltage dependence into three classes (Demidchik and Maathuis, 2007). (1) depolarization-activated NSCCs (DA-NSCCs) primarily catalyze Ca^{2+} conductance and have a role in controlling cytoskeletal organization (Demidchik *et al*., 2002). Moreover, they participate in catalysing K^+ release from root cells under salinity challenge. (2) hyperpolarization-activated NSCCs (HA-NSCCs) seem to have no role for Na^+ fluxes under saline condition. (3) voltage-insensitive NSCCs (VI-NSCCs) play a crucial role in catalysing Na^+ influxes from the soil solution into roots (Demidchik and Maathuis, 2007; Kronzucker and Britto, 2011). Additionally, NSCCs have been classified regarding to their responsiveness to certain ligands and physical stimuli including: (1) cyclic-nucleotide-gated NSCCs (CNGCs), (2) amino-acid-gated NSCCs (AAG-NSCCs), and (3) reactive-oxygen-species-activated NSCCs (ROS-NSCCs). However, these three types of NSCCs all belong into the VI-NSCCs subclass (Kronzucker

and Britto, 2011). The involvement of different K^+ transporters in Na^+ homeostasis has been studied by different research groups and will be discussed below in the section on K^+ homeostasis.

Plants control tightly the salt influx into the xylem stream in order to protect the actively growing cells. However, after penetration of salt into plant, it is preferential moved to older leaves, as a strategy to protect the growing (younger) leaves from harmful effects (**Fig. 8**) (Khelil *et al*., 2007). Thus, during leaf development there must be a sequential mechanism producing discontinuous patterns of salt partitioning (Cheeseman, 1988). When the salt has accumulated to toxic levels, the mature “sink” leaves are simply dropped down (Hasegawa *et al*., 2000).

Fig. 8: Symptoms of sodium and chloride toxicity on grapevine.

1.5.2.1.2 Na^+ exclusion

The ability of plant cells to maintain low cytosolic Na^+ concentrations is crucial for the ability of a plant to grow in high salt concentrations. The highly branched SOS signalling pathway plays a pivotal regulatory role in Na^+ homeostasis in salt-stressed plants. Under saline condition, Ca^{2+}-activated SOS3 interacts with SOS2 resulting in a SOS3/SOS2 signaling complex. The SOS1 (a plasma membrane Na^+/H^+ antiporter) was the first identified target of this SOS3/SOS2 pathway. Upon phosphorylation, SOS1 catalyzes Na^+ efflux across the plasma membrane reducing its harmful effects on cellular metabolism (Harper *et al*., 2004; Munns and Tester, 2008). SOS1 directly

signals to a putative K^+ transporter by-passing SOS2 and SOS3 and therefore was proposed to be necessary for safeguarding K^+ permeability of the plasma membrane during salinity stress (Qi and Spalding, 2004; Shabala *et al*., 2005). Additionally, the SOS1 has a very long tail and is assumed to function as Na^+ sensor (Zhu, 2002). However, role of the SOS pathway for plant salt tolerance seems to be not always only positively as critically reviewed by Kronzucker and Britto, 2011.

1.5.2.1.3 Na^+ compartmentalization

Plant vacuoles comprise some 90% of the cell volume and contribute to osmotic adjustment to maintain water uptake from saline solutions. The vacuole represents the main site of turgor generation since it functions as a depository compartment for the majority of cellular minerals, hormone conjugates, and water. In addition, it accumulates secondary metabolites and proteins involved in plant defence against pathogens and herbivores releasing them in response to attack and cellular damage. Vacuolar sequestration of xenobiotic and toxic components, such as Na^+, helps to reducing the harmful impact in the cytoplasm, where sensitive biochemical processes take place (Isayenkov *et al*., 2010). This strategy of intracellular compartmentalization of Na^+ is not important only for glycophytes that are stressed at 10-100 mM NaCl, but even for halophytes that require roughly 20 to 500 mM NaCl for optimal growth (Hasegawa *et al*., 2000). Flowers *et al*., 1977 reported that Halophyte cytosolic enzymes are neither remarkably salt resistant nor salt requiring, but display the same sensitivity to salt as enzymes from glycophytes (Flowers *et al.*, 1977). Cytosolic concentrations of Na^+ ranging from 10 to 30 and of K^+ above 100 mM have inhibitory effects on most enzymes (Munns and Tester, 2008). Thus, plant cells have to respond to elevated Na^+ concentrations by maintaining low cytosolic Na^+ concentrations through intracellular repartitioning of sodium into the vacuole. This transport of Na^+ into the vacuolar lumen uphill its electrochemical gradient requires energy originating from the proton gradient generated by the vacuolar H^+-ATPase and H^+-PP_iase (Blumwald *et al*, 2000). Therefore,

the capacity of the vacuole to sequester toxic cations is decisive for the ability of a plant cell to adapt to salinity (Darley *et al*., 2000).

The Na^+/H^+ exchanger (NHX) class of transporters, the plant homologue of the yeast Na^+/H^+ exchanger, were originally isolated and identified for their important roles in cellular ion homeostasis under drought and salt stresses (Apse *et al*., 1999; Gaxiola *et al.*, 1999). This class of transporters catalyzes the electroneutral exchange of Na^+ or K^+ with H^+ maintaining intracellular pH and Na^+ and K^+ homeostasis in all eukaryotes (Martinoia *et al*., 2012). *Arabidopsis thaliana* harbours six intracellular members of the NHX class that are divided into two groups, based on sequence homology. Unlike the vacuolar NHX1 to NHX4 which are localized in the tonoplast membrane, NHX5 and NHX6 reside in the endosome (Bassil *et al*., 2011). *AtNHX1* and *AtNHX2* transcript levels are not upregulated in response to NaCl in the ABA-free *aba2-1* but maintain their responsiveness to exogenous ABA indicating that the osmotic responsiveness of these genes is ABA-dependent (Yokoi *et al*., 2002). Data from the Arabidopsis *sos2* mutant showed that the tonoplast Na^+/H^+ exchanger in Arabidopsis is regulated by SOS2 (Qiu *et al*., 2004). NHX1, particularly, in concert with other members of this family of transporters, plays crucial roles in pH regulation and K^+ homeostasis, regulating processes from vesicle trafficking and cell expansion to plant development (Rodríguez-Rosales *et al.*, 2008). Overexpression of the vacuolar *AtNHX1* antiporter in Arabidopsis, canola or tomato plants increased the salt tolerance up to 200 mM NaCl (Apse *et al*., 1999; Blumwald, 2000; Chinnusamy *et al*., 2006).

1.5.2.2 Ca^{2+} homeostasis

Controlling Ca^{2+} levels is not only a crucial task under salinity but also relevant for all aspects of developmental and stress signaling. Therefore, plants have adopted different strategies to increase Ca^{2+}_{cyt} and to restore the initial low level after signalling has been completed. The balance between the ‘on’ reactions, by which the Ca^{2+}_{cyt} is elevated, and the ‘off’ reactions, through

which the Ca^{2} signal is damped by buffering, pumping and exchanging machineries, determines the intracellular Ca^{2+} levels at any time point (Berridge *et al*., 2003). The central plant vacuoles (equivalent to lysosomes of animal cells with regard to their degradation and autophagy functions) represent the major Ca^{2+} store in a mature plant cell. However, the Ca^{2+} sequestration into the vacuole is an energy-consuming process as the Ca^{2+}_{cyt} is lower than vacuolar Ca^{2+} (Pittman, 2011). High-capacity vacuolar Ca^{2+} exchangers (CAXs) genes play crucial roles in ion homeostasis and signal transduction (Hirschi, 2001), and are complemented by autoinhibited Ca^{2+}-ATPase pumps of the P_{IIB}-type (ACAs) on the tonoplast, similar to mammalian CaM-stimulated ATPases, are released from autoinhibition by binding of Ca^{2+}/CaM to the N-terminal autoinhibitory domain such that Ca^{2+}_{cyt} is reduced by a feedback regulation (Peiter, 2011).

1.5.3 Osmotic adjustment

Many plant species, especially those that are salt-tolerant, have the capacity to alter the intracellular osmotic potential when they experience osmotic stress, which could be imposed either directly (e.g. drought, salt and freeze-induced dehydration) or indirectly (e.g. chilling and hypoxia) (Nuccio *et al*., 1999). This active change of osmotic potential gave the rise to the widely accepted concept of osmotic adjustment (Bressan *et al*., 2008). For this purpose, plants mainly accumulate metabolically inert compounds in the cytoplasm as part of an overall adaptive mechanism to maintain both turgor and the driving gradient for water uptake by raising the cellular osmotic pressure. Additionally, these osmolytes represent a sink for unutilized products of primary metabolism (Bressan *et al*., 2008). They are mainly characterized by their low molecular-weight, highly solubility, and nontoxicity even at high concentrations. As organic osmolytes quaternary ammonium compounds (QACs), and tertiary sulfonium compounds (TSCs) have been observed in many organisms including bacteria, cyanobacteria, algae, animals, and higher plants (Rhodes and Hanson, 1993). They are advantageous, because they do not interfere with structure and function of

other macromolecules including proteins, even at comparatively high concentrations (Burg and Ferraris, 2008). The synthesis of compatible solutes consumes energy causing a reduction of plant growth, but allows the plant to survive under non-favorable conditions (Munns and Tester, 2008). Halophytes accumulate relatively high concentrations of osmolytes, whereas glycophytes achieve only low osmolyte concentrations. These osmolytes stabilize the tertiary structure of proteins, and scavenge stress-induced ROS species, thus providing osmoprotection(Türkan and Demiral, 2009).

A range of molecules such as sugars (e.g. fructose, sucrose and glucose), sugar alcohols (e.g. mannitol, glycerol and methylated inositols), complex sugars (trehalose, raffinose and fructans), amino acids (e.g. proline), methylated proline-related compounds (e.g. methyl-proline), betaines (e.g. glycinebetaine), and methylated sulphonio compounds (e.g. dimethylsulphoniopropionate, DMSP) have been proposed to fulfil osmoprotective functions in halophytes (Flowers and Colmer, 2008; Türkan and Demiral, 2009). Glycine betaine (GB), a fully N-methyl-substituted derivative of glycine, is considered as one of the major osmoprotectant osmolytes. Found in a wide variety of microoorganisms, higher plants, and animals, GB effectively stabilizes the quaternary structures of enzymes and folded protein structures, and at the same time preserves the thylakoid and plasma membrane integrity under stressed conditions (Chen and Murata, 2008; Rhodes and Hanson, 1993). In higher plants, GB is synthesized in chloroplasts from choline by the action of choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). Transgenic plants engineered to synthesize more GB were shown to exhibit improved tolerance to different forms of abiotic stress for different model plants such as *Arabidopsis thaliana*, rice, and rapeseed. Conversely, when GB was exogenously applied, low- or non-accumulating plants acquired stress resistance as shown for rice and maize (Chen and Murata, 2002; Tuteja, 2007).

There is evidence for a role of GB in signal transduction and ion homeostasis (Ashraf and Foolad, 2007). Transcription profiling in turfgrass and

Arabidopsis thaliana (John, 2002) revealed strong regulation of MAP kinase transcripts by GB, which is expected to modulate the SOS pathway. Additional candidates for downstream genes contributing to osmotic tolerance included a lipoxygenase, monodehydroascorbate reductase, osmotin, and several candidate 'master switch' genes including a WRKY DNA binding protein.

The amino acid proline (Pro) is a further major osmoticum that is accumulated under stress. Pro is involved in ROS scavenging, stabilisation of subcellular structures (membranes and proteins), and the buffering of cellular redox potential under stress conditions. Although, in plants, Pro can be synthesized from two different precursors, glutamate and ornithine (Verbruggen and Hermans, 2008), the glutamate pathway catalyzed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) represents the main pathway for stress-induced Pro accumulation, whereas the pathway catalyzed by ornithine δ-aminotransferase (OAT) and Δ^1-pyrroline-5-carboxylate synthetase (P5CR) is of minor relevance here (Stines *et al*., 1999). From the growth phenotypes of the *Arabidopsis thaliana* mutants *p5cs1* (mutant deficient in stress-induced Pro synthesis), *aba2-1* (ABA-deficient mutant), and *p5cs1/aba2-1* (double mutants) it can be inferred that Pro metabolism is required for ABA-mediated growth protection at low water potential (Sharma *et al*., 2011).

1.5.4 ROS scavenging

In addition to restoring osmotic gradients and ionic balance, adaptation to salt stress requires that ROS homeostasis is restored. Under normal growth conditions, ROS are continuously produced in plants as byproducts of aerobic metabolism such as photosynthesis, photorespiration and respiration (Abogadallah, 2010). However, ROS can be induced by a wide varity of stresses such as drought, salinity, pathogens and ozone. Salinity- or drought-stressed plants close stomata which in turn limits water loss (favourable effect) and the influx of CO_2 (unfavourable effect) (Hsu and Kao,

2003). Consequently, carbon reduction and NADPH (an electron acceptor in photosynthesis) consumption by the Calvin cycle are decreased, resulting in electron leakage from photosystem 1 to the alternative electron acceptor, O_2, and initiating the Mehler reaction (Türkan and Demiral, 2009). The resultant $O_2^{\bullet-}$ is considered as earliest ROS that consequently intiates other ROS, including the most noxious $OH^{\bullet}$. Additionally, the peroxisomal glycolate oxidase during photorespiration, plasma-membrane located NADPH oxidases, amine oxidases and cell-wall-bound peroxidases are important sources for ROS that are active to a certain extent even under normal conditions, but are activated in response to stress (Mittler, 2002).

Plants must strictly maintain ROS homeostasis to mitigate the toxicity of ROS throughout their lifecycle. Therefore, plants have employed different scavenging machineries that tightly control the ROS levels, both enzymatic and nonenzymatic (Apel and Hirt, 2004). Plant enzymatic antioxidant mechanisms include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), guaicol peroxidase (GOPX), and glutathione-S- transferase (GST). The metalloenzyme SOD is the most effective intracellular enzymatic antioxidant and acts by dismutating superoxide to H_2O_2 which in turn can then be detoxified by APX, GPX, and CAT (Apel and Hirt, 2004). The nonenzymatic antioxidants comprise ascorbic acid (ASH), glutathione (GSH), phenolic compounds, alkaloids, non-proteinogenic amino acids and α-tocopherols) (Gill and Tuteja, 2010).

1.6 Research on salinity tolerance of *Vitis*

1.6.1 Why grapevine as model?

Arabidopsis thaliana is frequently used as a genetic model in plant research for many reasons. First, it is a representative for the large group of dicotyledonous plants harbouring most of our crop plants. Second, extensive

genetic and genomic methods and resources are available for this model species that, in addition is easy to be transformed. Third, its genome represents one of the smallest known genomes among flowering plants. Forth, its small size and simple growth requirements make it easy to grow and handle in laboratory conditions. Fifth, the plant is a self-fertilizing diploid that produces thousands of seeds from a single individual with relatively short life cycle of ~8 weeks (Somerville and Koornneef, 2002). Based on these advantages, Arabidopsis has bee extensively studied as modelto dissect salt-stress pathways (Ma *et al*., 2006), including several genomic and proteomic analyses of salinity tolerance in sets of corresponding mutants (Jiang *et al*., 2007; Zhu, 2000). Sodium influx and accumulation in response to saline conditions were measured in the Arabidopsis ecotype C 24, also after treatment with pharmacological agents such as Gd^{3+} and Diethylepyrocarbonate (DEPC) (Essah *et al*., 2003). However, *Arabidopsis thaliana* is salt sensitive and does not provide a good model to study tolerance to salinity tolerance or Na^{+}. Moreover, the agronomic value of Arabidopsis itself is limited.

The decision to use grapevine as model in the current thesis, is mainly motivated by application.Grapevine is the economically most important fruit species worldwide and has been linked to agricultural and religious activities since the earliest writings and chronicles (Vivier and Pretorius, 2002). It has been domesticated more than 7000 years ago, and it has shaped human civilisation in the Near East and the Mediterranean regions (McGovern, 2003). In 2010, the worldwide grapevine areas comprised ~7100000 hectares of this intensely cultivated crop that produced 67000000 tonnes of fruits (FAOSTAT data, 2010. http://faostat.fao.org). In addition to its traditional, religious, and economic value, grapevine has been intensively studied with respect to functional genetics and, after *Arabidopsis* and rice, was the third crop, where a genome project had been completed. It has developed into the prime plant model for perennial crops and therefore shifted into the focus of applied plant research.

Specific for the study of salinity stress, grapevines are considered as moderately salt-sensitive and highly drought-tolerant crops: A soil EC of 2.5 $ds.m^{-1}$ (corresponding to 25 mM NaCl) results in both growth and crop reduction, more than 6.7 $ds.m^{-1}$ (corresponding to 67 mM NaCl) are required to cause vine death (Tattersall *et al*., 2007). Interestingly, chloride appears to be the more toxic component of NaCl in *Vitis* as is the case for other woody species, while sodium is the most dangerous ion for most annual plant species (Kronzucker and Britto, 2010), emphasizing the need for additional plant models outside *Arabidopsis thaliana*. In addition, grapevine is able to accumulate specific phytoalexins such as resveratrol at different developmental stages and upon pathogen infection (Derckel *et al*., 1999; Jeandet *et al*., 2002). These distinctive characteristics along with the availability of *Vitis* genome (Jaillon *et al.*, 2007) render grapevine an excellent model to study salinity signalling and salinity tolerance determinants.

1.6.2 Grapevine stilbenes

Stilbene phytoalexins are a class of low-molecular-weight plant secondary metabolites that are generated *de novo* in response to biotic (*e.g.* fungal infection) and abiotic stress factors (*e.g.* UV-irradiation and wounding), as well as during different developmental stages (Derckel *et al*., 1999; Petit *et al.*, 2009). Stilbens are a small family of phenylpropanoids produced in a limited number of unrelated plant species, such as grapevine (*Vitis vinifera* L and and *Vitis labrusca* L), peanut (*Arachis hypogaea*), Japanese knotweed, sorghum (*Sorghum bicolor*), and several species of *Pinus* and *Picea* (Parage *et al*., 2012). Grapevine stilbenes (resveratrol and viniferins) are constitutively present in the woody organs (roots, canes, stems). However, they are only produced upon induction in leaves and fruits (Bavaresco *et al*., 1999). Resveratrol (3, 5, 4'-trihydroxystilbene), in particular, is considered as the major component of the grapevine response to stress. It mainly accumulates in the skin of grape berries although it could be detected in other organs as well such as stem, axillary bud, shoot tip, petiole, root and leaf of grape plants (Wang *et al*., 2010).

Interestingly, in addition to its roles in plants, resveratrol was also found to have several benefits for animal and human health. Scientists were puzzled for many years about the fact that French people who consume high calory diets that clog the arteries, yet still maintain good health, a phenomenon termed "French paradox". The mortality rate from coronary heart disease is much lower in France than in other industrialised countries such as the USA and UK although the concentrations of serum cholesterol are similar to those of the USA and UK (Renaud and de Lorgeril, 1992). This discrepancy was explained by the daily glass of red wine accompanying the meals in France. Resveratrol performs multifunctional tasks. It acts as antioxidant, antimutagen, anti-inflammatory, inhibits cyclooxygenase and hydroperoxidase functions (antipromotion activity), and has antiprogression activity in human promyelocytic leukemia (Jang *et al*., 1997). Moreover, it works as an activator of Sir2, which encodes an NAD-dependent deacetylase proposed to underlie the beneficial effects of caloric restriction. Therefore, resveratrol has been shown to extend the lifespan of diverse species including yeast, Drosophila, and mouse (Guarente and Picard, 2005). The presence of resveratrol shifts the physiology of mice feeding high-calory diets towards that of mice on a standard diet, modulates known longevity pathways, and improves health, as indicated by a variety of measures including survival, motor function, insulin sensitivity, organ pathology, peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) activity, and mitochondrial number (Baur *et al*., 2006). However, in plant cells, resveratrol acts as signal triggering programmed cell death which makes sense in the context of a pathogen attack (Chang *et al*., 2011), but not in the context of salt stress. This implies that efficient salt adaptation must circumvent or quell the induction of defense-related responses that participate in PCD.

1.6.2.1 Biosynthesis and metabolism of stilbenes

Stilbenes are formed via the phenylpropanoid/polymalonate pathway. The phenylalanine ammonia-lyase (PAL) catalyses the first step of the phenylpropanoid pathway by converting the phenylalanine to cinnammonic

acid which subsequently produces ρ-coumaroyl acid via cinnamoate-4-hydroxylase (C4H) and the later introduce ρ-coumaroyl-CoA and three units of malonyl-CoA by the action of 4-coumarate: CoA ligase (**Fig. 9**). All higher plants seem to be able to conduct these three enzymatic steps, but only few plant species, such as grapevines, can produce stilbenes (Chong *et al*., 2009). The last steps of the phenylpropanoid pathway are catalyzed by Chalcone (CHS) and stilbene (STS) synthases that are related plant-specific polyketide synthases and are considered as key enzymes in the biosynthesis of flavonoids and of stilbene phytoalexins, respectively. STS is proposed to have evolved from CHS by a gene duplication followed by mutation of one amino acid in the active center. The advantage gained by this new enzymatic function favored the selection of plants with improved STS activity (Parage *et al*., 2012; Tropf *et al*., 1994). Resveratrol, after its synthesis, undergoes several modifications such as glycosylation to produce piceid (its inactive form) by the resveratrol glucosyltransferase (Hall and De Luca, 2007), but also methylation to the toxic pterostilbene, or oxidation and dimerization to the highly toxic δ and ε-viniferins (Morales *et al*., 1997, **Fig. 9**).

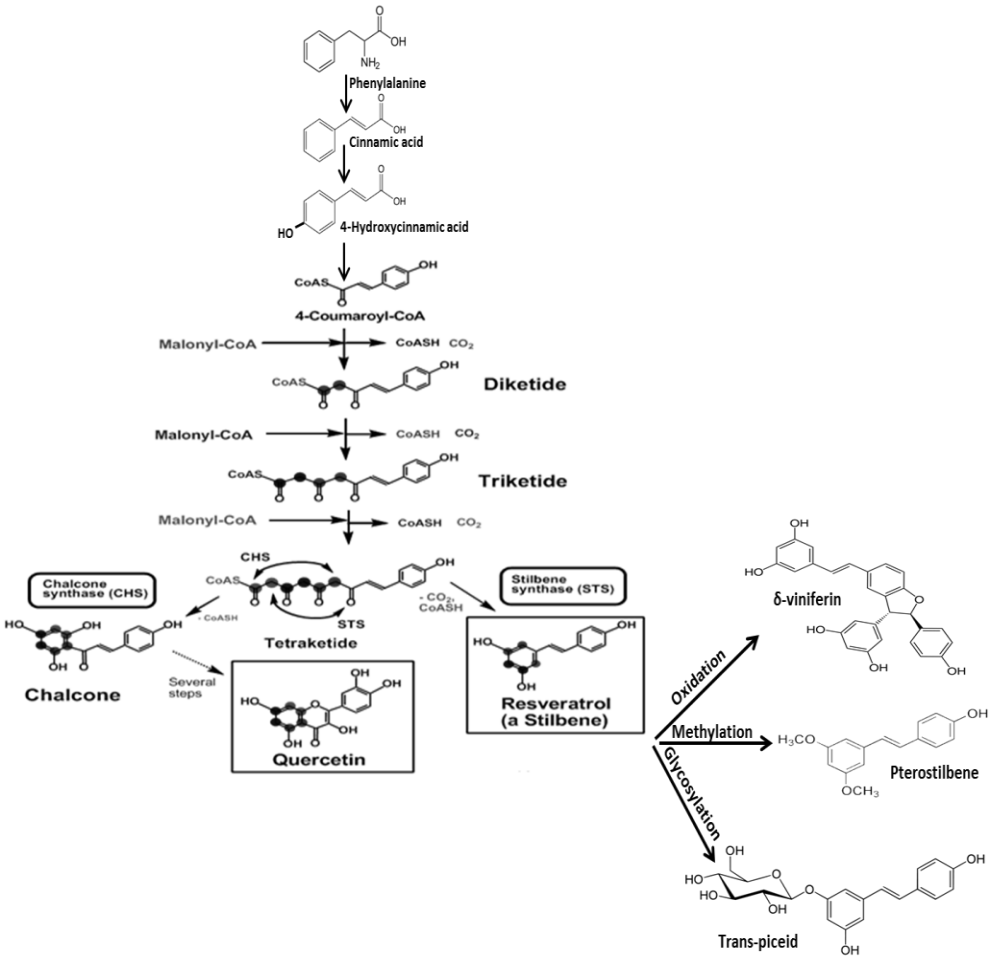


Fig. 10: Biosynthesis and metabolic pathway of grapevine stilbenes. PAL, Phe ammonium lyase; C4H, cinnamoate 4-hydroxylase; 4CL, 4-hydroxycinnamoate CoA ligase; CHS, chalcone synthase; STS, stilbene synthase. This pathway is adopted from Schröder Group website; http://www.biologie.uni-freiburg.de/data/bio2/schroeder/Resveratrol.html, with some modifications.

1.7 Scopes of this study

The elucidation of mechanisms by which plants sense, signal, and respond to salinity stress is of great interest to plant biologists. For instance, the phytohormones ABA and Jasmonates (JAs) play a central role in plant adaptation to stress. The role of ABA for osmotic adaptation has long been recognized and intensively studied. For example, ABA increases salinity tolerance by activating stress-responsive genes that encode enzymes for the biosynthesis of osmolytes (e.g. betaine), or protective proteins, such as dehydrins, and LEA-like proteins (Gao *et al*., 2004; Hasegawa *et al*., 2000).

However, for other hormones that accumulate in response of salinity, it is not clear, whether their increase is adaptive or simply a manifestation of *stress damage*: JAs, for instance have been shown to be induced by osmotic stress (see for instance Creelman and Mullet, 1995; Lehmann *et al*., 1995), and a correlation between salt tolerance and the steady-state levels of JA has been drawn for two tomato cultivars (Pedranzani *et al*., 2003). However, when this JA induction was investigated in a more quantitative manner in salt-stressed rice roots, JA was found to be induced much later and only modestly as compared to ABA. Only for very high concentrations of salt close to lethality, the induction of JA became dominant (Moons *et al*., 1997). Moreover, JA antagonized ABA with respect to the induction of adaptive genes. In our previous work, we have analyzed the role of JA for the induction of a osmoprotective PR10 protein (RSOsPR10) in the same system, rice roots (Takeuchi *et al*., 2011), and we could show that the RSOsPR10 transcript is also activated, in a manner similar to the wild type, in a rice mutant that cannot synthetize any JA. Thus, although strong activation of the JA pathway is accepted as key event of plant defense (Browse, 2009) required to induce typical defense proteins, such as proteinase inhibitors or enzymes involved in phytoalexin synthesis (Wasternack, 2007), adaptive responses to salt stress seem to be accompanied by a more constrained induction of the JA pathway. In fact, as pointed out in more details above, there exists a multimeric transcriptional corepression complex machinery to suppress JA-signaling: In *Arabidopsis thaliana*, 12 JAZ/TIFY proteins, the corepressor TOPLESS (TPL), TPL-related proteins (TPRs), and a Novel Interactor of JAZ (NINJA) participate in this machinery (Chini *et al*. 2007; Thines *et al*., 2007; Pauwels *et al*., 2010) indicating that JA signaling must be tightly controlled to avoid hazardous side effects. This complexity is further amplified by ramifications of the pathway into several bioactive derivatives of JA: Conjugation with isoleucine by the JAR1 (Jasmonate-Resistant 1) enzyme generates (+)-7-iso-Jasmonoyl-l-isoleucine (JA-Il) as endogenous JA-species with the highest bioactivity (Staswick and Tiryaki, 2004, Staswick, 2008, Fonseca *et al.*, 2009). The role of JA-Il, to our knowledge, so far has not been investigated in the context of salinity stress.

The overview in the introduction, which is far from complete, may suffice to demonstrate that adaptation to salinity is not provided by one single “master-switch”, but must have evolved from tuning the cross-talk of different regulatory circuits. To address these circuits, we decided to make use of natural biodiversity in a system, where osmotic adaptation can be studied in contrast to defense-related signaling. We employed two cell lines from two closely related grapevine species differing in osmotic tolerance (Ismail *et al*., 2012): *Vitis rupestris* inhabits rocky, sunny slopes, and therefore has evolved a considerable osmotic tolerance. In contrast, *Vitis riparia* occurs in alluvial woods and performs poorly under osmotic stress. During previous studies we could show that different genotypes of grapevine cell lines accumulate different levels and types of ROS-scavenging stilbenes in a defense context (Chang *et al*., 2011), and we have mapped these differences with respect to defense-related signaling (Chang and Nick, 2012). In addition to these experimental prerequisites, *Vitis* was chosen for its agronomical relevance. Grapevine is the economically most important fruit species worldwide, and since it is preferentially cultivated in semiarid regions, osmotic tolerance is a central issue. A conceptual problem is the fact that the same general players (such as calcium, protons, oxidative burst, jasmonate signalling) seem to convey different responses, depending on the *context*. Central questions were:

1. How can plant cells activate different (appropriate) outputs, although the input is of identical or similar in molecular quality?

2. What is the importance of signal signatures?

3. What is the role of NSCCs, where they function, and how they affect the plant response to stress?

4. How can the signatures of Ca^{2+}, protons and ROS shape the plant reaction under stress?

5. What are the precise roles of JA and its isoleucine conjugate (JA-Il, to our knowledge we show for the first time a response of this conjugate

in a stress context) in salinity adaptation?

6. How do stress signals, Ca^{2+}, H^{+} interact with phytohormones (JA, JA-Il, ABA, IAA, and SA)?

7. What is the role of grapevine phytoalexins stilbenes?

2 Materials and methods

2.1 Cell culture and chemical treatments

2.1.1 *Vitis* cell culture

Suspension cell cultures of *Vitis rupestris* and *Vitis riparia* generated from leaves (Seibicke, 2002) were cultivated in liquid medium containing 4.3 $g \cdot l^{-1}$ Murashige-Skoog salts (Duchefa, Haarlem, The Netherlands), 30 $g \cdot l^{-1}$ sucrose, 200 $mg \cdot l^{-1}$ KH_2PO_4, 100 $mg \cdot l^{-1}$ inositol, 1 $mg \cdot l^{-1}$ thiamine, and 0.2 $mg \cdot l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8. The microelements of the Murashige-Skoog medium are composed of 0.025 $mg \cdot l^{-1}$ $CoCl_2.6H_2O$, 0.025 $mg \cdot l^{-1}$ $CuSO_4.5H_2O$, 36.70 $mg \cdot l^{-1}$ FeNaEDTA, 6.20 $mg \cdot l^{-1}$ H_3BO_3, 0.83 $mg \cdot l^{-1}$ Kl, 16.90 $mg \cdot l^{-1}$ $MnSO_4.H_2O$, 0.25 $mg \cdot l^{-1}$ $NaMoO_4.2H2O$, and 8.60 $mg \cdot l^{-1}$ $ZnSO_4.7H_2O$, while the macroelements are 332.02 $mg \cdot l^{-1}$ $CaCl_2$, 170.00 $mg \cdot l^{-1}$ KH_2PO_4, 1900.00 $mg \cdot l^{-1}$ KNO_3, 180.54 $mg \cdot l^{-1}$ $MgSO_4$, 1650.00 $mg \cdot l^{-1}$ NH_4NO_3. Cells were subcultured weekly; 10 ml of stationary cells were inoculated into 30 ml of fresh medium in 100 ml Erlenmeyer flasks. The cell suspensions were incubated at 25°C in the dark on an orbital shaker (KS250 basic, IKA Labortechnik, Staufen, Germany) at 150 rpm.

2.1.2 Chemical treatments

To induce cellular responses, suspension cell cultures of *V. rupestris* and *Vitis riparia* were treated at day 5 after subcultivation with different concentrations of either sodium chloride (NaCl, diluted from a 5-M stock solution),, (±)-jasmonic acid (JA) (Sigma-Aldrich, Germany) diluted from an ethanolic stock solution, acetylsalicylic acid (aspirin) (Sigma-Aldrich, Germany) dissolved from a stock in dimethyl sulfoxide (DMSO), or an aqueous solution of the inhibitor 1-phenylpyrazolidinone (phenidone) containing 0.1% of polyoxy-ethylene-orbitan monolaurate (Tween 20) (both obtained from

Sigma-Aldrich, Germany). Gadolinium chloride ($GdCl_3$) (Sigma-Aldrich, Deisenhofen, Germany) was used as inhibitor of mechanosensitive calcium channels and diluted with H_2O to a 100 and 1000 mM stock solution. Negative controls contained the corresponding concentrations of solvent without the active ingredient. The fluorescent dye dihydroethidium (DHE), Sigma-Aldrich, Germany for detection of ROS was dissolved in DMSO in a 30-mM stock solution, covered with aluminum foil and stored in -20°C.

2.2 Measurement of packed cell volume (PCV)

Packed cell volume (PCV) as a measure of growth was recorded in response to different concentrations of NaCl (50, 85, 155 mM) or 0.75 mM $GdCl_3$ added directly at subculture. Equal aliquots of stationary cells were sub-cultivated in fresh medium in presence of different concentrations of NaCl, or equal volumes of the H_2O. In some experiments, 30 µM JA or 30 µM ABA, or 1 mM $CaCl_2$ was added alone or directly before 50 mM NaCl was applied. In addition, 0.75 mM $GdCl_3$ was applied to the cells with the same procedure.

The relative increment in PCV, $(\Delta V_t/V_t)/(\Delta t)$, was used as measure of the growth response. Three biological replicates of each cultivar were daily collected into 15 ml Falcon tubes, sedimented overnight at 4 °C, and then the cell growth rate was measured using the volume grading of the tube. Time courses of growth inhibition were followed by comparing the cell growth rate in presence of 50, 85, or 155 mM NaCl as compared to the solvent control. Additionally, growth rate of cells under 50 mM NaCl alone or in the presence of , 30 µM JA or 30 µM ABA, or 1 mM $CaCl_2$ was compared to application of 30 µM JA or 30 µM ABA, or 1 mM $CaCl_2$ alone and the water-treated samples. Moreover, the impact of GdC_{l3} on growth rate was monitored. Each data point represents at least the mean from 3 measurements collected each day after subcultivation over a week.

2.3 Determination of cell viability

To determine cell viability, cells were treated by NaCl, $CaCl_2$, JA, ABA, $GdCl_3$,

or water, as a control, directly after the weekly sub-cultivation for 8 days.

To differentiate between short and long term adaptation, and to test whether NaCl or with another treatments could induce cell death, cells from *V. rupestris* and *V. riparia* were stained with Evans Blue (Gaff and Okong'O-Ogola, 1971). Cells were transferred into a custom-made staining chamber (Nick *et al*., 2000) to remove the medium, and then incubated with 2.5 % Evans Blue for 3-5 min. After washing three times with distilled water, cells were mounted on a slide and observed under a light microscope (Zeiss-Axioskop 2 FS, DIC illumination, 20 × objective). Due to the breakdown of the plasma membrane, Evans Blue is capable of penetrating into dead cells, resulting in a blue staining of the cell interior. Frequency of cell death was calculated as ratio of the number of dead cells over the total number of scored cells. For each time point, 500 cells were scored in three dependent experiments.

2.4 Measurement of cellular Na^+- and Ca^{2+} content

Cells were treated with 155 mM NaCl at day 5 after subcultivation and incubated on a shaker for 2, 5, 10, 15, 30 min, 1, 2, 3, and 6 h at 150 rpm. At each time point, the medium components were three-time washed off with iso-osmotic mannitol solution using a Büchner funnel under vacuum (Babourina *et al*., 2000), and then dried at 80°C overnight. After determining dry weight, cells were digested according to Ippolito and Barbarick (2000) with minor modifications as follows: Dry cells of each biological replicate were transferred into digestion tubes (Gerhardt, UK), supplemented with 5 ml of concentrated nitric acid (HNO_3), and then incubated for at least 24 h at room temperature while vortexing at 6 and 24 h. Samples were placed on a water bath at 105°C for 2 h. After cooling, the final volume of each sample was adjusted to 10 ml with distilled water and vortexed. Na^+ and Ca^{2+} contents were measured by flame atomic absorption spectrometry (AAnalyst200, Perkin Elmer) in an air-acytelene flame (Institute of Mineralogy and Geochemistry, Karlsruhe Institute of Technology). Blank samples were

prepared by adding 5 ml concentrated nitric acid on an empty digestion vessel and processed as described above. Additionally, 155 mM NaCl was applied to the cells after pretreatment with 0.75 mM $GdCl_3$ for 2 min followed by the same procedure. In a third set of experiments, 1 mM $CaCl_2$ were added alone or directly prior to salt. Concentrations were calculated with reference to dry weight from three independent biological replicates.

2.5 Measurement and quantitative analysis of extracellular pH

Apoplastic alkalinization was followed by a pH meter (Schott handylab, pH 12) connected to a pH electrode (Mettler Toledo, LoT403-M8-S7/120), and recorded by a paperless recorder (VR06; MF Instuments GmbH, Albstadt-Truchtelfingen, Germany) at 1-s intervals. Before induction, 2 ml of suspension cells (4 d after subcultivation) were pre-adapted on an orbital shaker for ~90 min, until the pH was stable. To test the effect of salt on extracellular pH, cells were treated with 155 mM NaCl for 1 h. To block the induction of apoplastic pH, cells were pretreated with different concentration of $GdCl_3$ or water for 2 min before the addition of salt.

The pH data were exported to Microsoft Office Excel by the data-acquisition software Observer II_V2.35 (MF Instruments GmbH). The data were fitted based on a Michaelis-Menten equation with T_{pH50} as V_{max}, EC_{25} as K_m, and the concentration of NaCl as [S]. T_{pH50} was the time required to reach 50% of the maximal pH response. Consequently, the equation yielded K_m as an estimate for the concentration causing 25% of the maximal response (EC_{25}).

2.6 Detection of ROS species

ROS were detected under salt stress using dihydroethidium (DHE) according to Tarpey *et al.*, 2004. DHE detects essentially superoxide radicals ($O_2^{\bullet -}$). Shortly, 1 ml of suspension cells was stabilised for at least 1 h in the culture

shaker, and then 30 µM of DHE were added. Cells were incubated with the dye for 3 to 7 minutes in a dark chamber, on an orbital shaker at room temperature. They were washed 3 times in MS medium in a dark chamber, on an orbital shaker at room temperature. 155 mM NaCl were added immediately before observing cells under the fluorescent microscope (AxioImager Z.1, Zeiss, Jena, Germany) equipped with an ApoTome microscope slider through the filter set 43 HE (excitation at 550 nm, beamsplitter at 570 nm, and emission at 605 nm) over up to 6 hours.

2.7 Cloning and sequence analysis of *JAZ/TIFY* genes

Three putative full-length cDNA clones coding for the grapevine homologues of *AtJAZ1, 2*, and *3* (*TIFY10a*, *10b*, and *6b* respectively) were isolated from *V. rupestris* using full-length primers (**Table 2**). A high-fidelity PCR system (PhusionTM DNA Polymerase, NEB) was used with the following PCR parameters: 30 s template initial denaturation at 98°C, 10 s template denaturation at 98°C, 15 s primer annealing at 63, 64.1, 64.1°C respectively, and 40 s primer extension at 72°C for 35 cycles, with a final 5 min extension step at 72°C. The isolated sequences were compared with database sequence using the BLAST program (Altschul *et al*., 1997). The three putative grapevine *JAZ/TIFY* homolouges were inserted into the pJet1.2 vector (CloneJET™ PCR Cloning Kit, Fermentas) and transformed into competent *E. coli* DH5α according to the protocol of the manufacturer yielding pJet1.2 *JAZ1/TIFY10a*, *JAZ2/TIFY10b*, or *JAZ3/TIFY6b*, respectively. Upon isolation using the Roti®-Prep Plasmid MINI (Carl Roth, Karlsruhe, Germany) kit according to the protocol of the producer, the three inserts were sequenced by a commercial provider (GATC, Konstanz, Germany) and submitted to Genebank under the accessions JF900329 (*VrJAZ1/TIFY10a*), JF900330 (*VrJAZ2/TIFY10b*), and JF900331 (*VrJAZ3/TIFY6b*). Predicted

protein sequences were aligned and analysed using ClustalX (Jeanmougin *et al*., 1998), and GeneDoc (Nicholas and Nicholas, 1997), and the tree was visualized with the Treeview program (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

2.8 Quantification of gene expression

Aliquots (1.5 ml) of cells collected at day 5 after subcultivation were treated with different concentrations of NaCl (0-200 mM) for 1, 3 and 6 hour, sedimented by low-speed centrifugation (3000 rpm; 1 min), and shock-frozen in liquid nitrogen. Samples were homogenised with steel beads (Tissue Lyser, Qiagen/Retsch, Germany), and total RNA was extracted using the innuPREP Plant RNA Kit (analytikjena, Jena, Germany) following the protocol of the producer. The extracted RNA was treated with a DNA-free DNase1 (Qiagen, Hildesheim, Germany) to remove potential contamination by genomic DNA. The mRNA was transcribed into cDNA using the M-MuLV RTase cDNA Synthesis Kit (New England BioLabs; Frankfurt am Main, Germany) according to the instructions of the manufacturer.

To study gene expression by RT-PCR, transcripts were amplified by PCR primers (Table 2) with 30 cycles of 1 min denaturation at 94°C, 30 s annealing at 60°C except with *MYC2*, 63°C, and 1 min synthesis at 72°C. The PCR products were separated by conventional agarose gel electrophoresis after visualisation with SybrSafe (Invitrogen, Karlsruhe, Germany). Images of the gels were recorded on a MITSUBISHI P91D screen (Invitrogen) using a digital image acquisition system (SafeImage, Intas, Germany). The bands of the amplificons were quantified using the Image J software (http://rsbweb.nih.gov/ij/) and normalised relative to elongation factor 1α as internal standard. The results were plotted as fold increase of transcript abundance as compared with the untreated control. For the time-course, cells were treated with 155 mM NaCl for 1, 3, and 6 h, and then RNA was

extracted. *JAZ1/TIFY10a*, *COI1*, *MYC2*, *NHX1*, *STS*, and *RS* expression was measured by RT-PCR at each time point.

To test the effect of JA on the induction of the three putative *JAZ/TIFY* genes, cells were pre-treated with 0, 10, 20, 30, 40, and 50 µM JA for 1 h or corresponding concentrations of EtOH as a solvent control . Alternatively, 1 mM aspirin (Peña-Cortès *et al*., 1993) was added 6 h prior to addition of NaCl, and cells were harvested 1 h later. As solvent control, corresponding concentrations of DMSO were added instead of aspirin. In a third set of experiments, cells were pretreated with 2 mM of phenidone for 30 min (Bruinsma *et al*., 2010) prior to addition of 155 mM NaCl and then harvested 1, 3 and 6 h later. As a control, cells were pretreated with either 0.1% Tween 20 or phenidone 2 for 30 min before NaCl was added for 1 h. All quantifications of gene expression represent the mean from at least three independent experimental series.

Name	GeneBank accession no.	Primer sequence 5´–3´	Reference
EF1-α	EC959059	5´-GAACTGGGTGCTTGATAGGC-3´ 5´-AACCAAAATATCCGGAGTAAAAGA-3´	Reid *et al*. (2006)
JAZ1/TIFY10a 300bp	JF900329	5´-TGCAGTCTGTTGAGCCAATACATA-3´ 5´-CACGTTTCCGGACTTCTTTACAC-3´	this work
JAZ2/TIFY10b 360bp	JF900330	5´-CATCAACCATGTCATTTGACCTGT-3´ 5´-CGGTTGAGCTGCCTGTATTGATT-3´	this work
JAZ3/TIFY6b 333bp	JF900331	5´-AACAAGATCTCTGCACTTCCTCAT-3´ 5´- AGCACCAACCATGTTCTGACCAG-3´	this work
JAZ1/TIFY10a 807bp (full length)		5´-ATGTCGAGCTCCTCGGATATT-3´ 5´-CTACTTTGGAGATTTGGCAGCCA-3´	this paper
JAZ2/TIFY10b 861bp (full length)		5´-ATGTCGAATTCACCGGAGTTCTCT-3´ 5´-CTACAATCTAAGATCAAGTTGCTTTG-3´	this work
JAZ3/TIFY6b 1167bp (full length)		5´-ATGGAGAGAGATTTCATGGGTTTG-3´ 5´-CTACTCCTTGTTAGCTGAAAGAGC-3´	this work

COI1	TA2637_29760	5´-TGGTTTGACTGATGTGGGTCT-3´ 5´-CAGGGTCCAAGGGAGTAACA-3´	Moroldo *et al.,* 2008
MYC2	ABR23669	5´-CTGCTTCAAGGTCTACCATGTTG-3´ 5´-TTCTGGAGCTCGGTGATGTAAGT-3´	this work
STS	X76892	5´-GGAAATTAGAAACGCTCAACGTGC-3´ 5´-TGGTACAACATCACTCTTCTGACG-3´	this work
RS	AF274281	5´-ATGGCTTCAGTCGAGGAATTTAGA-3´ 5´-AAGATGGGTGATCTTGGACTTTGG-3´	this work
NHX1	AAV36562	5´-GCGACATTGTCATTTGTTGCTGAA-3´ 5´-GACTGGATGGTTCAGAAGAAATCA-3´	this work

Table 2: Designations, sequences, and literature references for the oligonucleotide primers used to amplify the marker sequences used in this study

2.9 Extraction and quantification of stilbenes

The production of stilbenes as ROS-scavengers was tested for both cell lines after challenge with 155 mM NaCl at different time points (0, 2, 4, 6, 8, 10, 24 or 48 h). The treated cells were harvested by centrifugation (5000 rpm, 5 min) to remove media, weighed, directly frozen in liquid nitrogen, and then stored at -80 °C until analysis. Stilbenes were extracted according to Tassoni *et al.* (2005) with minor modifications. 20 ml of 80 % (v/v) methanol in water were added to 3-5 g fresh weight. The mixture was then homogenized by an ultrasonic processor (UP100H, Hielscher, Germany) for 3 min. The homogenate was shaken for 2 h in the dark at room temperature and filtered through filter paper under vacuum with 500 pa. The filtrate was concentrated to a residual volume of 5 ml in a glass tube at 40 °C (Heating Bath B490, BÜCHI, Germany) at 280 rpm (Rotavapor R-205, BÜCHI, Germany), under a vacuum of 80 Pa (Vacuubrand CVC2, Brand, Germany). Water-soluble stilbenes were extracted by adding 2 ml of 5 % (w/v) $NaHCO_3$, and three aliquots of 5 ml ethyl acetate. The pooled ethyl-acetate phase was completely

dried leaving the stilbene residue on the bottom of the glass tube. The residue was resuspended in 2 ml of methanol for analysis by high performance liquid chromatograph (HPLC).

Stilbenes were analyzed by HPLC (Agilent, 1200 series, Waldbronn, Germany) using a Phenomenex Synergi hydro RP column (150 x 4.6 mm, particle size 4 µm, Phenomenex; Aschaffenburg, Germany), a DAD detector, and a quaternary valve. The flow rate was adjusted to 0.8 ml min^{-1}, and the injection volume was 20 µl. The UV-VIS spectra were recorded from 200 to 400 nm. The mobile phases included acetonitril (ACN), methanol and water in the following isocratic gradient: 2 min ACN/water (10/90 v/v); 15 min ACN/water (40/60 v/v); 30 min ACN/methanol (50/50 v/v); 32 min ACN/methanol (5/95 v/v); 35 min ACN/methanol (5/95 v/v); 39 min ACN/water (10/90 v/v); 42 min ACN/water (10/90 v/v). *Trans*-resveratrol, *trans*-piceid, and δ-viniferin were quantified and identified using an external standard on the basis of retention time and UV-VIS spectra. The standards for *trans*-resveratrol (Sigma-Aldrich, Deisenhofen, Germany), *trans*-piceid (Phytolab, Vestenbergsgreuth, Germany) and δ-viniferin (kind gift of Dr. Kassemeyer, State Institute of Viticulture, Freiburg, Germany) were dissolved in methanol to a concentration of 100 mg l^{-1}. Calibration curves determined using these standards were linear (r^2 >0.99) and used for quantification of the samples (Chang *et al*., 2011). At least four biological replicates were analyzed for each time point.

2.10 Hormone analysis

Phytohormone contents were quantified for both cell lines with three biological replicates at 1, 3, and 6 h after addition of 155 mM NaCl alone or preceded by pretreatment with 0.75 mM $GdCl_3$ (0.75 mM) for 2 min. As controls cells were treated in the same manner for 1 h with water or with 0.75 mM $GdCl_3$. Additionally, 1 mM $CaCl_2$ were applied in the absence of salt for

either 1 or 3 h. All samples were collected by removing the supernatant using a Büchner funnel under vacuum. Both, cells and supernatants, were shock-frozen in liquid nitrogen, freeze-dried at -50 °C for 2 days, and weighed. Plant hormones were extracted as described previously (Yoshimoto *et al*., 2009) with some modifications: Lyophilized cultured cells or culture media were homogenized in 4 ml of 80% acetonitrile (MeCN) containing 1% acetic acid, and extracted for 30 min with internal standards ($^{13}C_6$-JA-Ile, d_2-JA, d_6-SA, d_6-ABA and d_2-IAA). After centrifugation at 1663 g for 20 min, the supernatant was collected and the sediment extracted again with 4 ml of 80% MeCN containing 1% acetic acid. 1 ml (for cultured cells) or 4 ml (for culture media) of the supernatant were processed further for hormone analysis. After removing MeCN in the supernatant, the acidic aequous extract was loaded onto an Oasis HLB column cartridge (30 mg, 1 ml Waters, Milford, MA, USA), and washed with 1 ml of water containing 1% acetic acid to remove highly polar impurities. Plant hormones were eluted with 2 ml of 80% MeCN containing 1% acetic acid. Ten percent of the eluate were used for the analysis of salicylic acid (SA). After removing MeCN in the remaining eluate, the acidic water extract was loaded onto an Oasis WAX column cartridge (30 mg, 1 ml). After washing with 1 ml of water containing 1% acetic acid, neutral compounds were removed with 2 ml of 80% MeCN, and acidic compounds were eluted with 2 ml of 80% MeCN containing 1% acetic acid. Hormones were quantified by liquid chromatography-electrospray inonization-tandem mass spectrometry in the group of Dr. Seo, at RIKEN Yokohama as described in detail in Yoshimoto *et al*., (2009). For salt-induced hormones in the supernatant samples, see **figure 10**.

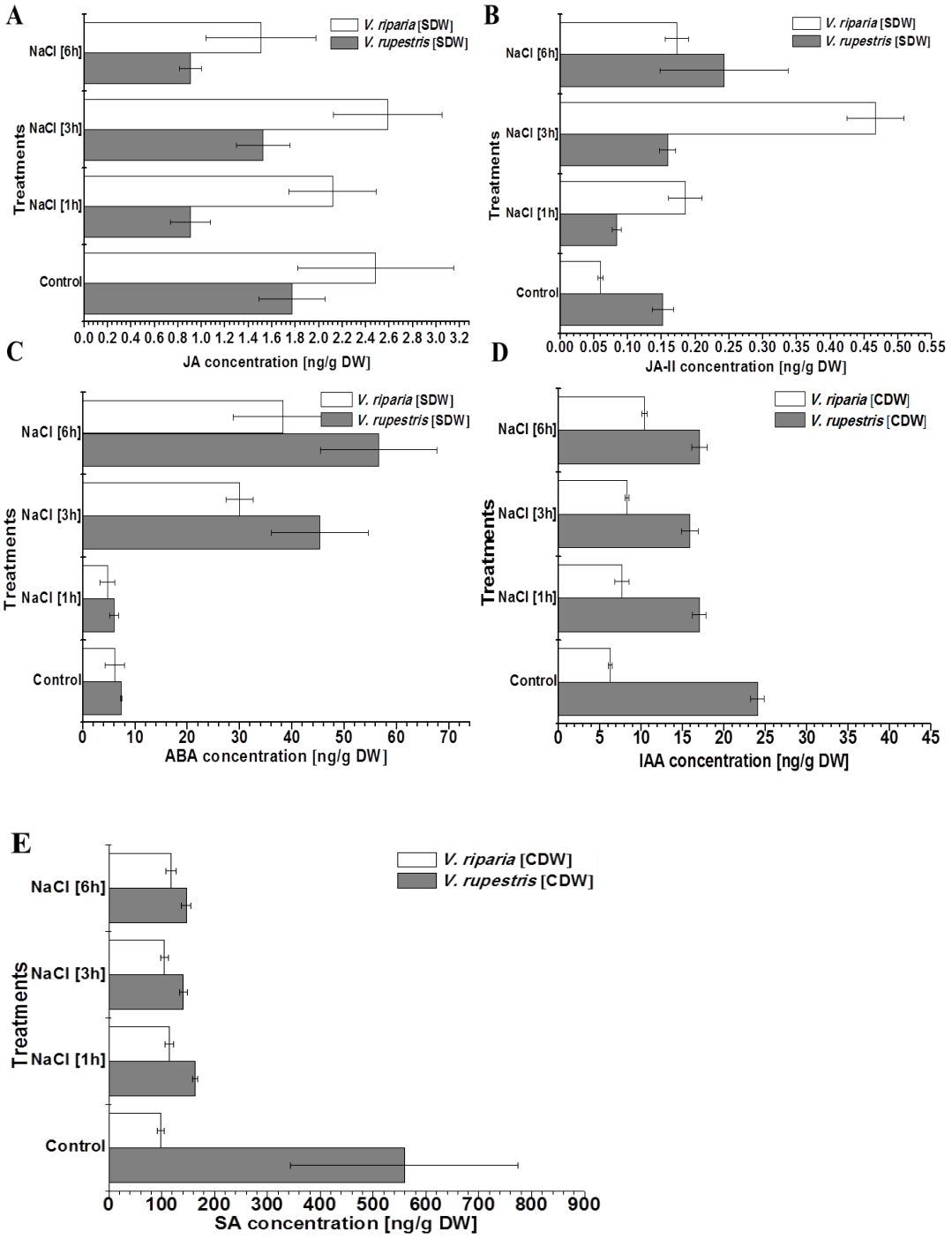


Fig. 10: Endogenous phytohormones under salinity treatment at different time points in the supernatant from *V. rupestris* or *V. riparia*. The effect of 155 mM NaCl at 1, 3, 6h on JA (**A**), JA-Il (**B**), ABA (**C**), IAA (**D**), and SA (**E**). Control samples were treated by H_2O for 1 h. Error bars represent SE, and different letters indicate significant differences among treatments (LSD (P<.05)). Supernatant dry weight (SDW).

2.11 Statistical analysis

The treatments of the current study were arranged as a factorial experiment in a completely randomized design (CRD). Three biological replicates were analyzed for each treatment. Comparisons among means were made via the Least Significant Differences LSD ($P<0.05$) multiple ranges by using the SAS (2000) software. Mean values and standard error of the mean were calculated using Microsoft Excel.

3 Results

3.1 Salinity signalling differs on the physiological level

3.1.1 Growth resumes under salinity in *V. rupestris*

As experimental system for the present study we employed two grapevine cell lines from genotypes that differ in their sensitivity to drought. The wild North American grape *Vitis rupestris*, used in viticulture as source for drought tolerant rootstocks was used as source for the first cell line, the second cell line was generated from the North American grape *Vitis riparia* that grows in alluvial forests and therefore is not adapted to drought. To test, whether the two lines differ in adaptation to salinity, we monitored the relative growth rate (using PCV as readout) under continuous challenge with three concentrations of NaCl (50, 85, and 155 mM) as shown in **Figures 11A** and **B**. The cell lines differed clearly in their growth behavior and in their response to salinity. Under control conditions, *V. rupestris* (**Fig. 11A**) steadily accelerated growth with a peak of 50% daily volume increase at day 4, slowing down during the subsequent days. Already the lowest concentration of salt (50 mM) made the cells shrink initially and reduced growth rate during the first days. However, for 50 mM of salt, growth rate recovered subsequently and even strongly excelled that of the control at day 6 (160% daily volume increase). Even for 85 mM NaCl, growth rate recovered peaking at day 7 with a value of 40% daily volume increase. This recovery contrasted with the situation observed in *V. riparia* (**Fig. 11B**). Here, under control conditions, growth rate increased to a peak of 40% daily volume increase at day 2, slowly decreasing during the subsequent days. For treatment with 50 mM, the initial shrinkage could be compensated by a recovery of growth rate peaking at day 5 with 45% daily volume increase. For 85 mM NaCl, no such

recovery was observed. To test, whether the reduced salt adaptation of *V. riparia* correlated with a higher mortality (%), we scored at day 8 cells stained by the non-permeable dye Evans Blue (Gaff and Okong'O-Ogola, 1971). As shown in **Figure 11C**, mortality at 50 mM and 85 mM NaCl was significantly increased in *V. riparia* over that found in *V. rupestris*, indicating a higher level of salinity-induced damage. However, these differences could not account for the reduced (50 mM NaCl) or lacking (85 mM NaCl) adaptation of growth rate in *V. riparia*. For the highest concentration used (155 mM NaCl), the majority of cells (~80%) was dead in both cell lines, which explains the failure to recover growth rate.

Thus, for 50 mM and 85 mM NaCl, *V. rupestris* displays a clear adaptation of growth after a lag of a few days, whereas this adaptive response is weaker (50 mM NaCl) or even absent (85 mM) in *V. riparia*.

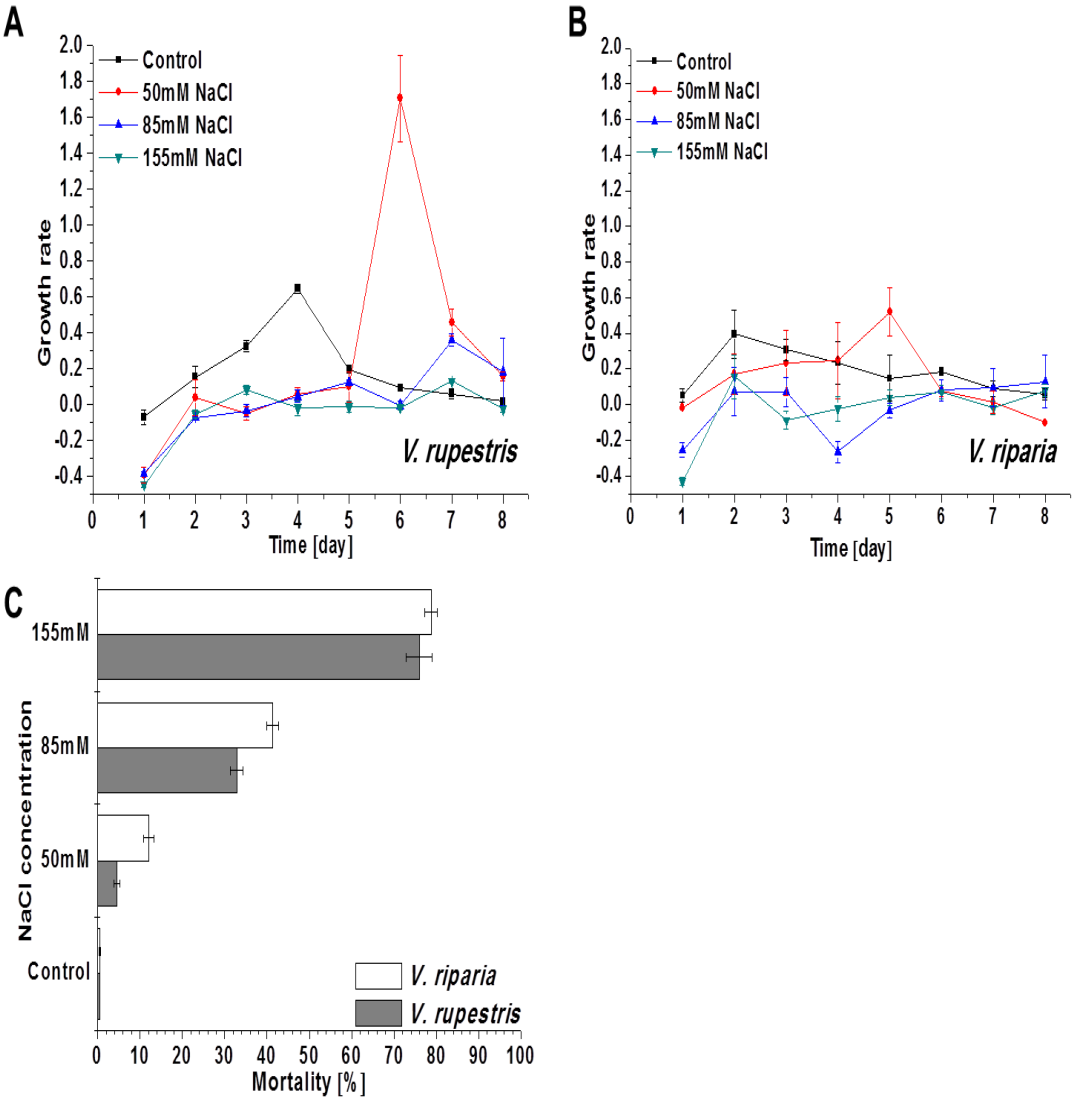


Fig. 11: Time course of relative growth rate ($\Delta V_t/V_t$ over Δt) during salt stress in *V. rupestris* (**A**) versus *V. riparia* (**B**) for different concentrations of NaCl. (**C**) Mortality (%) at day 8 for the two genotypes. Data represent mean values and standard errors from 3 independent experimental series.

To investigate the role of JA on growth rate alone or with 50 mM NaCl, 30 µM of JA was applied to both cell lines (**Fig. 12A, B**). JA in the absence of salt stress reduced growth by about 50% in *V. rupestris* and *V. riparia*, at day 4 and 2, respectively, as compared to the control. In both cell lines, exogenous JA caused significant delay in growth recovery in response to salt, after 7 and 8 days. In addition, JA application with salinity did not show induction to cell mortality in comparison to NaCl alone (**Fig. 12C**). This suggests that JA directs cells to defense over than growth metabolism which negatively impact

cell growth.

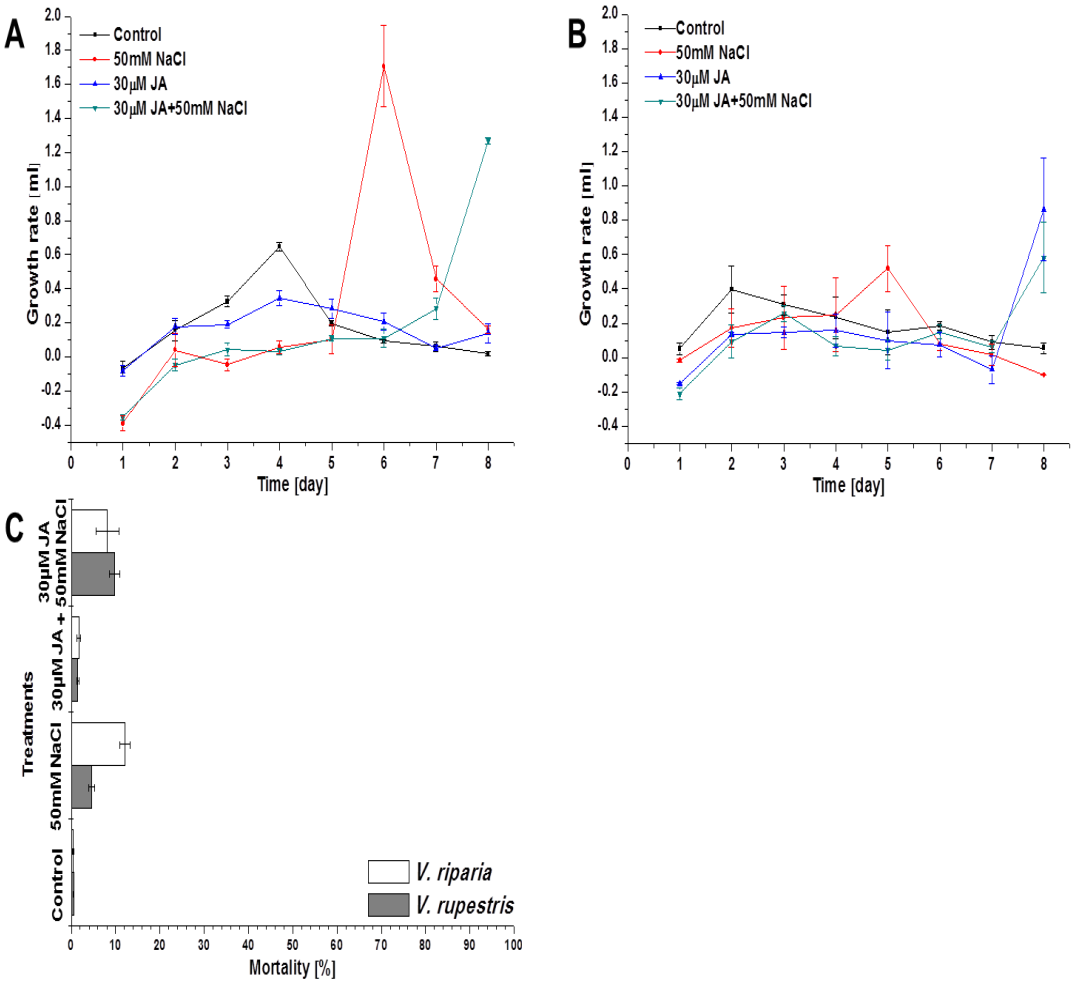


Fig. 12: Relative growth rate ($\Delta V_t/V_t$ over Δt) during 30µM JA alone or with 50mM NaCl in *V. rupestris* (**A**) versus *V. riparia* (**B**). (**C**) Mortality (%) at day 8 for the two genotypes. Values are means ± SE (n = 3).

The impact of ABA on cells growth rate with or without 50 mM NaCl was also studied in both lines (**Fig. 13A, B**). Similar to JA, ABA delayed growth recovery after treatment with 50 mM NaCl. Similar to JA ABA did not cause any significant mortality by itself, but it reduced salt triggered mortality in *V. riparia*, but not in *V. rupestris* (**Fig. 13C**).

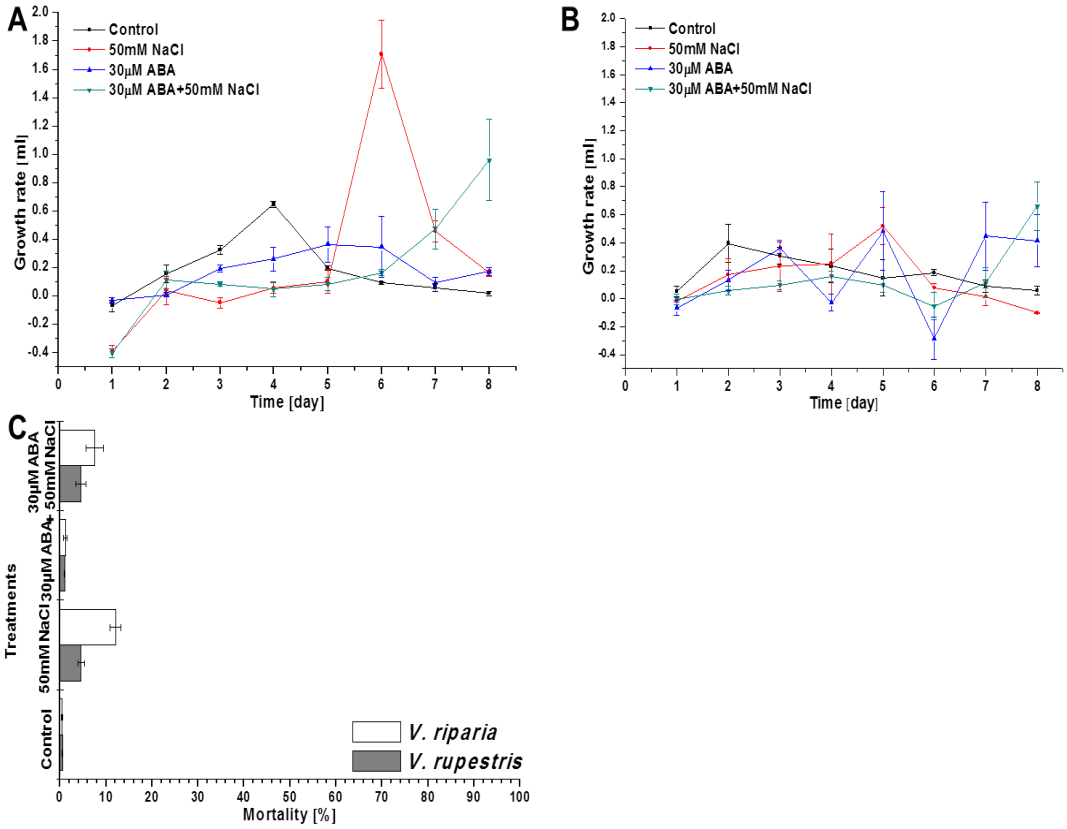


Fig. 13: Relative growth rate ($\Delta V_t/V_t$ over Δt) during 30µM ABA alone or with 50mM NaCl in *V. rupestris* (A) versus *V. riparia* (B). (C) Mortality (%) at day 8 for the two genotypes. Values are means ± SE (n = 3).

To test a the effect of $CaCl_2$ on ameliorating salt damage, $CaCl_2$ was added alone or directly before treating cells with 50 mM NaCl (**Fig. 14A, B**). While $CaCl_2$ alone had no negative impact on *V. rupestris* growth (**Fig. 14A**), it reduced the growth of *V. riparia* showing the same pattern as 50 mM NaCl did (**Fig. 14B**). Interestingly, 1 mM $CaCl_2$ was able to ameliorate salinity impact on *V. rupestris* cells starting from the forth day. However, it decreased the *V. riparia* growth when it added with NaCl (50 mM) more than NaCl alone. In addition, $CaCl_2$ pretreatment induced cell mortality to about 45 % in NaCl-stressed *V. riparia*, but not in *V. rupestris* (**Fig. 14C**).

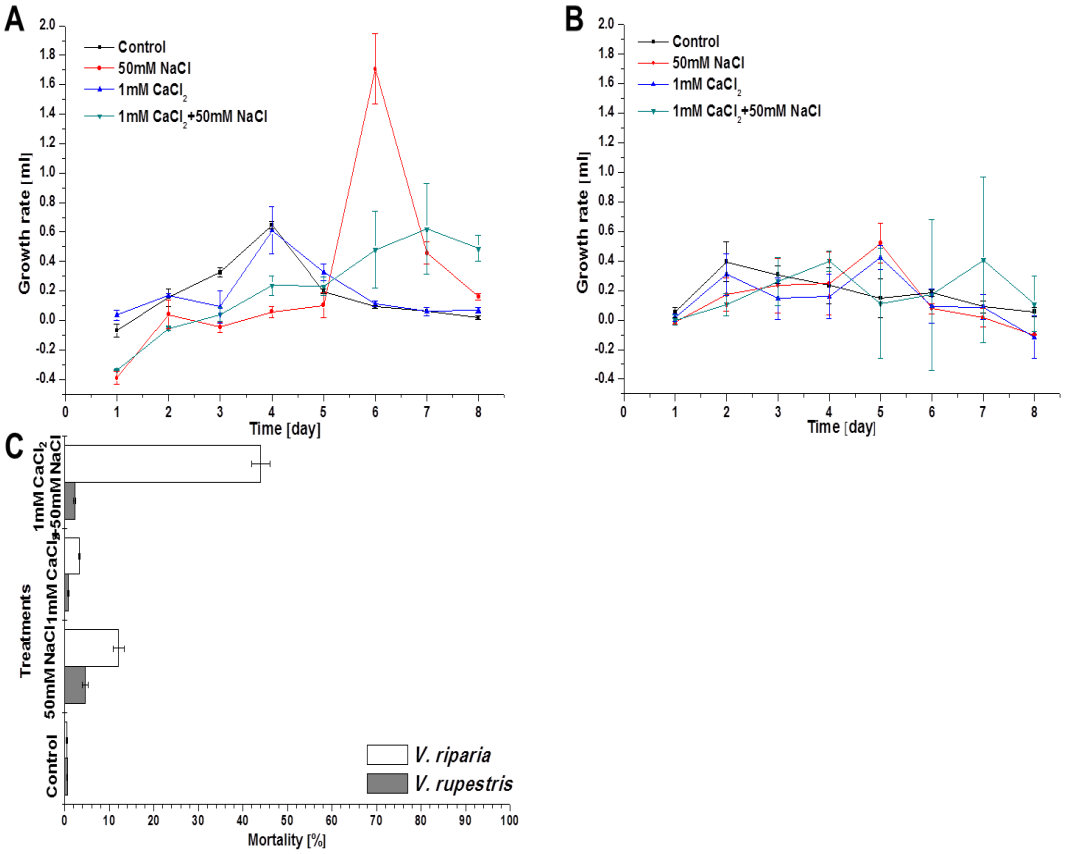


Fig. 14: Relative growth rate ($\Delta V_t/V_t$ over Δt) during 1mM $CaCl_2$ alone or with 50mM NaCl in *V. rupestris* (**A**) versus *V. riparia* (**B**). (**C**) Mortality (%) at day 8 for the two genotypes. Values are means ± SE (n = 3).

Finally, the impact of $GdCl_3$ (an inhibitor of stretch-activated ion channels) on *Vitis* cell growth was investigated by adding 0.75 mM $GdCl_3$ to both cell lines (**Fig. 15A, B**). $GdCl_3$ was able to decrease the growth rate in both cells, however, the effect was much more pronounced in *V. rupestris.* In addition, was able to raise cell mortality to 80% in *V. rupestris*, not in *V. riparia* (**Fig. 15 C**).

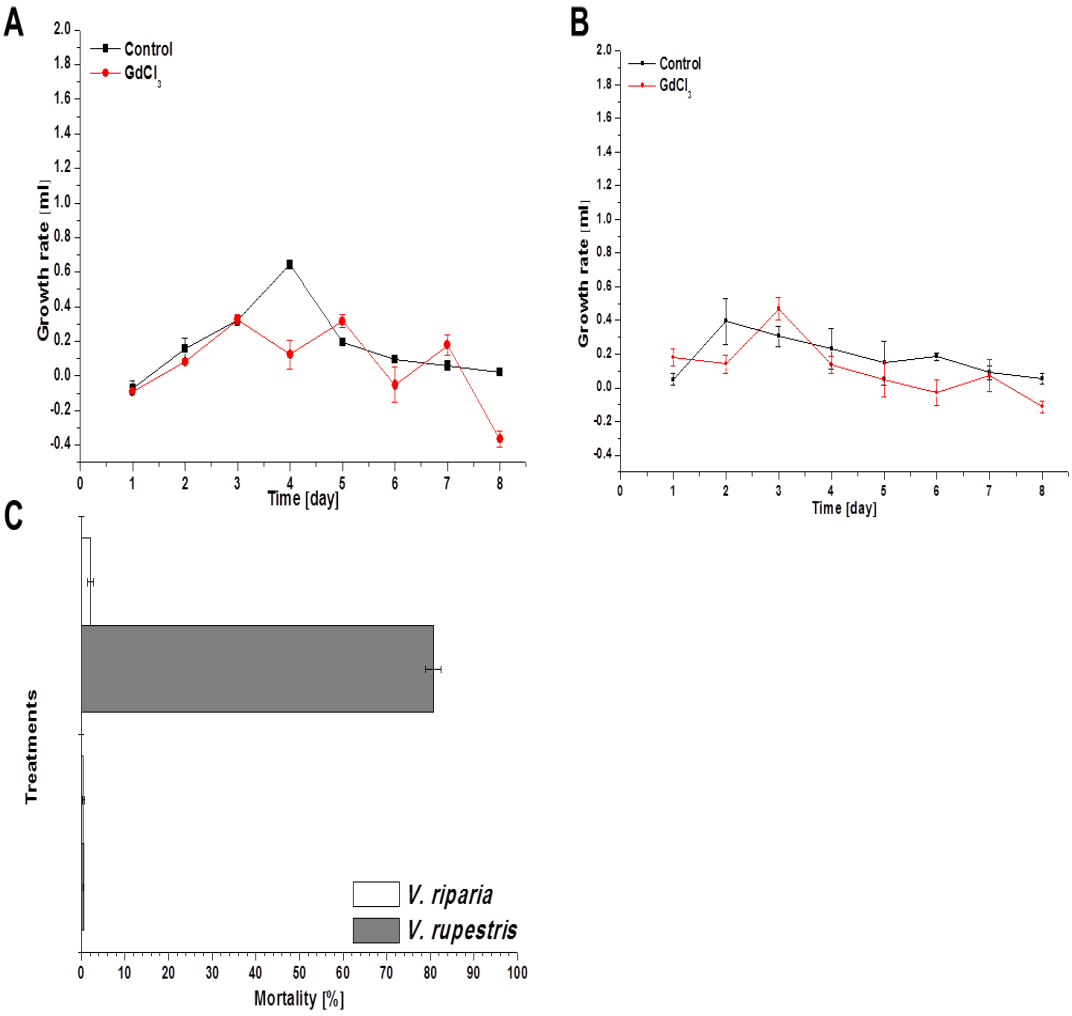


Fig. 15: Relative growth rate ($\Delta V_t/V_t$ over Δt) during 0.75mM $GdCl_3$ or H_2O in *V. rupestris* (A) versus *V. riparia* (B). (C) Mortality (%) at day 8 for the two genotypes. Values are means ± SE (n = 3).

3.1.2 The kinetics of Na^+ uptake consist of three phases

To test the uptake of Na^+ over time and to address the point, whether the different adaptation correlates with differences in uptake, the two cell lines were treated with 155 mM NaCl or with H_2O as a control, and sampled at 2, 5, 10, 15, 30 min, and 1, 2, 3, and 6 h to quantify Na^+ ions. **Figure 16A** shows the time course of Na^+ uptake and reveals that Na^+ concentration does not increase at a constant rate. An initial phase I of rapid uptake is followed by a halting phase II (delineated by the arrows in **Fig. 16A**), and a further phase III of rapid uptake. The early uptake (phase I) has been completed already at

the first sampling point (2 min), and could not be resolved further due to the limitations in handling the samples. Interestingly, phase I results in a twofold higher Na^+ content in *V. rupestris* (~6 mg/g) as compared to *V. riparia* (~3 mg/g). The two lines mainly differ in phase II: For *V. rupestris*, the concentration of salt oscillates around a steady-state level of ~7 mg/g, and, from 10 min, even drops to 5 mg/g indicating that more Na^+ is extruded from the cell than penetrates from outside (phase Ⅱ). This process is subsequently fading and a new wave of salt increase initiates, such that Na^+ concentration increases again to 8 mg/ml (phase Ⅲ). In *V. riparia*, the halting phase II is barely manifest as a slower increase leading to a shoulder in the curve between 2-15 min. In contrast to *V. rupestris*, Na^+ content is not dropping in phase II, but just growing slower. Finally, also for *V. riparia,* the cells "give in", such that Na^+ steadily increases arriving at the same final level as in *V. rupestris.*

Thus, the two lines differ in the timing of Na^+ influx – whereas in the first 15 min Na^+ levels are higher in *V. rupestris* than in *V. riparia*, this is reverted by active export of Na^+ against a considerable concentration gradient. This export is so efficient in *V. rupestris* that, between 15 min and 360 min, the salt concentration can be kept lower than in *V. riparia*.

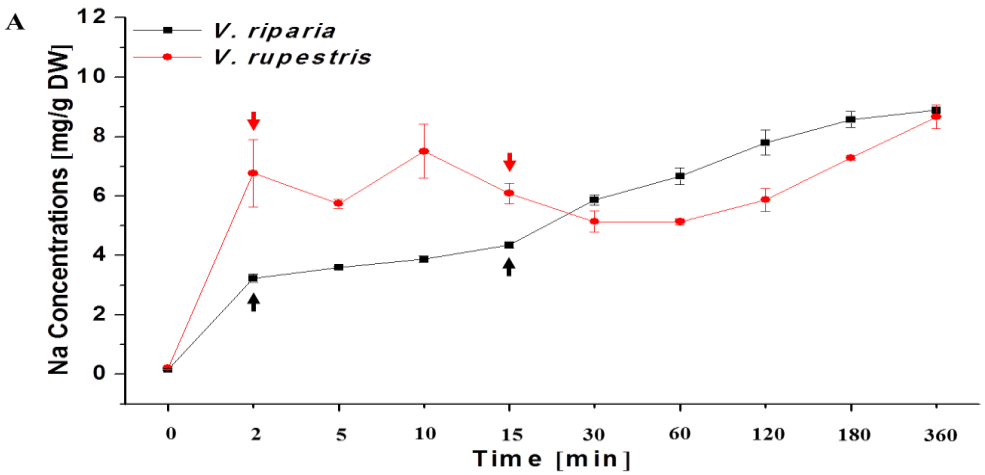


Fig. 16: Time course of Na^+ uptake upon continuous challenge with 155 mM NaCl in control cells of *V. rupestris* (red) versus *V. riparia* (black). Data represent mean values and standard errors from 3 independent experimental series. The arrows in **A** indicate begin and end of the phase II defined in the text. To resolve the early time points, the time axis is plotted in a non-linear scale.

3.1.3 Ca^{2+} influx alters Na^{+} uptake kinetics

Activation of Ca^{2+} influx channels located in the plasma membrane has been proposed as primary signal for salinity signaling (Knight et al., 1997). In addition, Ca^{2+} can block the NSCCs discussed as primary gate of Na^{+} influx (Demidchik and Tester, 2002). We therefore tested the impact of external Ca^{2+} on Na^{+} uptake. Both cell lines were pretreated with 1 mM $CaCl_2$ directly before adding 155 mM NaCl (**Figs. 17A, B**). The effect of Ca^{2+} was qualitatively different depending on the respective phase of Na^{+} uptake: the increase of Na^{+} during the early phase I (<2 min) as well as during the intermediate phase II (2-15 min) was inhibited in both lines (more pronounced in *V. rupestris* than in *V. riparia*). In contrast, the uptake during phase III (>15 min) was promoted (more pronounced in *V. riparia* than in *V. rupestris*). To test, which part of the Ca^{2+} effect is based on influx through the plasma membrane, $GdCl_3$ was used as *bona-fide* inhibitor of both Ca^{2+} influx (Knight et al., 1997) and NSCCs (Demidchik et al., 2002) in a concentration that had been defined to be saturating for the inhibition of defense-related signaling in the same cell system (Qiao et al., 2010). Similar to treatment with exogenous Ca^{2+}, the effect of $GdCl_3$ was dependent on the respective phase of salt uptake (**Figs. 17C, D**). During phase I, $GdCl_3$ reduced salt uptake slightly (similar to Ca^{2+}). During phase II, the slope of uptake was higher than that observed for Ca^{2+}. For phase III, $GdCl_3$ was almost ineffective in *V. rupestris* (**Fig. 17C**), but clearly inhibitory in *V. riparia* (i.e. antagonistic to Ca^{2+}, **Fig. 17D**).

Thus, the uptake of Na^{+} can be dissected into at least two mechanisms that differ with respect to their dependency on external Ca^{2+}: the uptake before 15 min (phases I and II) is inhibited by Ca^{2+} (but also by $GdCl_3$, indicating that Ca^{2+} influx is not the decisive factor here). In contrast, the uptake after 15 min (phase III) is promoted by Ca^{2+} (antagonized by $GdCl_3$, indicating a role for Ca^{2+} influx channels).

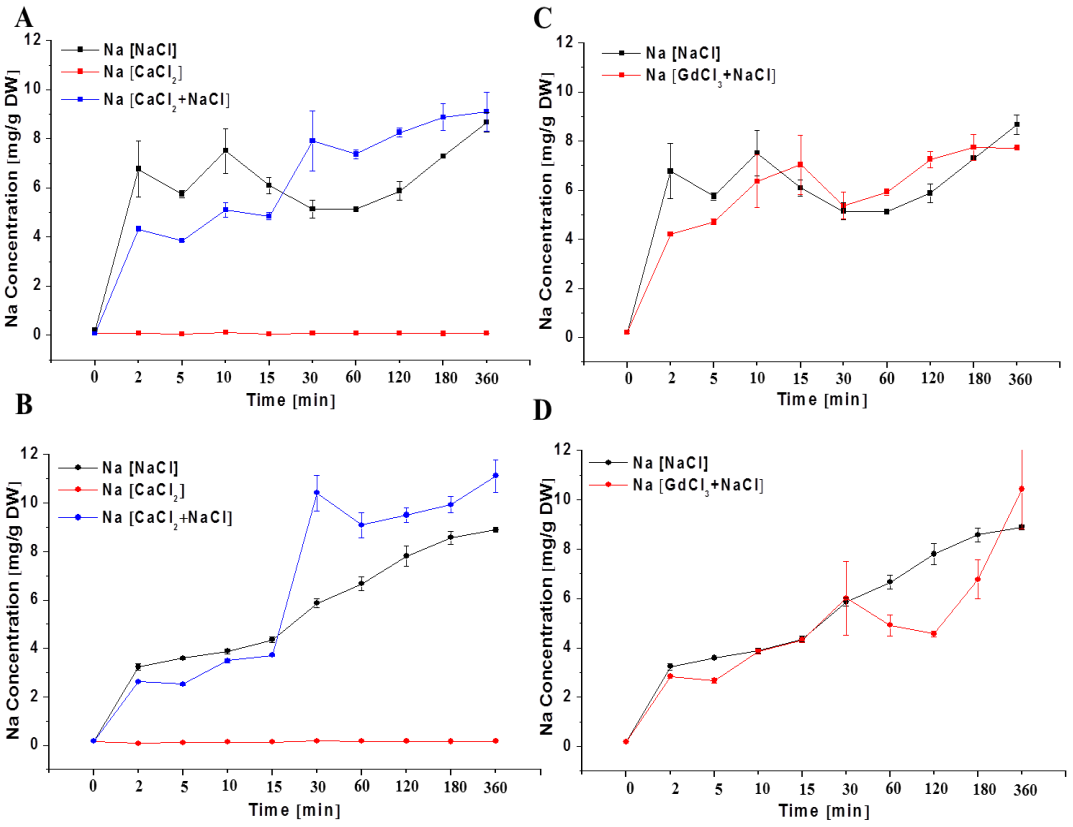


Fig. 17: Time course of Na^+ uptake upon continuous challenge with 155 mM NaCl alone or after pretreatment with 1 mM $CaCl_2$ (**A, B**) or 750 µM $GdCl_3$ (**C, D**) for *V. rupestris* (**A, C**) and *V. riparia* (**B, D**). Data represent mean values and standard errors from 3 independent experimental series. As negative control in **A** and **B**, Na^+ content after pretreatment of Ca^{2+} without subsequent salinity exposure was recorded (red lines). To resolve the early time points, the time axis is plotted in a non-linear scale.

3.1.4 Sign reversal in salt-induced changes of Ca^{2+} content

To interpret the Ca^{2+} effect on Na^+ uptake (**Figs. 17A, B**), we followed the time course of Ca^{2+} content during incubation with 155 mM NaCl. The time courses are shown in **Figure 18** and differ qualitatively between the two grapevine cell lines. In *V. rupestris* (**Fig. 18A**), Ca^{2+} content basically follows the temporal pattern of Na^+ uptake (**Fig. 16**) with a rapid increase during phase I of Na^+ uptake, a plateau and a decrease during phase II, and a second wave of increase in phase III of uptake. Since here only the Ca^{2+} in the MS-medium (0.32 g/l $CaCl_2$) was available, potential release of cell-wall bound Ca^{2+} should be equilibrated, such that these increases probably reflect the activity of Ca^{2+} influx channels. The pattern for *V. riparia* was basically a

mirror-image (**Fig. 18A**) with a sharp drop of Ca^{2+} during phase I of Na^{+} uptake, a low plateau during phase II and a partial recovery during phase III. This means that, in *V. rupestris,* the pattern of Ca^{2+} content parallels that for Na^{+} content, whereas in *V. riparia* the two ions show an inverted behavior. This difference between the two cell lines represents a clear sign reversal.

Calcium content might change either by the activity of ion channels or by binding of Ca^{2+} ions to the pectic components of the cell wall. To discriminate between these two components, we repeated the time courses in the presence of $GdCl_3$. For *V. rupestris*, the temporal pattern of Ca^{2+} content was not significantly altered by $GdCl_3$ (**Fig. 18B**), whereas for *V. riparia*, the Ca^{2+} content was strongly reduced (**Fig. 18C**). To probe for the contribution of Ca^{2+} binding to unsaturated pectic binding sites in the cell wall, in a third set of experiments Ca^{2+} content was followed in the presence of either 1 mM $CaCl_2$ (to saturate binding sites in the cell wall), or of a combination of 1 mM $CaCl_2$ with 155 mM NaCl. In *V. rupestris*, incubation with 1 mM $CaCl_2$ established a high plateau of Ca^{2+} content within 2 min (about twofold as compared to 155 mM NaCl), this increase could be almost eliminated, when 155 mM NaCl were administered together with this Ca^{2+} treatment (**Fig. 18D**). In *V. riparia*, there was a distinct biphasic increase of Ca^{2+} content under these conditions. Prior to 15 min, 1 mM $CaCl_2$ alone caused a first wave of increase, which was followed from 15 min by a second rise leading to roughly the same Ca^{2+} levels as found in *V. rupestris* (**Fig. 18E**). When 155 mM NaCl were administered together with this high Ca^{2+} content, there was virtually no difference to the situation observed for 155 mM NaCl alone. However, from 15 min, the Ca^{2+} levels increased parallel to the situation without NaCl, but to lower amplitudes.

In summary, the two cell lines not only show a sign reversal with respect to Ca^{2+} levels in response to salinity, they also differ with respect to a late Ca^{2+} influx (>15 min) present in *V. riparia*, but not in *V. rupestris*, and probably with respect to the presence of free binding sites for Ca^{2+} in the cell wall.

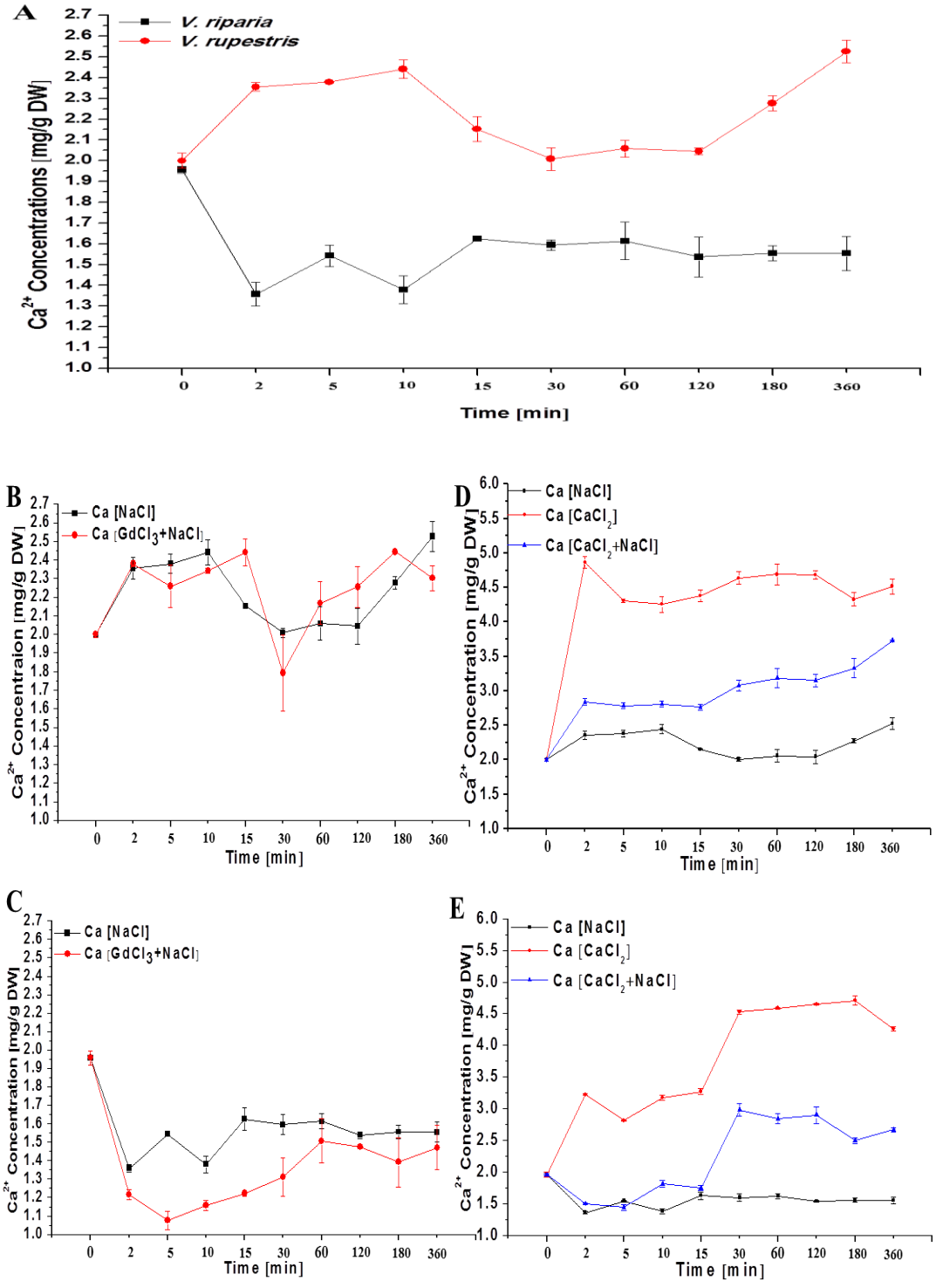


Fig. 18: Time course of Ca^{2+} uptake upon continuous challenge with 155 mM NaCl in control cells (**A**) of *V. rupestris* (red) versus *V. riparia* (black), and after pretreatment with 750 µM $GdCl_3$ (**B, C**) or 1 mM $CaCl_2$ (**D, E**) for *V. rupestris* (**B, D**) and *V. riparia* (**C, E**). Data represent mean values and standard errors from 3 independent experimental series. As negative control in **D** and **E**, Ca^{2+} content after Ca^{2+} application was followed (red lines). To resolve the early time points, the time axis is plotted in a non-linear scale.

3.1.5 NaCl-induced extracellular pH differs in both cell lines

Extracellular alkalinisation is considered to be one of the earliest defense-related responses as the apoplast is seen as the first plant compartment challenged by environmental signals (Felix *et al*., 1993; Hoson, 1998). However, it is also an early signal in the response to salinity stress (Geilfuß and Mühling, 2013). This response covers two underlying mechanisms – a rapid influx of Ca^{2+} and protons (Jabs *et al*., 1997), followed by an efflux of anion-exporters that are activated by Ca^{2+} signaling (Felle et al., 1998). We therefore followed the response of extracellular pH [pH_{ext}] to salinity in the two cell lines as shown in (**Fig. 19**). Both cultivars first showed a transient reduction of pH_{ext} recovering the initial level and then, from around 3 min after addition of salt, produced a strong alkalinization that was maximal about 10 min later and then very slowly declining. The lag time for the initiation of proton transport was shorter in *V. rupestris* (15 sec) as compared to *V. riparia* (36 sec). *V. rupestris* cells also showed a more pronounced alkalinization (ΔpH_{ext} ~ 0.4) (**Fig. 19A**) as compared to *V. riparia* (ΔpH_{ext} ~0.25) (**Fig. 19B**). At higher concentrations, the maximum was reached later as compared to lower concentrations. In *V. rupestris*, the peaks were much more pronounced as compared to *V. riparia* (**Fig. 19C**).

To characterise the difference between the two cell lines on a quantitative level, time courses were recorded by varying the concentration of salt. The results were fitted using a Michaelis–Menten equation with pH_{max50} (the time when the pH response reached the half-maximum) as indicator of velocity. When T_{pH50} was plotted over the concentration of NaCl (**Fig. 19D**), saturable curves were found that could be well fitted by a Michaelis-Menten function (R^2 0.840 for *V. rupestris* and R^2 0.882 for *V. riparia*). From these functions, effective concentrations (EC_{25}, inducing 25% of the maximal response) could be calculated to be 80 mM NaCl for *V. rupestris* and 122 mM NaCl for *V. riparia*, respectively.

To test the role of NSCCs on inducing pH_{ext}, $GdCl_3$, as inhibitor of mechanosensitive Ca^{2+} influx channels, but also of NSCCs, was applied for 2 min before adding NaCl (**Figs. 19A, B**). $GdCl_3$ sharply reduced pH_{ext} of cells. This was followed by a slow, but steady increase of pH in *V. rupestris* restoring the initial level, whereas no such recovery was observed in *V. riparia*. Even when the concentration of $GdCl_3$ was increased to 0.75 mM in *V. rupestris*, pH_{ext} could recover partially. When $GdCl_3$ was administered in the absence of salinity, no recovery of pH was observed even in *V. rupestris*, indicating that this recovery resulted from the salt-induced alkalinisation on the background of a more acidic pH.

The comparison of **Figs. 16, 17** and **18** reveals that apoplastic pH decreases during the early phase I of rapid Na^+ uptake, but increases preceding phase II of Na^+ uptake. This increase is more pronounced in *V. rupestris* (were during phase II even more Na^+ is extruded than enters the cells) as compared to *V. riparia* (were Na^+ uptake during phase II is just slowed down slightly). $GdCl_3$ delayed / reduced salt-triggered alkalinization in *V. rupestris* (**Fig. 19A**), and impaired the efficiency by which the uptake of Na^+ could be halted during phase II (**Fig. 17 C**). In *V. riparia*, where $GdCl_3$ had almost no effect on Na^+ uptake (**Fig. 17 D**), salt-triggered alkalinization was completely suppressed (**Fig. 19B**).

Thus, there is a correlation between phase II of Na^+ uptake and the amplitude / robustness of extracellular alkalinization.

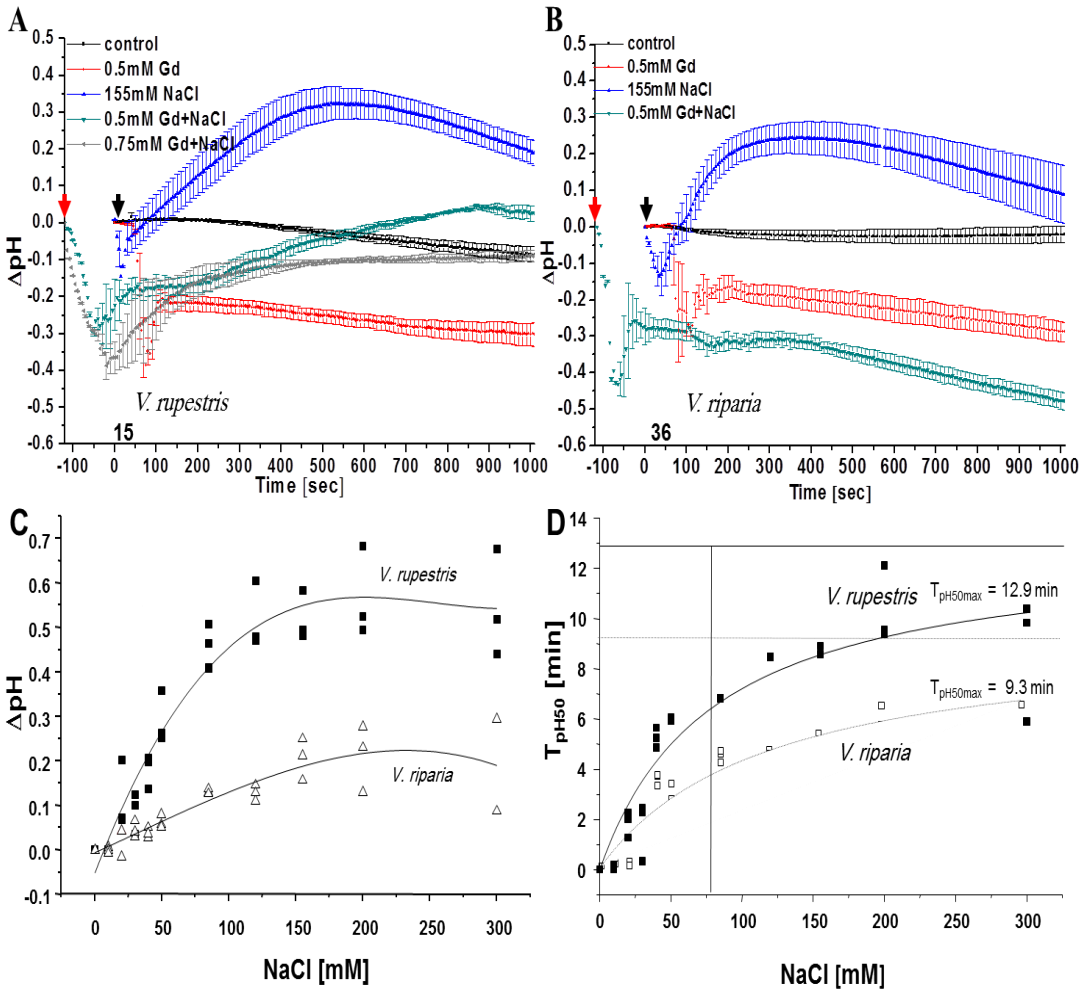


Fig. 19: Response of apoplastic pH to NaCl in grapevine cells. Representative time course of the response of apoplastic pH to 155 mM NaCl alone or after adding 0.5 and 0.75mM of $GdCl_3$ (an inhibitor of NSCCs) for 2 min for *V. rupestris* (A) or *V. riparia* (B). Red-colored arrow for adding $GdCl_3$ before NaCl was applied after 2 min (black-colored arrow). (C) Dose response curves for the maximal pH change over salt concentration, (D) kinetic analysis of pH responses in *V. rupestris* (closed squares, solid lines) and *V. riparia* (open squares, dotted lines). The half-maxima of pH to increasing concentrations of NaCl were fitted by a Michaelis-Menten function. T_{pH50} i is defined as time to reach 50% of the maximal response.

3.1.6 NaCl induces ROS species

To test for the negative impact of NaCl on *Vitis* cells, ROS as indicator of cellular stress were visualised by dihydroethidium (DHE). DHE is a cell-permeable compound that upon entering the cells interacts with $O^{2\bullet-}$ and is considered as earliest ROS to be generated, to form oxyethidium, which in

turn interacts with nucleic acids to emit a bright red color detectable as red fluorescence (Tarpey *et al.,* 2004). The induction of ROS species was monitored over 6 h in both cell lines. Elevated levels of ROS species were induced by salt stress in both cultivars (**Fig. 20**). However, ROS induction commenced earlier (from 1 h) and reached much higher levels in *V. riparia* as compared to *V. rupestris*, where earliest indications of elevated ROS levels were not visible before 3 h after addition of salt.

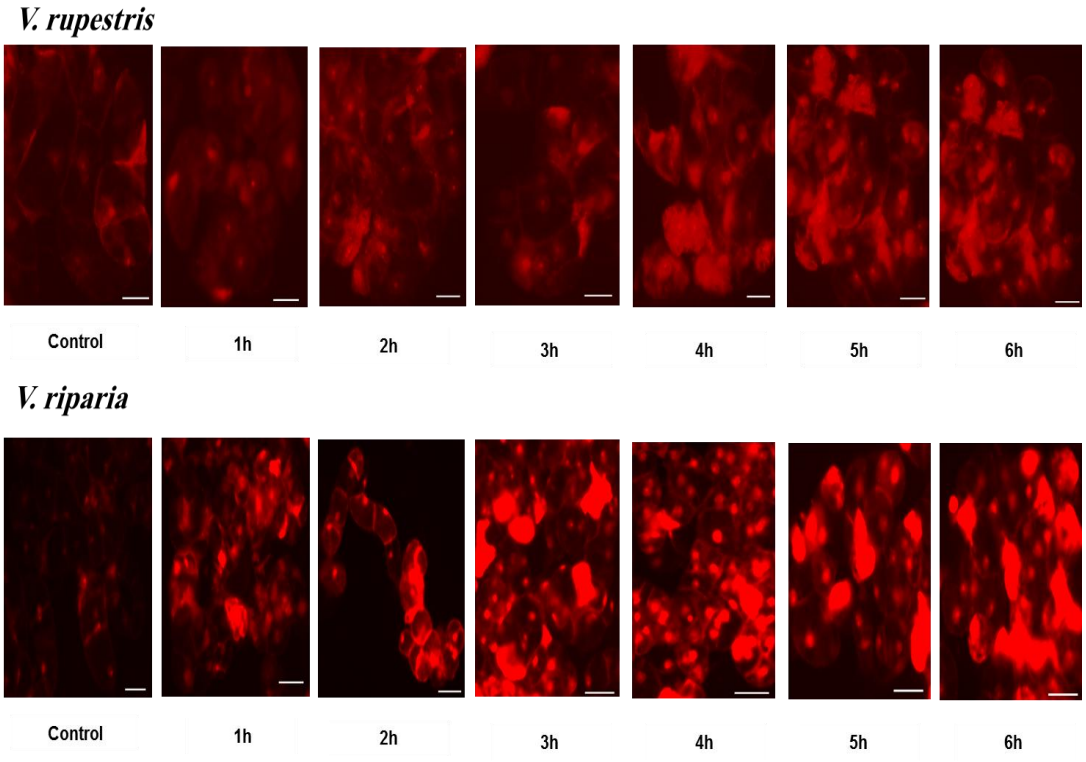


Fig. 20: Effect of 155 mM NaCl on ROS induction in 5 day-old suspension cells of *V. rupestris* an *V. riparia*. Cells were stained with dihydroethidium (DHE), which records superoxide radicals ($O_2^{\bullet-}$)155 mM NaCl was added to cells preloaded with the dye immediately before microscopic observation, and the signal was followed over 6 h. Size bar 20 µm.

3.2 Salinity signalling differs on the molecular level

3.2.1 Isolation and identification of *JAZ/TIFY* genes

Based on the available sequences of *Arabidopsis thaliana* JAZ/TIFY proteins,

putative homologues in *V. vinifera* cv. “Pinot Noir” were identified by a BLAST search. The phylogenetic tree based on the 12 available JAZ/TIFY protein sequences of *Arabidopsis thaliana* and their 9 putative JAZ/TIFY-LIKE (JAZL/TIFYL) homologues in *Vitis vinifera* cv. “Pinot Noir”. (**Fig. 21A**) showed five branches (I to V). Four of those contained members from both plant species, branch III did not contain a member from *Vitis*. All *Vitis* JAZL/TIFYL proteins contain a putative N-terminal ZIM/tify-domain, and a putative C-terminal Jas-domain as conserved domains. The consensus sequence for the ZIM/tify and Jas domains (**Fig. 21B**) was created using http://weblogo.berkeley.edu/logo.cgi. We succeeded to clone three of these putative *JAZ/TIFY*-LIKE genes from the *V. rupestris* cell line using RT-PCR with degenerated primers (**Table 2**). Alignment of the three proteins (**Fig. 21C**), showed that two of those, *VrJAZ1/TIFY10a* (gene bank accession JF900329), and *VrJAZ2/TIFY10b* (gene bank accession JF900330) were fairly similar and belonged to the same branch of the tree (I, **Fig. 21A**), whereas the third, *VrJAZ3/TIFY6b* (gene bank accession JF900331) was longer and fell into a different branch (IV, **Fig. 21A**). The sequences from *V. rupestris* were almost identical with those from *Vitis vinifera* cv. “Pinot Noir” (only 2-3 amino acid exchanges in the variable regions of the JAZ/TIFY-LIKE proteins.

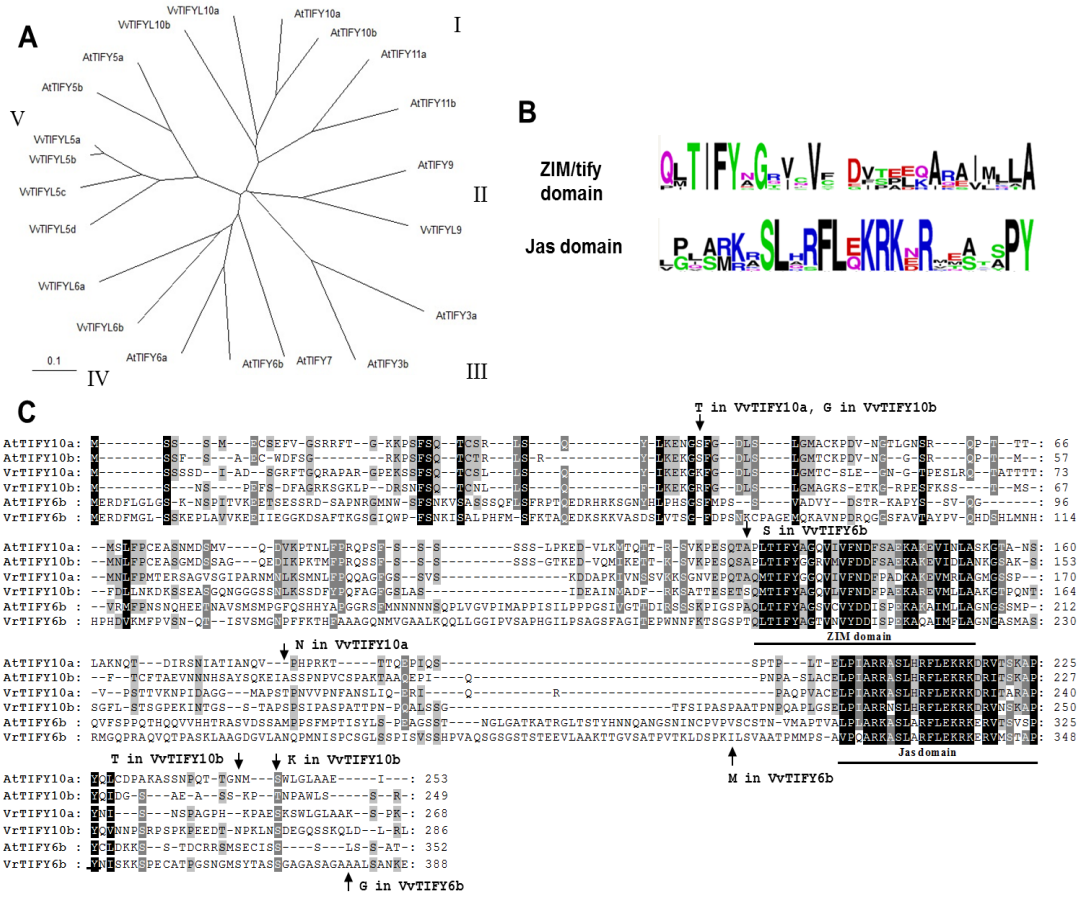


Fig. 21: In silico-analysis of Vitis *JAZ/TIFY*-homologues. A Phylogenetic tree based on NJ of available *JAZ/TIFY* sequences from *Arabidopsis thaliana* and their JAZ-LIKE/TIFY-LIKE (*JAZL/TIFYL*) homologues in *Vitis vinifera* cv. "Pinot Noir". (NJ), bootstrap values 0.1. The *JAZ/TIFY*-members analysed in this study are highlighted by coloured boxes, B consensus motives, C alignment with *AtJAZ/TIFY*-homologues (sequences of Vr and Vv are almost identical), arrows indicate amino-acid differences between *V. rupestris* and *V. vinifera* cv. "Pinot Noir".

3.2.2 Impact of NaCl, JA, and aspirin on the *JAZ/TIFY* genes expression

Since these three proteins (termed as JAZ 1-3/TIFY10a, b, 6b based on their homology to AtJAZ/TIFY proteins) seemed to be the dominant members in *V. rupestris* cells, their expression was investigated under salt-stress by reverse transcription-PCR (RT-PCR) using elongation factor 1α as internal reference for quantification. The dose-response dependency of transcript abundance was determined 1 hour after the addition of NaCl. The results were in agreed

with the expression of defence genes under Harpin treatment (Qiao *et al.,* 2010). In contrast, all three genes were induced after addition of salt for 1h (**Fig. 22A** shows a representative concentration series) with a peak at concentrations around 80-120 mM and a decline for higher salt concentrations. *JAZ1/TIFY10a* showed significant (**Fig. 22A**) induction in *V. rupestris* under salt stress as compared to *V. riparia*. The same was observed for *JAZ3/TIFY6b*. *JAZ2/TIFY10b* was slightly induced by salt to a similar factor in both cultivars (**Fig. 22A**). These results show a significant (by about a factor of 2) salt-induction of *JAZ1/TIFY10a* and *JAZ3/TIFY6b* in *V. rupestris*, which parallels the salt-tolerance of growth and the induction of extracellular alkalinisation.

To test whether these *JAZ/TIFY* genes respond to JA, cells were treated for 1 h with different concentrations (0 to 50 µM) of JA. As solvent control, cells were treated with a corresponding concentration of EtOH (**Fig. 22B** shows a representative dose series). *JAZ1/TIFY10a* as well as *JAZ3/TIFY6b* was induced with the increase of JA concentrations to 30 µM with a tendency to decrease at 40 and 50 µM in both cell lines (**Fig. 22B**). *JAZ2/TIFY10b* was only very weakly expressed. Both cell lines responded in a similar way, the difference in induction observed for NaCl (**Fig. 22A**) was not manifest in their response to JA.

To test, whether the induction of *JAZ/TIFY* transcripts by salt can by suppressed by JA antagonists, cells were treated with the same concentrations of NaCl for 1 h after pre-treatment with 1 mM aspirin or corresponding amount of its solvent solution (DMSO) as a control for 6 h. The induction of these *JAZ/TIFY* genes by salt treatments was completely suppressed by pretreatment with aspirin (**Fig. 22C** shows a representative dose series).

Thus, *JAZ1/TIFY10a* and *JAZ3/TIFY6b* are significantly induced by NaCl in a concentration-dependent manner. This induction is more pronounced in *V. rupestris* as compared to *V. riparia*, especially in the physiologically relevant range around 100 mM NaCl. Both *JAZ/TIFY*-transcripts are induced by JA with highest expression at 30 µM and without any significant difference between the two cell lines. Third, aspirin

can quell the induction of *JAZ/TIFY* transcripts by salt indicating that the response of transcription requires JA-signaling.

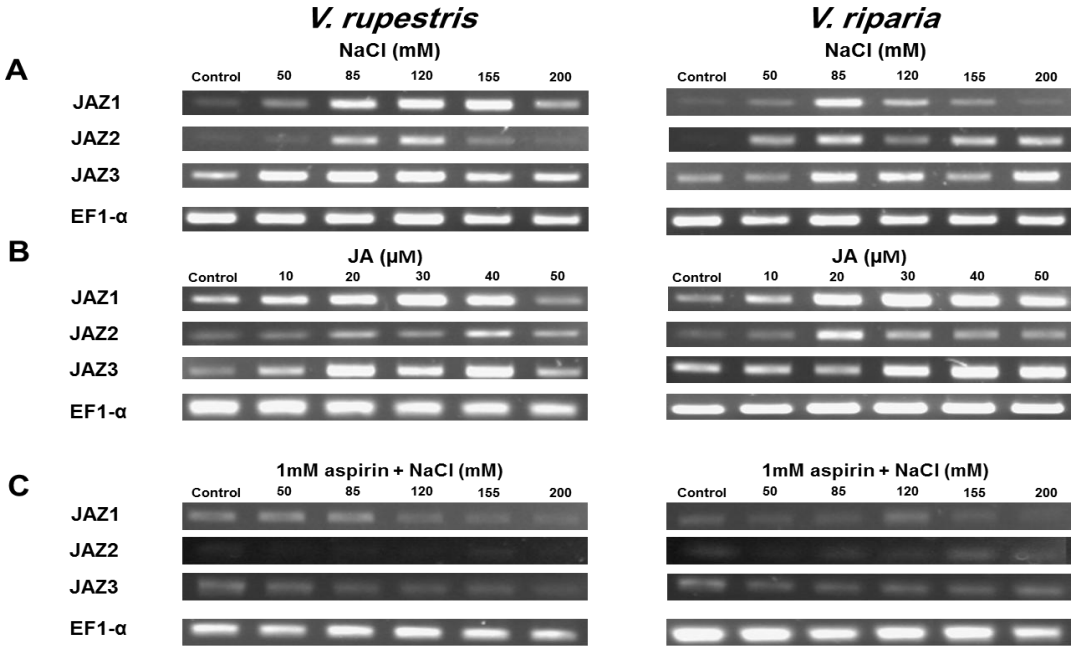


Fig. 22: The expression of *JAZ/TIFY* under different treatments. Response of *JAZ/TIFY* genes in *V. rupestris* and *V. riparia* to different concentrations of NaCl (A) and JA (B) for 1 h, or to NaCl for 1 h after pretreatment with aspirin (1 mM) (C) for 6 h. Elongation factor 1α (*EF1-α*) was used as internal standard. One representative example of three independent experiments is shown.

3.2.3 Time courses of salt tolerance genes

To get insight into the sequence of cellular events, the expression of three marker genes for jamsomate signaling (*JAZ1/TIFY10a*, *MYC2*, and *COI1*), a marker for salt adaptation (*NHX1*), and two markers of biotic defense (phytoalexin synthesis, *STS, RS*) was measured at a concentration of 155 mM NaCl, where *JAZ1/TIFY10a* was significantly expressed (**Fig. 23** shows a representative time series). In both cell lines, induction of *JAZ1/TIFY10a* was clearly manifest at 1 h after addition of NaCl. However, it was significant in *V. rupestris*, and even doubled during the following two hours, declining subsequently, while in *V. riparia* the induction was much weaker and did not increase after 1 h (**Figs. 23**). In contrast, *MYC2* slightly increased after 1h

and then declined over time in both cell lines. *COI1*, encoding for the jasmonate receptor, seemed to be increased between 1-3 h after addition of salt (**Fig. 23**), but this response was not significant. In contrast, *NHX1* as marker for salt adaptation was clearly induced in *V. rupestris* from 3 h (roughly 3 fold at 6 h) which was not observed in *V. riparia*. The stilbene-related genes *STS* and *RS*, widely used as markers for biotic defense in *Vitis*, did not respond to salt in *V. riparia.* However, they were clearly induced in *V. rupestris* from 3 h after addition of salt especially *RS*. These results in comparison between the two cell lines show a stronger induction of *JAZ1/TIFY10a* correlated with elevated induction of *NHX1* (salt adaptation) in *V. rupestris* as compared to *V. riparia*.

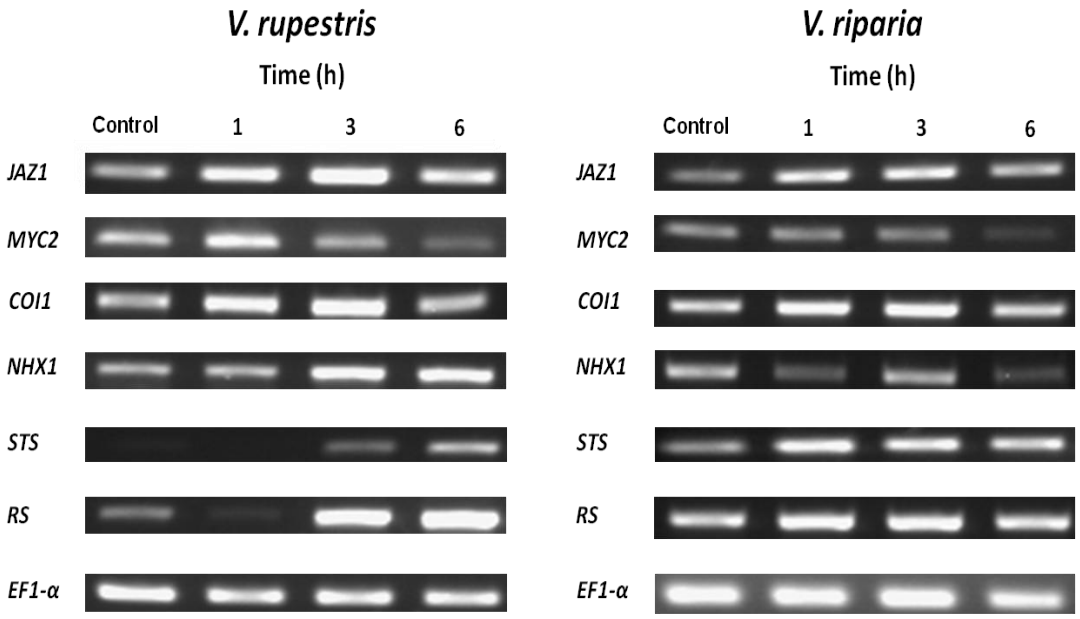


Fig. 23: Time course of induction for transcripts of *JAZ1/TIFY10a*, *MYC2*, *COI1*, *NHX1*, *STS*, and *RS* by 155 mM NaCl in *V. rupestris* and *V. riparia*. Elongation factor 1α (*EF1* α) was used as internal standard. One representative example of three independent experiments is shown.

3.2.4 Phenidone can inhibit the induction of *JAZ1/TIFY10a* genes by salt

To further investigate, whether the induction of *JAZ1/TIFY10a* by salt requires

JA, the synthesis of JA was blocked by phenidone, an inhibitor of lipoxygenases that trigger early steps in the octadecanoid pathway. *Vitis* cells were pretreated with 2 mM phenidone for 30 min before adding 155 mM NaCl for 1, 3, and 6h. As a control cells were pretreated with 2mM phenidone or 0.1% Tween 20 for 30 min before 0mM NaCl was added for 1h (**Fig. 24**). Phenidone inhibited the induction of *JAZ1/TIFY10a* by salt stress very efficiently, especially in *V. rupestris*. Similar to the results obtained for aspirin (**Fig. 24C**), this result suggests that JA signaling (more specifically: JA synthesis) is necessary to mediate the induction of *JAZ1/TIFY10a* expression in response to NaCl.

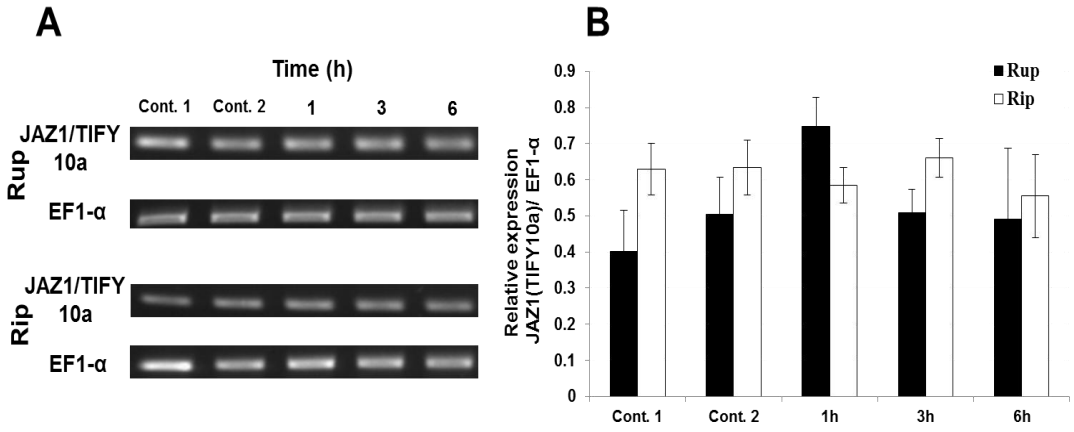
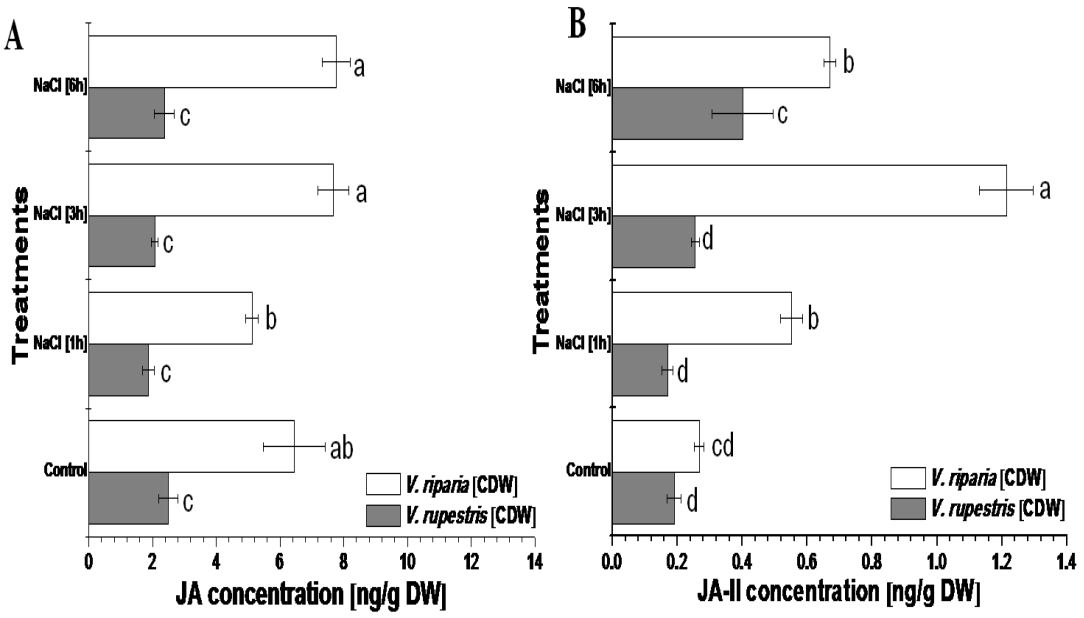


Fig. 24: Effect of phenidone on the induction of *JAZ1/TIFY10a* transcripts under salt stress at different time points in *V. rupestris* (Rup) and *V. riparia* (Rip). A Representative time course of transcript abundance of cells pretreated with phenidone for 30 min in response 155 mM NaCl for 1, 3, and 6 h. As a control cells were pretreated with 0.1% Tween 20 as a control 1 or phenidone as a control 2 for 30 min before 0 mM NaCl was added for 1h. B Time course of transcript abundance of *JAZ1/TIFY10a* in both cultivars relative to elongation factor 1α. The data represent averages from three independent experimental series; error bars represent standard errors.

3.3 Accumulation of phytohormones under salinity in *Vitis*

3.3.1 Phytohormones accumulate differently during salinity stress

To clarify the role of the JA pathway in comparison to the adaptive role of ABA, endogenous levels of JA, JA-Il, and ABA were followed in response to 155 mM NaCl in cells at day 5 after subcultivation as shown in **Figure 25**. For JA, the ground level in *V. riparia* was more than twice of that found in *V. rupestris* and increased further by about 25% from 3 hours after the onset of salt treatment (**Fig. 25A**). In contrast, *V. rupestris* maintained a very low level of JA, even after prolonged salinity. The difference was even more pronounced, when the highly bioactive JA-Il was measured (**Fig. 25B**). Here, salt induced a fourfold increase for *V. riparia*, whereas *V. rupestris*, after 6 h of salt stress had just reached the level of JA-Il found in unchallenged control cells of *V. riparia*. For ABA (**Fig. 25C**), the situation was reversed – here, in *V. rupestris*, the induction was more pronounced as compared to *V. riparia*, although both lines accumulated comparable levels of ABA from 3 h after the onset of treatment. Thus, the improved salinity adaptation in *V. rupestris* correlated with a clear reduction in salt-induced formation of JA, and most pronounced JA-Il. In contrast to JA, IAA and SA were much higher in unchallenged *V. rupestris* as compared to *V. riparia* (**Fig. 25 D and E**). Salinity made both hormones decline in *V. rupestris*, whereas in *V. riparia* there was no significant change.



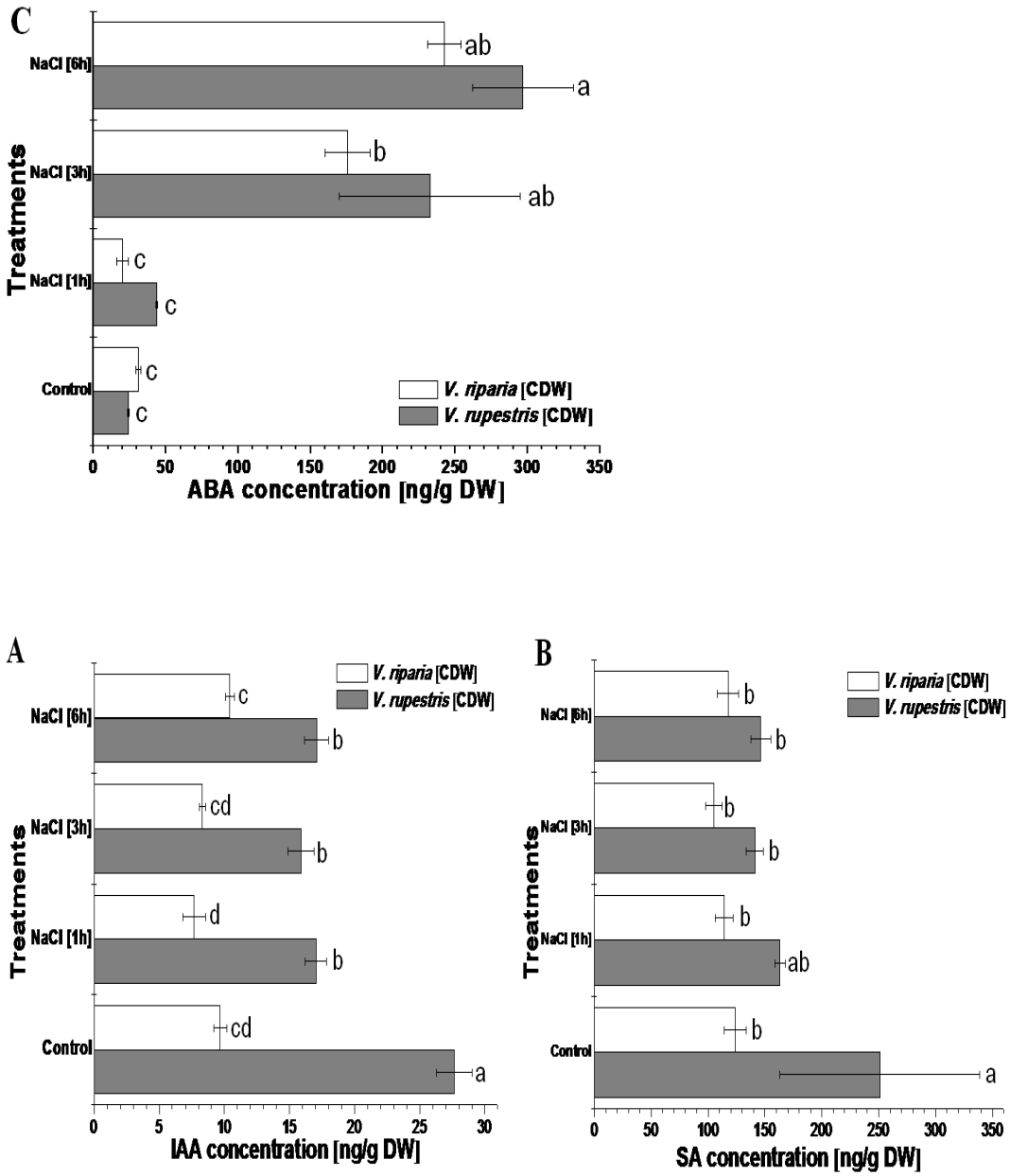


Fig. 25: Representative time-course of endogenous JA (A), JA-Il (B), ABA (C), IAA (D), and SA (E) under 155 mM NaCl at 1, 3, 6h in both cell lines. Control samples were treated by H_2O for 1h. Error bars represent SE, and different letters indicate significant differences among treatments (LSD (P<.05)).

3.3.2 Sign reversal in the calcium effect on salt-induced hormone levels

Since the two cell lines showed qualitative differences in Ca^{2+} accumulation (**Fig. 18**), we investigated the effect of either $GdCl_3$ or $CaCl_2$ on salt-induced

accumulation of phytohormones as shown in **Figure 26**. In *V. riparia*, 0.75 mM of $GdCl_3$ triggered a strong accumulation of JA and JA-Il within 1 hour (**Figs. 26A, B**). When salt was combined with the $GdCl_3$ treatment, this progressively quelled the $GdCl_3$ triggered increase such that the JA and JA-Il levels from 3 to 6 h after induction had returned to the ground levels (**Figs. 26A, B**), whereas they remained elevated when the salt treatment was administered in the absence of $GdCl_3$ (**Figs. 25A, B**). Similar to $GdCl_3$, 1 mM $CaCl_2$ increased the ground levels of JA and JA-Il. For ABA, neither $GdCl_3$ nor $CaCl_2$ caused any significant accumulation (**Fig. 26C**). Moreover, the salt-induced accumulation of ABA (**Fig. 25C**) was suppressed by $GdCl_3$. In *V. rupestris*, where JA did not accumulate in response to salt (**Fig. 25A**), $GdCl_3$ and $CaCl_2$ reduced the level of JA (**Fig. 26A**) and JA-Il (**Fig. 26B**) even further, however, $GdCl_3$ significantly increase the accumulation of JA-Il after 3 h in response to salt. In the same line, the level of ABA was not elevated in *V. rupestris* by $GdCl_3$ as well as by $CaCl_2$, but the strong accumulation of ABA in response to salt in this cell line (**Fig. 25C**) was even further promoted by $GdCl_3$ (**Fig. 26C**). In summary, the response of salinity-triggered accumulation of JA/JA-Il shows differential sensitivity to $GdCl_3$ between the two cell lines, and for salinity-triggered accumulation of ABA, there is even a sign-reversal.

A pretreatment with $GdCl_3$ cancelled the salt-induced decrease of IAA and SA in *V. rupestris* (compare **Figs. 25D and E** with **Figs. 26D and E**). However, IAA was significantly induced when $GdCl_3$ was applied alone or with NaCl for 1 h. In contrast, *V. riparia* displayed no changes in both hormones comparing to NaCl alone. $CaCl_2$, on the other hand, resulted in IAA reduction only after 3 h of application *V. rupestris* (**Fig. 26A and B**).

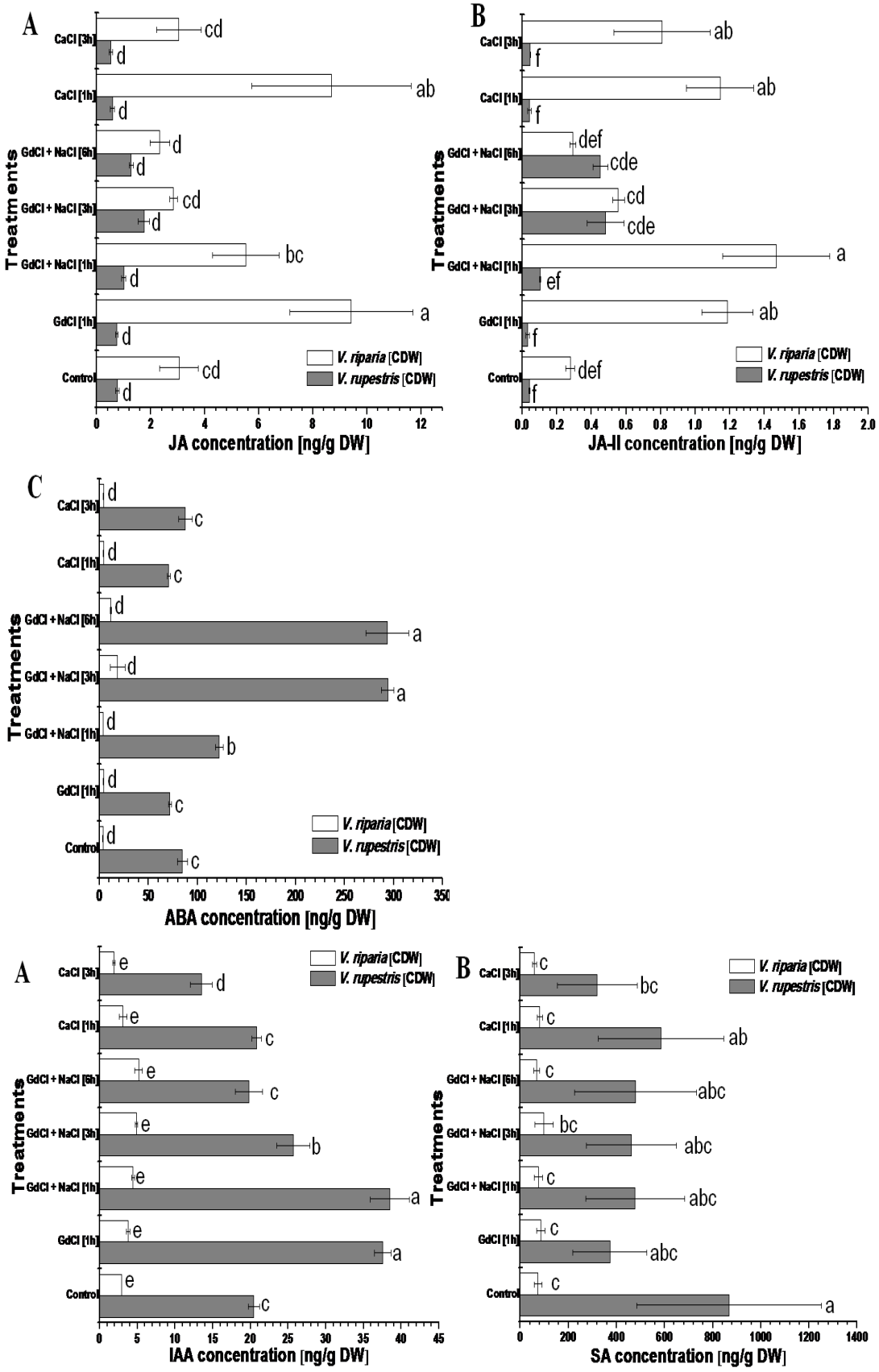


Fig. 26: Endogenous JA (A), JA-Il (B), ABA (C), IAA (D), and SA (E) under different

treatments at different time points in vitis cells elicited by 155 mM NaCl after 0.75mM of $GdCl_3$ pretreatment for 2 min or by 1 mM $CaCl_2$ alone for 1 and 3h. Control samples were treated by H_2O or 0.75mM of $GdCl_3$ for 1h. Error bars represent SE, and different letters indicate significant differences among treatments (LSD ($P<0.05$)).

3.4 Stilbene accumulation in response to salt

In grapevine, the accumulation of stilbenes represents an important event in defense. For the grapevine cell system used in the current study, especially the accumulation of the highly cytotoxic δ-viniferin has been shown to herald defense-related cell death (Chang *et al*., 2011). Since defense-related signaling and salinity-induced signaling share several events (Ismail *et al*., 2012), we used salinity triggered accumulation of stilbenes as indicator for damage-related signaling. **Figure 27** shows the accumulation of the stilbene-glycoside α-piceid (**Fig. 27A**), the aglycone *trans*-resveratrol (**Fig. 27C**), and the highly toxic oxidative dimer δ-viniferine (**Fig. 27B**) in response to salinity. Salt-stressed *V. riparia* cells accumulated piceid in levels comparable to the piceid triggered preceding defense-related PCD (Chang and Nick 2012). In contrast, *V. rupestris* did not accumulate α-piceid (**Fig. 27A**). *V. riparia* cells also accumulated δ-viniferin (the toxic oxidative dimer of resveratrol) whereas *V. rupestris* cells did not (**Fig. 27B**). Compared to the situation in defense-related cell death (Chang and Nick, 2012), the levels of salt-induced δ-viniferin were lower, however (around 15%). For *trans*-resveratrol, only very low levels (around 0.020-0.030 µg/g) were measured with higher accumulation in *V. riparia* (**Fig. 27C**), which is about two orders of magnitude lower than the values obtained for defense-related cell death (Chang and Nick, 2012). These results show that *trans*-resveratrol is almost absent under salinity stress. Furthermore, the stilbene pattern for salinity (α-piceid, δ-viniferin) differs from that for defense-related cell death (*trans*-resveratrol, δ-viniferin). Finally, stilbene output (a response to oxidative stress, see Chang and Nick, 2012) is quelled in *V. rupestris*, but not in *V. riparia*.

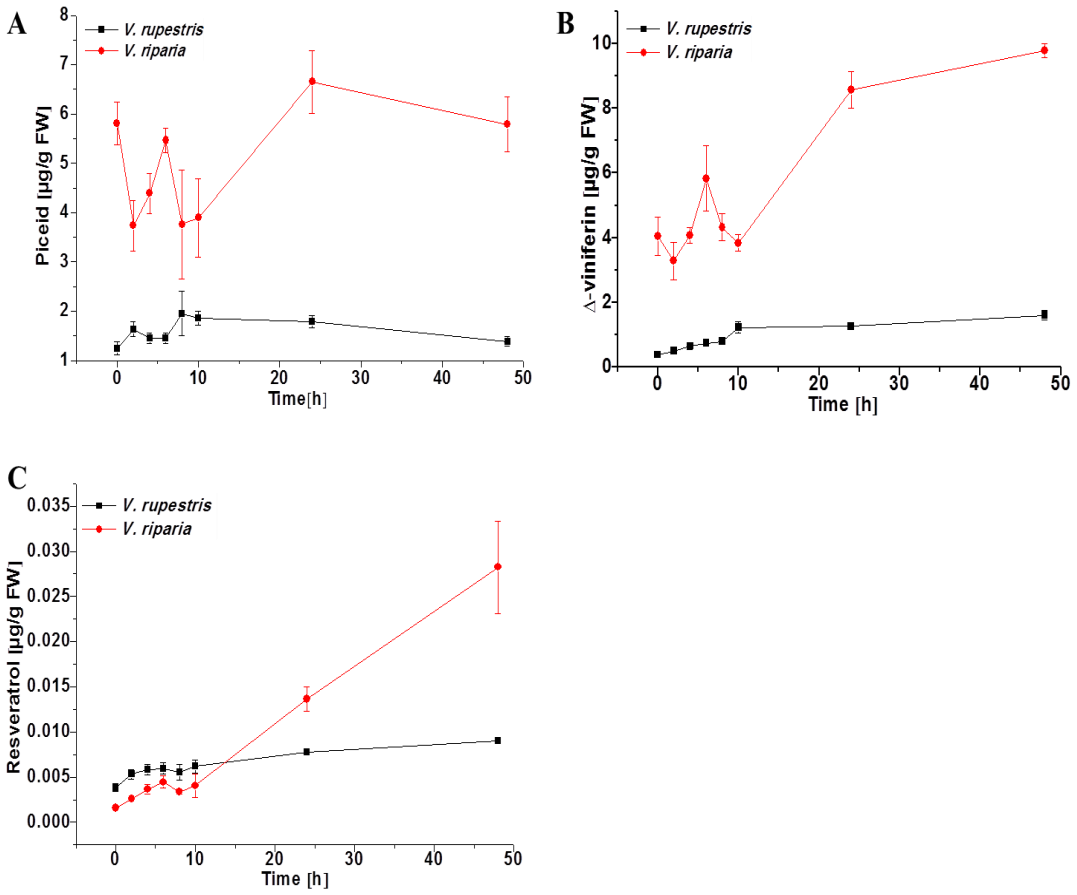


Fig. 27: Grapevine stilbenes under salinity. The induction of Piceid (A), δ-viniferin (B), and Resveratrol (C) (µg/g) in response to 155 mM NaCl in both cell lines at different time points. Values are means ± SE (n = 3).

4 Discussion

Salinity does not stress only plant but also biologists who sincerely try to discriminate its tangled elements. To disentangle plant stress signaling represents a scientific challenge: Numerous events overlap between *stress adaptation* and *stress damage*, on one hand, and the large number of only partially identified players make it difficult to delineate a clear-cut line between both events. In the present work, we investigated, in parallel, two grapevine cell lines differing in their performance under salinity. Our basic approach was to correlate differences of early salinity-induced responses with the differential adaptation of the two cell lines. This should allow to assign these events to either *stress adaptation* or *stress damage*.

4.1 Adaptive response versus cellular damage

The following cellular events were observed in response to salt stress: extracellular alkalinisation, which can be completely or partially inhibited by Gd^{3+} ions, induction of *JAZ1* and *JAZ3*, (weak) induction of *MYC2* and *COI1*, induction of *NHX1*, induction of *RS*, formation of ROS, and salt-tolerance monitored as increase in PCV despite challenge by NaCl. These responses could either be adaptive or they could merely report cellular damage caused by NaCl.

For instance, both biotic and abiotic stress factors induce an oxidative burst. The resulting reactive oxygen species are highly destructive and can damage DNA, proteins, lipids, and carbohydrates (Gill and Tuteja, 2010). On the other hand, ROS can be important signal transduction molecules acting in stress adaptation (Miller *et al*., 2010).

To discriminate between adaptive and damage events, we used two grapevine cell lines that were expected to differ in their salt tolerance, because they originated from two species with different humidity requirements. In fact, for 50 mM and 85 mM NaCl, *V. rupestris* displays a

clear adaptation of growth after a lag of a few days, whereas this adaptive response is weaker (50 mM NaCl) or even absent (85 mM) in *V. riparia* (**Fig. 11**). Based on this reference, adaptive responses should be more pronounced in *V. rupestris*, whereas events found to be stronger in *V. riparia* are likely to report damage rather than adaptation. In fact, extracellular alkalinisation was stronger in *V. rupestris*. The same was true for the induction of *JAZ1* and *JAZ3*, *NHX1*, and *RS*. In contrast, generation of ROS was stronger in *V. riparia*, suggesting that this oxidative burst represents a manifestation of cellular damage rather than an adaptive response.

In this work, three representatives of the *Vitis JAZ* family were cloned (**Fig. 21**) and their expression under NaCl stress was followed (**Fig. 23A**). We observed a transient induction of two *JAZ*-members (*JAZ1* and *JAZ3*) that was more pronounced in *V. rupestris* (**Fig. 22**). In parallel, transcripts of *MYC2*, a transcription factor that in the absence of jasmonate-Ile is inhibited by complexation with JAZ-proteins, are slightly elevated as well as those of *COI1*, a jasmonate signaling factor that responds to jasmonates (**Fig. 23**). Since the activity of jasmonate signaling is primarily regulated at the posttranslational level (e.g. by proteolytic degradation of the inhibitory JAZ-proteins), these transcript responses of *JAZ1* and *JAZ3* as well as of *MYC2* and *COI1* are probably not directly involved in the signaling *per se*, but must be interpreted as elements in the complex feedback regulation that tunes the competence for JA-signaling with the activity of the signaling pathway. Nevertheless, these responses can be used as readout to monitor the activity of jasmonate signaling.

The differential responsiveness of the *JAZ* in the two cell lines could either be caused by differential activation of jasmonate synthesis or, alternatively, by differential jasmonate-sensitivity of signaling. To differentiate between these two possibilities, the cell lines were treated with exogenous JA. The application of JA could mimick the effect of salt stress on the induction of *JAZ* transcripts (**Fig. 22B**) with virtually the same response observed for both cell lines. This suggests that the signaling evoked by JA proceeds in the same manner and intensity. Thus, the differences in salt-induced *JAZ* induction

must reside upstream of JA-signaling, for instance, in different levels of JA-synthesis.

Pre-treatment with aspirin (**Fig. 22C**) or phenidone (**Fig. 24A,B**) was able to suppress the induction of *JAZ* genes in response to salt stress suggesting that salt-induced JA-signaling is necessary for the *JAZ*-response. Both agents have been used to silence JA-dependent signaling, but have a different mode of action: Salicylic acid and its acetylated derivative, aspirin, seem to act as antagonists of JA-signaling (Lorenzo and Solano, 2005; Balbi and Devoto, 2008), whereas phenidone is thought to block jasmonate synthesis through inhibition of the lipoxygenase that converts α-linolenic acid (Bruinsma *et al*., 2010).

The concentration of aspirin used in the present study (1 mM) is in the range of those used during most studies on defence signaling (e.g. Mur *et al*., 1996). For phenidone, a classical inhibitor of lipoxygenases, 2 mM have been shown to block the accumulation of jasmonates in Brussels sprout (Bruinsma *et al*., 2010). This inhibitor has first to be converted by lipoxygenase into a semioxidised one-electron derivative that is responsible for the inhibition (Cucurou *et al*., 1991). Due to this end-product dependent mode of action, the molar concentration of phenidone has to exceed that of its target by a factor of 10-30 times. In addition to JA synthesis, phenidone blocks the formation of volatile compounds such as hexanal and cis-hexenylacetate that function in the chemical communication with herbivoral insects and their parasitoids (Bruinsma *et al*., 2010), a side effect that is not of relevance in the context of a cell culture.

Salt signaling shares several events with biotic defence including apoplastic alkalinisation (**Fig. 19**) that is sensitive to Gd^{3+}, and transient induction of *JAZ* transcripts (**Fig. 23**). Apoplastic pH has been used to monitor rapid defence responses upstream of gene expression (Felix *et al*., 1993; Felle and Hanstein, 2002). A widely accepted model (Scheel, 1998) proposes that pathogen-derived elicitors bind to a host receptor activating Ca^{2+} influx. Together with Ca^{2+}, protons can permeate from the acidic apoplast into the neutral cytoplasm (Jabs *et al*., 1997). The resulting apoplastic alkalinisation is

therefore a readout for the activity of the Ca^{2+} channel, but does not convey defence signaling itself. In the *V. rupestris* cell line, the activation of apoplastic alkalinisation by the Harpin elicitor could be blocked by gadolinium, a classical inhibitor of mechanosensitive Ca^{2+} channels (Qiao *et al*., 2010). Gadolinium sensitivity could be confirmed also for salt induced alkalinisation (**Fig. 19**) suggesting that the same mechanosensitive Ca^{2+}-channel is at work. Consistent with this conclusion, self-reporting Arabidopsis thaliana cells expressing GFP-based pH and Ca^{2+} monitors either salt and drought stress were shown to trigger apoplastic alkalinisation as well as Ca^{2+} influx (Gao *et al*., 2004).

Whereas early responses of transcription to salt and Harpin (*JAZ1* and *MYC2*) showed similar pattern (Ismail *et al*., 2012), there were clear differences in the expression of downstream genes. For instance, transcripts for the phytoalexin synthesis genes *STS* and *RS* were induced much later after salt stress (from 3 h) as compared to a much earlier induction (about 0.5 h) after Harpin treatment (Qiao *et al*., 2010). Moreover, the pattern of transcript abundance is inverted – whereas in case of Harpin, *STS* is most responsive, for salt stress it was *RS* that showed the strongest induction. These differences suggest that both kinds of stresses use separate pathways.

4.2 The Faster the Early Response, the Better the Adaptation

We observed that the fluxes of Na^+ (resulting in stress damage), and Ca^{2+} and H^+ (acting as stress signals) proceeded in both lines, however, with different spatiotemporal pattern (signatures). When Na^+ ions are administered to a plant, they enter by passive transport through the plasma membrane NSCCs (mainly, DA-NSCCs and VI-NSCCs) within seconds (Tester and Davenport, 2003). The two studied cell lines showed different Na^+ flux pattern (**Fig. 16**). Since the elevated intra- and extracellular Na^+ partially inhibits the K^+ outward rectifiers (KORs), the higher initial uptake of Na^+ during phase I in *V. rupestris* might prevent the loss of cellular K^+, maintaining cellular K^+/Na^+ homeostasis under stress (Shabala *et al*., 2006).

The rapid uptake of Na^+ in *V. rupestris* during phase I would also contribute to overcome osmotic loss of water (Munns and Tester, 2008) and might contribute to the pronounced drought tolerance of this species.

The earliest cellular response to salinity seems to be a rapid increase of free cytosolic Ca^{2+} within 1 to 5 sec either through influx channels situated in the plasma membrane or through release from internal stores, especially the vacuole (Donaldson *et al*., 2004; Knight *et al*., 1997). Interestingly, *V. riparia* exhibited a sharp drop of cellular Ca^{2+} content concomitantly with phase I of Na^+ uptake (**Fig. 18A**) consistent with a release of Ca^{2+} from the apoplast. A similar decrease of cellular Ca^{2+} content had been described for mesophyll tissue of *Vicia faba* L. leaves and for barley roots, but was absent in protoplasts derived from these cells, a phenomenon which was attributed to Na^+/Ca^{2+} and H^+/Ca^{2+} ion exchange in the cell wall (Cuin and Shabala, 2005; Shabala and Newman, 2000). A pretreatment with $GdCl_3$ even amplified this salt-induced Ca^{2+} drop as compared to NaCl alone (**Fig. 18C**). This inhibitor experiment suggests that a certain salinity-induced Ca^{2+} influx does exist. However, it seems to proceed with low efficiency and is overrun by the Ca^{2+} loss from the cell wall. In fact, when excess Ca^{2+} was applied alone or in combination with NaCl to saturate binding sites in the cell wall, Ca^{2+} influx became visible in *V. riparia* but its activity was low (**Fig. 18E**) correlated with a low rate of Na^+ and H^+ influx under salt stress (**Fig. 16 and Fig. 19B**).
In contrast, the pattern of Ca^{2+} influx in NaCl-treated *V. rupestris* showed a mirror-image: Here Ca^{2+} content changed in a manner similar to Na^+ uptake, with a clear induction in the first 10 min. This correlates with the kinetic efficacy of NSCCs and their fast uptake of both Na^+ (**Fig. 16**) and H^+ (**Fig. 20A**), which might sequester them from occupying potential Ca^{2+}-binding sites in cell wall. This effect would be complemented by a more efficient influx of apoplastic Ca^{2+} into the cytoplasm (**Fig. 18A, B, and D**). Similar to the elevated Na^+ uptake during phase I, this fast Ca^{2+} uptake might be linked to the pronounced drought-tolerance of *V. rupestris*, and thus be correlated more with osmotic rather than ionic stress (Knight *et al*., 1997).

Salinity-induced cytosolic Ca^{2+}, in turn, activates the plasma membrane ATPases mediated by Ca^{2+}-CaM-dependent protein kinases, restoring membrane voltage after Na^{+}-induced depolarization, and maintaining membrane integrity, and ionic homeostasis (Klobus and Janicka-Russak, 2004; Shabala *et al*., 2006). Additionally, the elevated cytosolic Ca^{2+} promotes H^{+} influx and inhibits AHA1 (a P-type proton ATPase catalyzing H^{+} efflux) resulting in apoplastic alkalinization (Wolf *et al*., 2012). Kinetic differences in cytosolic Ca^{2+} spiking should therefore become manifest as kinetic differences of apoplastic pH. In fact, salt stress induces a rapid apoplastic alkalinisation that differs between the two cell lines with respect to kinetics (pH_{max50} 12.9 and 9.3 min for *V. rupestris* and *V. riparia*, respectively) and dose-dependency (**Fig. 19C and D**). Consistent with the more active early Ca^{2+} influx in response to NaCl (155 mM) the lag time for apoplastic alkalinisation was only 15 sec in *V. rupestris*, whereas the sluggish Ca^{2+} influx in *V. riparia* was correlated with a longer lag of alkalinisation of 36 sec (**Fig. 19**). As to be expected, salinity-induced Ca^{2+} fluxes act upstream of proton fluxes. The lanthanoid $GdCl_3$ completely blocked (*V. riparia*) or at least impaired (*V. rupestris*) apoplastic alkalinisation. Cytoskeleton-tethered mechanosensors participate in the perception of osmotic stress signals (Türkan and Demiral, 2009) and trigger a rapid increase of cytosolic Ca^{2+} within 1 sec, followed by apoplastic alkalinization within 12 sec. Both ion fluxes are blocked by a pretreatment with the lanthanoid La^{3+} (Monshausen *et al*., 2009). Thus, the earliest events of the cellular salinity response are shared with those triggered by mechanic challenge. Apoplastic alkalinization might promote adaptive events including activation of wall-consolidating enzymes such as pectin methylesterase, or, on the other hand, inhibition of expansins involved in cell-wall expansion (Wolf *et al*., 2012). In *V. rupestris*, alkalinization was much more pronounced (ΔpH_{ext} ~ 0.4) (**Fig. 19A**) compared to *V. riparia* (ΔpH_{ext} ~0.25) (**Fig. 19B**) suggesting that adaptive arrest of cell expansion may underlie the initial decrease of growth rates in NaCl-stressed *V. rupestris* in contrast to *V. riparia* (**Fig. 11A and B**). Additionally, the Ca^{2+}-induced H^{+} influx might feedback on Ca^{2+} signaling by

affecting Ca^{2+} affinity for CaM (Busa and Nuccitelli, 1984).

As additional adaptive event, the salt-induced free cytosolic Ca^{2+} promotes the SOS3/SOS2 which phosphorylates the membrane-bound Na^+/H^+ antiporter, SOS1 causing Na^+ efflux (Qiu *et al*., 2002). The more efficient Ca^{2+} uptake in *V. rupestris* should therefore cause a subsequent decline in cellular Na^+ content, which would be a mechanistic explanation for the drop in Na^+ content during phase II (**Fig. 16**). Since SOS2 also regulates the vacuolar Na^+ transporter NHX1, in concert with other members of this family of transporters maintaining Na^+ and K^+ homeostasis even under drought and salt stress (Apse *et al*., 1999; Gaxiola *et al.*, 1999; Qiu *et al*., 2004), the more active Ca^{2+} influx in *V. rupestris* should be followed by induction of NHX1 transcripts. We found strong induction of NHX1 transcripts after 3 h of salinity stress in *V. rupestris*, but not in *V. riparia* (**Fig. 23**). The induction of the vacuolar NHX1 transporter in conjunction with the sensitivity of Na^+/H^+ antiporters to cytosolic pH (Padan *et al*., 2001) indicates that, in *V. rupestris*, the Na^+ entering the cell during phase III of uptake is efficiently compartmentalized into the vacuole, whereas in *V. riparia*, more Na^+ would remain trapped in the cytoplasm.

On the other side, elevated levels of apoplastic Ca^{2+} strongly and partially block the main gates of Na^+ entry, VI-NSCCs and DA-NSCCs, respectively (Demidchik and Maathuis, 2007). When external Ca^{2+} was added to the *Vitis* cells, it significantly reduced Na^+ influx in both cell lines, with higher efficacy in *V. rupestris* (**Fig. 17A and B**). However, this positive effect was reverted in phase III, where Ca^{2+} significantly increased Na^+ uptake in both cell lines, especially in *V. riparia*. Since the two lines showed different Ca^{2+} influx pattern under all treatments (**Fig. 18**), elevated levels of cytosolic Ca^{2+} at the beginning of phase III (from 15 min) in both cell lines might activate the HA-NSCCs that are weakly selective for monovalent cations with a late activation ~40–60 min (Davenport and Tester, 2000; Demidchik *et al*., 2002). Again, when $GdCl_3$ was applied 2 min before salt treatment, Na^+ uptake was reduced during both phases I and II in both lines (being less effective than Ca^{2+}). However, unlike the Ca^{2+} signature, this inhibitory effect was pronounced during phase III only in *V. riparia*, but absent in *V. rupestris* (**Figs.**

17C and D). In Arabidopsis root epidermal protoplasts, already 100 µM $GdCl_3$ were sufficient to suppress 95% of Ca^{2+} influx carried by HA-NSCCs (Demidchik *et al*., 2002) indicating that the HA-NSCCs may be the predominant type of channels on *V. riparia* plasma membranes. In contrast, the two more effective NSCCs (DA-NSCCs and VI-NSCCs) might represent the major type of channels in *V. rupestris.* This work hypothesizes that the apparent strong impact of plasma membrane NSCCs and their kinetic activities on the early stress responses of plants which has to be tested through electrophysiological approaches. Since these channels transport Ca^{2+} and H^+ and thus determine the signatures of these signals, NSCCs activity acts upstream of Ca^{2+} and H^+ flux and signaling activity and thus represent the earliest events of adaptive signaling.

4.3 The Tighter the control of JA/JA-Ile accumulation, the better the adaptation

ABA and JAs play central roles in plant adaptation to stress, constraining root growth, cell cycle, photosynthesis and transpiration, and thus prioritizing defense metabolism over growth. Furthermore, they play essential and overlapping roles for the induction of stomatal closure (Suhita *et al*., 2004). Upon osmotic stress, ABA accumulates resulting in upregulation of osmotic stress-responsive genes such as the ABA-responsive element/complex (ABRE) (Ishitani *et al*., 1997). This ABA accumulation is clearly adaptive, since the ABA deficient *aba*-mutants of Arabidopsis perform poorly under drought or salt stress or even die (Zhu, 2002). However, ABA seems to be not the only adaptive signal. ABA-deficient mutants of maize and tomato showed generally the same leaf growth rates as wildtype plants in drying or saline soils, and Arabidopsis *DREB1A* and its functional rice ortholog *OsDREB1A* promote tolerance to drought, high-salt, and freezing stresses independently of ABA (Dubouzet *et al*., 2003). As candidate for this ABA-independent signal, gibberellins (GAs) have been suggested (Munns and Tester, 2008). However, recently, OsDREB1A was found to by upregulated downstream of OsbHLH148 in a JA-dependent fashion, whereby OsbHLH148 is suppressed

by OsJAZs (especially OsJAZ1 and 3). Under JA signaling, OsbHLH148, OsJAZ, and OsCOI1 assemble into a complex resulting in the degradation of OsJAZ proteins via the 26S proteasome and thus the release of OsbHLH148 (Seo *et al*., 2011). In the current study, the two cell lines accumulated comparable levels of ABA (**Fig. 25C**). However, in the salt-sensitive *V. riparia* the accumulation of JA and JA-Il was fourfold and sixfold, respectively, as compared to the more osmotic-tolerant *V. rupestris* (**Fig. 25A and B**). Moreover, in *V. riparia*, JA-Il was induced earlier (after 1 h), while in *V. rupestris* 6 h were required until a first significant induction became visible and even then just reached the ground level of JA-Il observed in *V. riparia*. Synthesis and accumulation of JA have been linked with PCD in infected tobacco leaves (Kenton *et al*., 1999), and Arabidopsis protoplasts (Zhang and Xing, 2008), and both leaves and cell-suspension cultures of *Vitis vinifera* L. cv. Lemberger (Repka *et al*., 2004). Interestingly, JA does not promote PCD only in plant cells, but also in cancer cells and sharing several similar effects in both cells including ROS generation, MAPK induction, cell cycle arrest, and PCD (Flescher, 2007). In the *V. rupestris* cell line, the elicitation of cell death by the bacterial effector Harpin was preceded by accumulation of both resveratrol and its toxic oxidative dimer δ-viniferin (Chang and Nick, 2012). However, the same line produced very little of these stilbenes in response to salinity stress (**Fig. 27**). In contrast, salt-stressed *V. riparia* cells accumulated high amounts of δ-viniferin following the accumulation of JA and JA-Il (**Fig. 27B**). Cellular damage monitored by fluorescent detection of superoxide radicals was clearly ameliorated in *V. rupestris* as compared to *V. riparia*, where superoxide radicals were dramatically increased from two hours after the onset of salt stress (**Fig. 20**). Again, this response clearly diverges from the situation observed for biotic stress: In the *V. rupestris* cell line, Harpin treatment was found to induce a massive oxidative burst. This seems to be connected to the oxidation of resveratrol into its highly toxic derivative δ-viniferin (Chang *et al*., 2011), probably involved in the execution of hypersensitive cell death. Programmed cell death is an efficient strategy to block a biotic intruder, but would be completely inappropriate for salt

adaptation. The findings from the current study link salt tolerance in *V. rupestris* with suppression of oxidative burst, reduced accumulation of JA and JA-Il, early induction of *JAZ1*, induction of *NHX1*, suppression of stilbene accumulation, and reduced cell death. In contrast, the salt-sensitivity of *V. riparia* is linked with an early oxidative burst, massive accumulation of JA and JA-Il, accumulation of stilbenes, and cell death, whereas induction of *JAZ1* transcripts and *NHX1* are suppressed (**Fig. 23**). Since MeJA (10 µM) promotes the accumulation of *cis*- and *trans*-resveratrol and their glycosides, piceid, in *Vitis vinifera* cell suspension (Krisa *et al*., 1999; Tassoni *et al*., 2005). Resveratrol, in turn, can efficiently induce, in grapevine suspension cells, oxidative burst, actin bundling, accumulation of cell-death related PR5, and cell death (Chang *et al*., 2011), suggesting that the JA pathway triggers PCD via stilbenes.

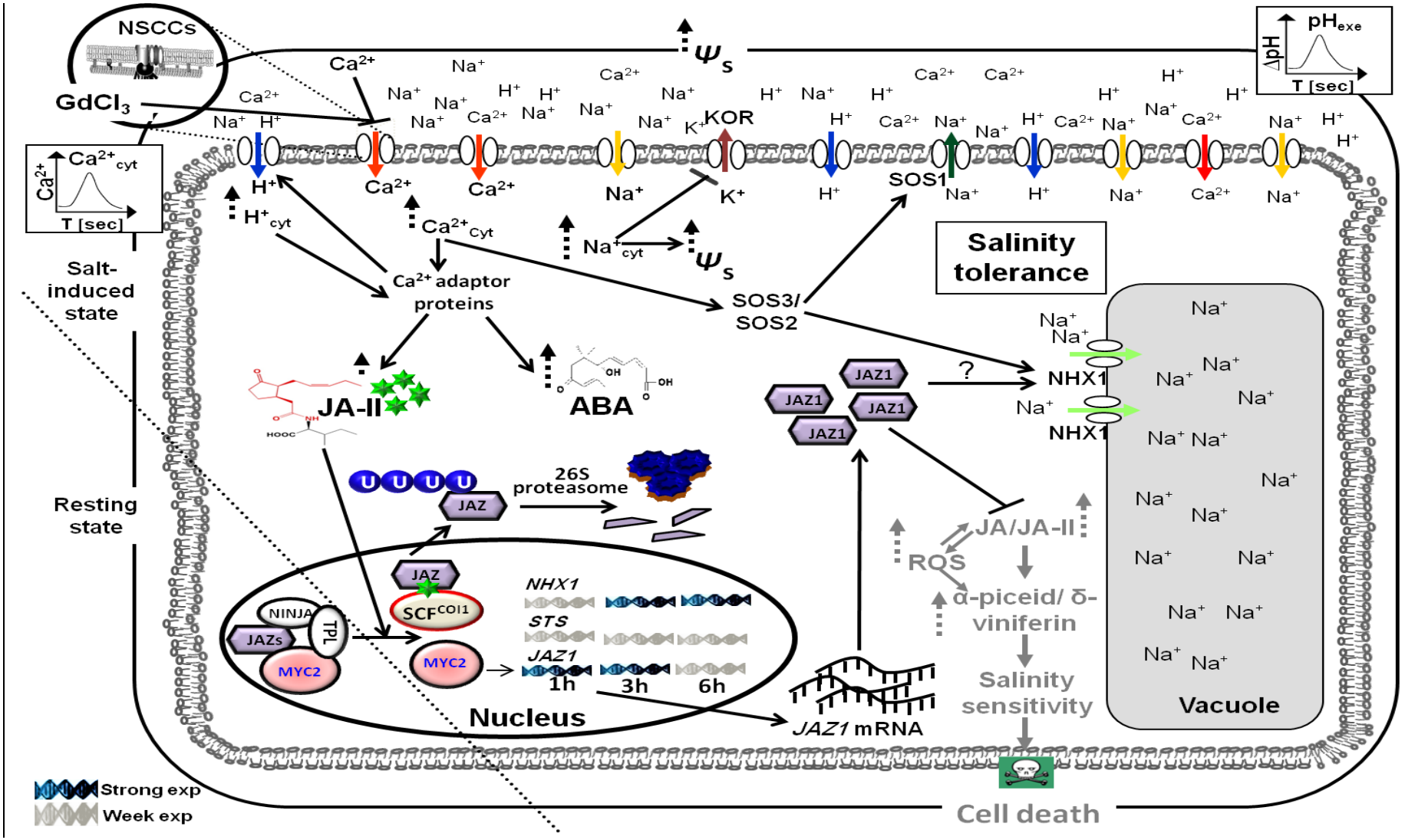
This leads to a model, where constrained JA accumulation and signaling are a precondition to escape salinity-induced cell death and to activate salinity adaptation. As expected from this model, the JAZ proteins as negative regulators of JA signaling are crucial for the tolerance salinity and drought: When *GsJAZ2* from *Glycine soja* was over-expressed in Arabidopsis, the resultant mutant performed better than wildtype under salinity with a significant accumulation of *NHX1* after 6 h (Zhu *et al*., 2012). Furthermore, in a rice mutant over-expressing *OsbHLH148*, both *OsDREB* and *OsJAZ* genes were highly induced upon drought stress (Seo *et al*., 2011). Over-expression of *OsJAZ9* significantly improved salt and drought tolerance of rice (Ye *et al*., 2009). Interaction of JAZ/TIFY proteins with multiple transcription factors that play central roles of plant hormones: for JA; such as MYC2, MYC3, MYC4, for GAs; DELLA, for Ethylene; EIN3 and EIN3-LIKE 1(EIL1), and probably other transcription factors (Kazan and Manners, 2012) gives these JAZ proteins a key roles in orchestrating cross-talk among plant hormones and therefore their actions that exclusively affecting all aspects of plant cellular events. Of course, the crucial roles of JAs in improving salinity or drought stress, such as inducing stomatal closure, OsbHLH148 activity, and even the induction of *JAZ* genes cannot be ignored. However, from the published

record and the results of our current study, the existence of efficient suppression machinery, like JAZ proteins, that tightly control JAs levels should be considered as a pivotal factor for stress adaptation. The accumulation of JA, JA-Il, and ABA depends on Ca^{2+} homeostasis, since in *V. riparia* both excessive Ca^{2+} as well as inhibition of Ca^{2+} influx by $GdCl_3$ can induce JA and JA-Il in the salt-sensitive *V. riparia*, whereas in the salt tolerant *V. rupestris* they induce ABA instead with some cross-talk on the accumulation of SA (**Fig. 25 D, E and Fig. 26 A-C**). Calcium homeostasis might be the factor that links the early salinity-induced events (ion-fluxes, apoplastic alkalinization) with later events of *stress damage* (JA, JA-Il, stilbenes, dominating in *V. riparia*) versus *stress adaptation* (ABA, SA, *NHX1*, dominating in *V. rupestris*)

4.4 Towards a model for salinity tolerance or sensitivity

In the current study, we addressed the role of JA-signaling in the context of salinity stress. As biological template to assign the investigated cellular events to either salt damage or salt adaptation we compared two grapevine cell lines differing in their ability for salt adaptation. The successful adaptation to salinity stress in *V. rupestris* is correlated with an efficient extrusion of Na^+ during phase II (between 2 and 15 min) accompanied by apoplastic alkalinisation. This is followed by accumulation of ABA, whereas the accumulation of JA and JA-Il were quelled. As a consequence, stilbenes that accumulate in this system in the context of oxidative burst and PCD are suppressed. In contrast, the sensitive *V. riparia* is less efficient in the export of Na^+ during phase II correlated with a lower activitiy of apoplastic alkalinisation. Subsequently, JA and especially JA-Il accumulate as well as the highly cytotoxic oxidative stilbene dimer δ-viniferin. Our data show clearly that JA and especially the biologically potent JA-Il accumulate in the context of salt damage and are quelled in the context of salt adaptation. **Figure 28** summarizes, on the phenomenological level, some of positive or negative events (**Figs. 16, 17, 18, 19, 20, 21, 24, 26, and 27**), that accompany salt adaptation in *V. rupestris* or salt sensitivity in *V. riparia*, respectively.

Since several players relevant for the uptake of sodium and the adaptation to Na^+ stress such as VI-NSCCs, DA-NSCCs, HA-NSCCs, and SOS are regulated by Ca^{2+}, we probed the individual salinity-triggered events with respect to their modulation by exogenous Ca^{2+} or gadolinium ions as *bona-fide* inhibitors of Ca^{2+} channels (Demidchik and Maathuis, 2007; Knight *et al*., 1997). Moreover, we followed the changes of cellular calcium content in response to salinity. This “calcium/gadolinium signature” derived from these experiments can now be used as phenomenological framework to test molecular candidates and to assign their function to early and late events of *stress damage* versus *stress adaptation*.


NSCCs
GdCl3
Ca2+
Na+
H+
K+
KOR
SOS1
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Ca2+cyt
H+cyt
Na+cyt
Salt-induced state
Resting state
Ca2+ adaptor proteins
SOS3/ SOS2
Salinity tolerance
JA-II
ABA
JAZ
26S proteasome
SCFCOI1
NINJA
JAZs
TPL
MYC2
NHX1
STS
JAZ1
1h
3h
6h
Nucleus
JAZ1 mRNA
JA/JA-II
ROS
α-piceid/ δ-viniferin
Salinity sensitivity
Vacuole
Cell death
Strong exp
Week exp

Figure 28: Model for Salinity Tolerance or Sensitivity: In the control situation, JA signaling is suppressed by a multimeric transcriptional corepression complex (JAZ/TIFY, TPL, TPRs, and NINJA). However, upon salinity stress, fluxes of Na^+, Ca^{2+}, and H^+ occur with different spatiotemporal signatures channeling plant cells to either salinity adaptation or cell death. Details are given in the discussion. Colored and Black arrows indicate activation by salinity stress and internal triggers, respectively. On the plasma membrane, arrows with blue, red, and orange represent H^+, Ca^{2+}, and Na^+ influx, while dark green and brown show Na^+ and K efflux, respectively. Light green arrows represent Na^+ influx into vacuole. Dashed arrows (▲ , ▲ , ▲) refer to non-significant (later significant after 6h), significant, and highly significant induction. Black arrows indicate activated signaling pathways while gray ones for inactivation. Black lines indicate repression action. Abbreviations: ABA, abscisic acid; JA, jasmonic acid; JA-Il, jasmonoyl isoleucine; ROS, reactive oxygen species; SA, salicylic acid; *STS*, stilbene synthase; Ψs osmotic potential; KOR, K^+ outward rectifiers; NSCCs, non-selective cation channels; SOS, salt overly sensitive; NHX1, vacuolar Na^+/H^+ exchanger 1; JAZ1, jasmonate ZIM-domain protein 1; TPL, the corepressor TOPLESS; TPRs, TPL-related proteins; NINJA, a Novel Interactor of JAZ (NINJA); U, ubiquitination.

4.5 Conclusion

Stress signalling in general, and salinity signalling in particular, are of great impact for agriculture. A conceptual problem is the fact that the same general players (such as Ca^{2+}, protons, oxidative burst, JA signalling) seem to convey different responses, depending on the context. How can plant cells activate different (appropriate) outputs, although the input is of identical or similar molecular quality? To illustrate the importance of signal signatures, we have chosen two closely related experimental systems (two genotypes of grapevine) that respond differently to salinity stress. We investigate the uptake of ions (Na^{+}, Ca^{2+}), apoplastic alkalinisation as early events, and phytohormones (jasmonates, ABA) and the grapevine phytoalexins stilbenes as late events. We conclude that a tight constraint of jasmonate signalling is required to escape cell death (which would be an adaptive response in a defense context) and to undergo salinity adaptation. To our knowledge we show for the first time a response of the jasmonate isoleucine conjugate in a stress context.

4.6 Outlook

The mechanism of salt tolerance is complex and includes several levels of signaling and adaptation (Parida and Das, 2005) including ion fluxes across the plasma membrane. To dissect their biological roles, patch-clamp technique should be employed. JA-signaling represents a pivotal role in the adaptation to both abiotic and biotic stress factors. JAZ proteins represent an important target to modulate the activity of JA-signaling. Transgenic Arabidopsis thaliana truncated versions of JAZ proteins (where the jas domain had been deleted) produced a dominant JA-insensitive phenotype (Chini et al., 2007; Thines et al., 2007). We suggest that generating transgenic grapes overexpressing JAZ1Δjas will give a huge chance to study

the link between JA-signaling and stress adaptation on the functional level. An interesting aspect will be the link between plasma-membrane located signaling (Ca2+ influx) and plastid-located activation of jasmonate synthesis.

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Publications:

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