CRF2 and its role in cytokinin response and abiotic stress
by

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Abstract

Cytokinin Response Factors are a small family of plant transcription factors originally discovered in a screen for genes whose expression is controlled by cytokinin. Cytokinin is a plant hormone known to be involved in developmental processes such as root-shoot patterning, and delaying senescence. It functions through a modified two-component signalling cascade, which is reviewed in Chapter II. Cytokinin has also been implicated in stress response, which has been examined in both Arabidopsis and tomato with specific CRFs.

Cytokinin has been shown to attenuate signs of physiological stress, such as decrease in photosynthesis and production of reactive species. In studying this, cytokinins have either been externally applied or genes creating cytokinin are overexpressed *in planta*. A key element of this research has been to examine the plant's native levels of cytokinin with response to stress. We see levels increasing when WT seedlings are exposed to salt (150 mmol NaCl). When *crf2* is exposed to the same treatment, we see a direct contrast; there is a decrease in CK levels. RNA-seq was run at the same time and showed differential expression of 8,014 genes between WT and *crf2* when comparing transcripts expressed in control vs salt treatment.

In tomato, cytokinin measurements and RNA-seq were done to analyze differences in cytokinin levels and gene-expression between two types of abiotic stress: salt and oxidative. Salt showed an increase in CK levels while hydrogen peroxide showed a decrease. Additionally, transcriptomes showed 6,643 DE transcripts between treated tissues. These data indicate that

although abiotic stress is frequently thought to underlie similar reactions physiologically, this may not be the case.

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TF	Transcription Factors
CK	Cytokinin
BA	Benzyl Adenine
CRF	Cytokinin Response Factor
DE	Differentially Expressed
AHK	Arabidopsis Histidine kinase
AHP	Arabidopsis Histidine Phospotransfer Protein
AP2	Apetala 2
ERF	Ethylene Response Factor
ARR	Arabidopsis Response Regulator
CKX	Cytokinin Oxidase
DAG	Days After Germination
GUS	β-glucuronidase
H2O2	Hydrogen Peroxide
HK	Histidine Kinase
HPt	Histidine phosphotransfer protein
IPT	Isopentenyl transferase
LOG	Lonely Guy
ROS	Reactive Oxygen Species

RR	Response Regulator
SlCRF	*Solanum lycopersicum* cytokinin response factor
TCS	Two-Component Signaling

Chapter 1: Introduction

Background

Much of the world's arable land is faced with limited crop growth due to poor soil conditions and of that, saline soils comprise a large part. High levels of salt can cause stunted growth, limited yield and even death of the plant (Abdel Latef et al, 2017). It can prevent seedlings from germinating and decreased yield later on. My research was done with two types of plants, Arabidopsis and tomato. *Arabidopsis thaliana*, sometimes called thale cress or mouse-ear cress, but increasingly referred to as Arabidopsis, is a relatively new model species that has taken well to genetic manipulation for research purposes. It is of the order Brassicales, and although related to mustard and broccoli, it is not consumed. It is European in origin and considered a weed, something one may see growing out of sidewalk cracks or in rocky, mountain soil. Its use in labs dates to the late 1970s, but gained greater use in the following ten years. For a comprehensive review, see Koornneef and Meinke (2010). It became widespread for a number of reasons. Previously-used model organisms such as snapdragon, petunia, and corn have a much slower developmental timeframe, taking months to reach maturity. Arabidopsis takes around eight weeks from germination to seed set, after which it senesces quickly. It self-pollinates, which is convenient when examining genetics. It is also easy to outcross for experimental purposes; the stigma and stamens are easily accessible, so there is little risk of damaging the plant during manual pollination.

In laboratory settings where space is limited, and light and environmental conditions are controlled in growth chambers, the plant is compact, around 30 cm high and 10 cm across, and large numbers can be grown for purposes of replication and experimental treatments. Perhaps the most useful thing with Arabidopsis is its extremely high quantity of seeds. A single plant can

generate 1000s of seeds, which are easily harvested and compact to store because a single seed averages .17 mm in diameter (Rashotte Lab, unpublished data). As a research organism, it has many tools at the ready – knockout, inducible, and reporter lines, kits, assays, and media specifically geared towards optimal results, and an abundance of tools that allow us to analyze data quickly and easily. Another benefit of working with Arabidopsis is that it is diploid, so genetic analyses can be done with relative ease. It has a fully sequenced genome which allows clarity when examining genetic problems. Because it has been so heavily used as a research organism, its development and physiology are easy to work with. When using the most common ecotype, dubbed Columbia, after its development in Columbia, MO, we know when to expect emergence of true leaves, and when bolting of the central stem should occur. Additionally, growth is highly synchronous. The seeds geminate within hours of each other on the same day and germination occurs with almost 100% of seeds. This makes it easy to study ontological parameters of organism development, such as delayed germination, flowering or senescence. In the other plant species with which I research, these things can be variable and harder to track.

Tomato, *Solanum lycopersicum*, does not have all the tools that Arabidopsis does but it is a good plant to use as an applicable crop species. It is a fruit, and its size, quantity, and quality is of central importance in agriculture. Microtom is an ideal species to use in lab, as it has been bred to have a smaller size and smaller fruits, making it more manageable in laboratory settings. There are existing transgenic lines of this, and specific knockout or knockdown lines can be made with RNAi, and now CRISPR. The tomato genome is diploid and completely sequenced, although not as well annotated as Arabidopsis, and there are fewer tools readily available for genome-wide or single-gene analyses.

Abiotic stress and Cytokinin Response Factors

This research is looking at basic questions about growth and development in the plant, and how stress conditions play a role in that. Plant growth is demonstrated to suffer under biotic stress conditions, such as pathogen attack and herbivory, as well as abiotic, environmental factors like drought, freeze, high heat, humidity, and poor soil conditions. This can particularly impact the development of fruits and seeds, which are often the agronomically significant, consumable portion.

General physiological parameters have been examined related to this, as well also how specific genes alter these responses. The genes looked at belong to a small group of transcription factors called Cytokinin Response Factors. They are part of a larger family of transcription factors known as APETALA2/Ethylene Response Factors (AP2/ERF) (Sakuma et al, 2002). The CRFs were originally pulled out of a screen for cytokinin response, noticeable because many of its members were strongly induced (Rashotte et al, 2006). The CRFs have a conserved region of about 60 amino acids at their N-terminus of the protein and can be broken into five distinct clades via homology in a CRF domain at the C-term. Upon further examination of all clades, only three of those, I, III, and V, are found to respond to cytokinin (Zwack et al, 2012). The CRFs are conserved across all land plants with at least one member from each clade being represented in a given species; the notable exception is Brassicales which lack a clade IV representative.

Cytokinin-responsive clade I contains two genes in Arabidopsis, CRF1 and CRF2, and one in tomato, SlCRF2. Original focus was spent on examining both CRF1 and CRF2 in Arabidopsis, expecting them to show similar physiological characteristics in the assays carried out, but frequently experiments with CRF2 would yield results differing from wild type (WT), but those from CRF1 would be indistinct from WT plants. For this reason I primarily focused on

CRF2 in my dissertation research. The link between abiotic stress and CRFs was first established in an experiment exposing plants to cold (4°C) stress. CRF4 overexpressors were more resistant to cold than WT, while mutants were more sensitive (Zwack et al, 2016). CRF4 belongs to clade II, which is not cytokinin responsive. Other experiments have shown a more definitive link to stress and cytokinin. CRF6, a cytokinin-responsive clade III member, was shown to directly mediate expression levels of multiple genes involved in cytokinin signaling after exposure to H_2O_2 (Zwack et al, 2016).

In Chapter 2 of this dissertation cytokinin signaling is reviewed, with a section including the role of CRFs within this pathway. In Chapter 3, endogenous cytokinin levels are examined in response to salt, with a clear difference in response between wild-type and *crf2*. Chapter 4 again looks at cytokinin levels after abiotic stress, this time in microtom, examining the differing results seen between salt and hydrogen peroxide.

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Chapter 2: Plant Cytokinin Signaling

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Abstract

Cytokinin is an essential plant hormone involved in a wide range of plant growth and developmental processes controlled through its signaling pathway. Cytokinins are a class of molecules that are N^6-substituted adenine derivatives, such as isopentenyl adenine, *trans*- and *cis*-zeatin that are common in most plants. The ability to perceive and respond to this hormone occurs through a modified bacterial two-component pathway that functions via a multi-step phosphorelay. This cytokinin signaling process is a crucial part of almost all stages of life, from embryo patterning, to apical meristem regulation through organ development, and eventually during senescence. The cytokinin signaling pathway involves the coordination of three types of proteins: histidine kinase receptors to perceive the signal, histidine phosphotransfer proteins to relay the signal, and response regulators to provide signal output. This pathway contains both positive and negative elements that function in a complex co-ordination to control the cytokinin hormone regulated plant responses. While much is known about how this cytokinin signal is perceived and initially regulated, there are still many avenues left to explore before we fully understand cytokinin control of plant processes.

Key Words: Cytokinin, Two-component signaling, Multi-step phosphorelay, Histidine Kinase, Histidine Phosphotransfer Protein, Response Regulator, Cytokinin Response Factor

Introduction

Cytokinins are a critically important class of chemicals in plants. Cytokinin is connected to and often essential for necessary processes in nearly every growth stage of a plant and in most, if not all organs and tissues. Cytokinin is one of the classic 5 plant hormones. It is also readily used in research, farming, and industry contributing to 40% of the plant growth regulation

market in 2013 and accounting for $1.25 billion (USD) in sales. Despite this there is still much to learn about cytokinin and how a plant perceives and responds to this required hormone. This review will cover cytokinin signaling and the advances that have been made in this field, along with emerging topics of study for this hormone.

Cytokinin was originally discovered as a plant hormone in 1955 as part of a large push to be able to regenerate plants in the lab through tissue culture. Prior to this time it was known that plant cell tissue cultures required the hormone auxin to allow cells to enlarge, but there was an additional undefined substance that needed to be added to the media before cells would start dividing. A variety of unusual mixtures were used along with auxin to regenerate plant tissue, including autoclaved herring sperm. Later, kinetin was identified as an rtefactual product of heating, and was the first recognized cytokinin, still widely used today (1). Native cytokinin was discovered decades later, isolated from maize, *Zea mays*, and was called zeatin (2). This general class of hormones was named cytokinin, after the word cytokinesis, for cell division from which this hormone had its origins.

Cytokinin affects many processes in the plant and perhaps primary among them is cell division and proliferation in the shoot apical meristem (SAM) responsible for the production of all above ground organs. There it has been shown to define boundaries between the meristematic cells of the SAM and emerging leaves. In the root, cytokinin also plays a role in cell differentiation, helping to determine cell size and as well as being critical to the formation of vasculature tissues. As noted in tissue culture regeneration, cytokinin and auxin often play balancing roles in organ determination and patterning: an increased auxin:cytokinin ratio leads to root tissue while an increased cytokinin:auxin ratio leads to shoot tissue. In addition, cytokinin has a strong role in response to light and sink/source regulation, and it can delay senescence in leaves when applied exogenously (3). It has a role in tolerance to abiotic stresses such as drought, heat, and salt (4, 5). Newer information implicates cytokinin in dealing with biotic stress and plant immunity under pathogen attack (6). Agronomically, increased cytokinin levels have shown greater grain yield in rice (7). It has also improved fruit yields in tomato plants grown under high-salt conditions, indicating potential benefit for crop production in saline soil environments (8). Further information about the functional roles of cytokinin in growth and development is well-reviewed elsewhere (3, 5, 9).

The Structures of Different Cytokinins and their Activities

Cytokinin does not exist as a single molecule, but comes in various forms. Native plant cytokinins are N^6-substituted adenine derivatives, which vary in the addition and structure of their side groups (Figure 1). The biological activity of cytokinin in plant processes is linked to the presence of these different side groups. The most frequently occurring cytokinins tend to be trans-zeatin, tZ, isopentyladenine, iP, cis-zeatin, cZ, and dihydrozeatin, DZ (9). The cis- isomer, cZ, is a less active form than tZ, performing weakly in bioassays, but it may be of primary importance in specific organs or certain plant species such as maize, rice, and garbanzo (3, 10, 11). In Arabidopsis it was found that tZ is associated with periods of rapid growth, whereas cZ predominated in ontological periods of slower growth (12). There are also many synthetic cytokinins, existing both as adenine-type, like kinetin, and phenylurea compounds (Fig. 1), most notably N-phenyl-N'-(2-chloro-4-pyridyl)urea, CPPU, and thiadiazuron, TDZ, which can be used as a potent herbicide. No native plant phenylurea compounds have yet been discovered (9). Some natural adenine-based aromatic compounds have been found, but only in a few plant species and they are generally present in very small quantity so as not to play a large role in cytokinin functioning (9).

The biological activity of different cytokinin forms appears to relate directly to the ability of the molecule to bind the receptors. This may be best shown by the work of Hothorn, et al. (13) where they examined the crystal structure of the Arabidopsis cytokinin receptor AHK4 and binding of different adenine-based cytokinins, as well as the phenylurea-based cytokinin thiadiazuron. The protein receptor binding pocket for cytokinin is comprised largely of nonpolar amino acids with a few, significant polar residues that contribute to the stabilization of the molecule, such as aspartic acid 262, which forms hydrogen bonds with the adenine ring (13). For tZ binding, an additional hydrogen bond is formed with threonine 294 and the hydroxyl oxygen on the substituted isoprenoid side chain producing a more stable connection, and likely responsible for tZ being a more biologically active cytokinin form. Water molecules in the active site also serve to stabilize cytokinin, interacting with both it and other amino acid residues that are too far away to directly bond with the cytokinin itself. For the phenylurea-based thiadiazuron, the phenolic portion of the molecule behaves largely like an adenine ring, and is stabilized by the same aspartic acid 262 residue. This work and future experiments should help to clarify this initial and crucial part of the cytokinin signaling cascade.

Cytokinin Signaling: Multi-step Phosphorelay

Almost 20 years ago one of first genes involved in carrying out cytokinin response was cloned and identified as a histidine kinase (HK), very similar to the HK receptors involved in two-component prokaryotic signaling (14). Later it was found that bacterial homologs of response regulators could also respond to cytokinin addition (15), and accumulating evidence caused cytokinin signaling to be based around a two-component model (Figure 2 top). The bacterial two-component pathway is generally composed of a histidine kinase receptor which perceives the signal and a response regulator (RR) protein which mediates signal response to it (16, 17). The receptor histidine kinase (HK) is an integral membrane-spanning protein found as a homodimer. One side of the membrane holds an input domain where an environmental signal is perceived, allowing activation in an ATP-dependent reaction on the other side of the membrane. One HK catalyzes the phosphorylation of a conserved histidine residue on the opposing HK molecule. This phosphate is then relayed to a conserved aspartic acid on the receiver domain of a RR protein (17). The activated RR then carries out a given response in this signaling pathway, such as inducing or repressing genes as a transcription factor.

The cytokinin signaling pathway functions like a modified bacterial two-component system with several distinct differences, including altered and novel components such as the relay of a signal into the nucleus. Cytokinin receptors are hybrid histidine kinases (CHKs), containing both a histidine kinase domain and a receiver domain (Figure 2 bottom; Figure 3). When cytokinin is bound to the receptor, the histidine kinase domain is activated and autophosphorylates, and the phosphate is relayed from a conserved histidine to a conserved aspartic acid located in the receiver domain of the molecule. The phosphorelay proceeds and the phosphate is transferred to a new pathway component, a histidine phosphotransfer protein (HPt), which moves into the nucleus, where the response regulators reside. Once in the nucleus, a HPt relays the phosphate to the receiver domain of a response regulator, which controls the output of cytokinin signaling (18).

The Receptors – Histidine Kinases

Cytokinin is perceived by membrane-bound hybrid histidine kinase receptors. In Arabidopsis, where the majority of the cytokinin signaling pathway work has been conducted, there are three major CHK receptors, AHKs 2, 3, and 4 (19), although this appears to be true for most other diploid angiosperm species too (20). The CHK receptors are able to perceive cytokinin through their cyclase/histidine kinase associated sensory extracellular (CHASE)

domain, a conserved 200-230 amino acid domain that can perceive low molecular weight ligands, such as cytokinin derivatives (21). It is thought that upon binding with cytokinin, a conformational change occurs and across the membrane, the receptor dimer autophosphorylates, then transfers a phosphate group from a specific histidine of the CHK domain to an aspartic acid of its receiver domain, the first step in the canonical phosphorelay. Cytokinin receptors were originally thought to be solely on the plasma membrane with an extracellular CHASE domain as this is the analogous set-up in bacteria, and homologous expression experiments allowed them to complement a plasma membrane receptor with an AHK in unicellular yeast (22, 23; Fig. 3). Only recently it was found that cytokinin binding was associated with endomembranes. The CHK receptors were tagged for identification, gradient-centrifuged, and immunoblotted, and were found to be associated with the endoplasmic reticulum. Bimolecular fluorescence complementation also showed strong fluorescence at the ER. As such CHK receptors are now thought to primarily span the endoplasmic reticulum membrane with the CHASE domain located in the lumen of the ER (Fig. 3; 19).

Each CHK receptors has been shown to have different *in planta* expression patterns and binding affinities for different cytokinins (24). While these factors vary some among species, generalizations to related receptor orthologs are often made from detailed examinations of the receptors in Arabidopsis. The AHKs have been found to have the following cytokinin binding affinities: in order of highest to lowest, AHK2 affinity is iP>tZ>iPR>tZR>DZ, AHK3 affinity is tZ>tZR>DZ>iP>cZ, and AHK4 affinity is tZ>iP>tZR>iPR (24, 25). The determination of receptor localization has been largely made from observations of AHK GUS reporter lines: AHK2 and AHK3 are primarily expressed in aerial tissues, while AHK4 expression is greatest in roots. Perhaps not surprisingly, the different functional roles for each receptor largely parallel these expression patterns. For example, the link between cytokinin signaling and regulation of leaf senescence is found in AHK2 and AHK3, which are more strongly expressed in this organ. Review of functional connections has been covered by others previously (5,26), including the redundancy seen with CHKs and throughout the cytokinin signaling system. It is predicted to act as a buffer for this important hormone signaling system. Redundancy has been illustrated in analyses of Arabidopsis mutants, where single and double mutants have few morphological defects, although cytokinin sensitivity is clearly altered (26). Extreme morphological defects in

size, vascular formation, and fertility are only seen when all three AHK receptors are knocked-out (26).

Relaying the Signal – Histidine Phosphotransfer Proteins

Since the receptors are ER membrane-localized and RRs are primarily nuclear-localized, another molecule is needed to help relay the signal through the pathway: the histidine phosphotransfer proteins or HPts. HPts are proteins of about 150 amino acids long with a conserved histidine residue in the reveiver domain that accepts the phosphate group relayed from the CHK (25). HPts function as intermediates and move the phosphate from the cytosolic face of the ER into the nucleus, where they will phosphorylate response regulators (Fig. 3). Experiments done to examine the intracellular localization of HPts show both a cytosolic and nuclear distribution, which is independent of both the addition of cytokinin and their phosphorylation status. While originally thought to move to the nucleus only after being phosphorylated, it is now believed that HPts are constantly cycling in and out of the nucleus between the receptors and response regulators (27).

In Arabidopsis there are five true histidine phosphotransfer proteins (AHPs) that function in cytokinin signaling, AHPs 1, 2, 3, 4, and 5 (28; Fig. 4), as well as AHP6, a pseudo-HPt that lacks the conserved histidine necessary for phosphorylation. Phylogenetic examination of the HPts reveals that AHPs 2, 3, and 5 are most closely related, with AHP1 in a separate clade with other dicot HPts, AHP4 falling into a pseudo-HPt clade with rice pseudo Hpts, and AHP6 outside of these groupings. The true HPts are generally thought of as positive regulators of cytokinin signaling, since they are forwarding the signal to response regulators. In contrast, pseudo-HPts act as negative regulators of cytokinin signaling by interacting with receptors and competitively preventing phosphotransfer and thus positive signaling (29). As noted for the CHK receptors, HPts also have redundant functions. When AHP mutants are created, no single or double *ahp* mutants show phenotypic variation, although higher order mutants show increasing phenotypic abnormalities and the quintuple mutant *ahp1-5* shows abnormal silique development, lower seed set, diminished xylem and phloem development, an extremely shortened primary root, and an increase in adventitious roots, indicating their importance in plant development.

Response Regulators

The Response Regulators (RRs) are a group of proteins in the cytokinin signaling pathway that function just as their name suggests, regulating plant response or output in the perception of cytokinin. All RRs contain a two-component receiver domain, which includes a conserved N-terminal aspartic acid, a central phospho-acquiring aspartic acid, and a C-terminal lysine (30). The RRs can be further divided into three classes based on the presence or absence of an additional GARP transcription factor domain, and transcriptional regulation by cytokinin: type-A RRs, type-B RRs, and type-C RRs (Fig. 3; 30). Both type-A and type-C RRs lack a transcription factor domain and function as negative regulators of cytokinin signaling, interacting and interfering with proteins in the pathway that are positive signalers. These classes can be distinguished by their transcriptional induction by cytokinin, wherein type-As are highly induced and type-Cs are unaffected. Type-B RRs in contrast have a transcription factor domain, function as positive regulators of cytokinin signaling and are not induced by cytokinin. RRs are the largest group of proteins in the cytokinin signaling pathway. There are 24 Arabidopsis Response Regulators or ARRs, which can further be divided into each RR class: ten type-As, eleven type-Bs, and two type-Cs (Fig. 4). There is also one additional RR, ARR23 which doesn't belong to any class; it has a truncated receiver domain and may not perform its phospho-accepting function (31). Type-As and type-Bs are the major groups of RRs in all plants and are discussed in greater detail below. Most of the work on type-Cs has been conducted using those found in Arabidopsis, ARR22 and ARR24. ARR22 has been experimentally shown to interact with AHP 2, 3, and 5 in both plant and yeast cells. It has been shown to express in developing seed chalaza, but not at later life stages (32). Constitutive overexpression of ARR22 results in a severely dwarfed phenotype; however, the *arr22* mutant shows no observable phenotypic defect (31, 32). ARR 24 is expressed in pollen, and shows transcript amplification in buds, flowers, and siliques, but not in leaves or stems. Single *arr24* mutants show no phenotype, and neither do a*rr22 x arr24* double mutants (33). Overall it is unclear exactly how type-Cs function, but it has been suggested that they work as negative regulators by taking phosphoryl groups meant for type-Bs out of the signaling system without a cytokinin regulated output. Their overexpression would then reduce type-B positive signaling.

It should be noted that in addition to the classically defined RRs, there is a pseudo response regulator family, PRRs, that contains a receiver domain, but lack the conserved phospho-accepting aspartic acid, for example replaced with glutamic acid. PRRs are not part of

the cytokinin signaling pathway, instead have been shown to play a role in circadian functions (30).

Type-B Response Regulators

The type B response regulators have both a receiver domain as well as a C-Terminal GARP transcription factor domain, named after the genes/gene families that are known to contain it: GOLDEN2 in *Zea mays*, the ARRs, and Psr1, a protein found in *Chlamydomonas reinhardtii* (34, 35). The GARP domain is a Myb-related transcription factor domain (Fig. 3). Detailed characterization of the GARP domain in the type-B RR, ARR10, revealed a domain around 60 amino acids long, which forms three alpha helices and a flexible arm toward the N-terminus. In complex with DNA this GARP domain binds to the major groove of DNA with a helix-turn-helix motif, and the arm binds the neighboring minor groove (35). There are eleven total type-Bs in Arabidopsis that can be divided into three subfamilies (Fig. 4). Subfamily I contains the major players in cytokinin signaling: ARR1, ARR2, ARR10, ARR11, ARR12, ARR14, and ARR18 (34). Subfamily II contains ARR13 and ARR21, and in subfamily III are ARR19 and ARR20. Neither of these is as well-studied as subfamily I, but would appear to play more minor roles based on work conducted to date (36, 37).

The major type-B RR subfamily I members in Arabidopsis, ARRs 1, 2, 10, and 12, known to be involved in downstream cytokinin responses, have largely redundant functions, similar to other cytokinin signaling components. Higher-order type-B mutants show reduced sensitivity to cytokinin with greater root elongation, and increased number of lateral roots among other effects as compared to wild type. The regulation of known cytokinin primary response genes is also affected (38). The ARR1 protein has been shown to bind to the nucleotide sequence AAGAT(T/C) or GAT(T/C)TT which is repeatedly found in the promoter region of many cytokinin regulated genes, such as every type-A ARR, a cytokinin oxidase (which degrades cytokinin) and a cytokinin hydroxylase (involved in the biosynthesis of trans-zeatin) among others (39). As noted above the addition of exogenous cytokinin does not directly regulate the transcription of type-B RRs, but does induce the transcription of the genes that type-B transcription factors are binding to and regulating. This has been shown in a triple mutant, *arr1,10,12* background, where the addition of cytokinin shows no increase in transcripts of these type-B gene targets, affirming that these genes are a crucial part of the cytokinin signaling pathway (40).

Type-A Response Regulators

Type-A RRs are primarily composed of the conserved aspartic acid phospho-receiver domain that is present in all RRs, but lacks any other domain, including the GARP domain, and do not function as transcription factors (Fig. 3). Instead type-A RRs functions as negative regulators of cytokinin signaling. Most plants have a number of type-As; there are ten in Arabidopsis that have highly similar amino acid sequences and group in pairs based on this: ARR5 and ARR6, ARR7 and ARR15, ARR8 and ARR9, and ARR16 and ARR17 (41; Fig. 4). Like other abundant parts of the cytokinin signaling pathway, type-A RRs have redundant functions in the plant and show increasingly severe phenotypes in higher order mutants, such as in Arabidopsis show an increased sensitivity to cytokinin. For example, calli of the *arr3, 4, 5, 6, 8, 9* mutant show greater than WT shoot production and fewer roots with increasing cytokinin concentration, as well as greater inhibition of lateral roots than wild-type seedlings. All type-A RRs have been shown to contain at least one type-B binding sequence in their promoters and can function downstream of the type-B ARRs, although they have also been shown to interact directly with the AHPs (40, 42). The type-As are some of the most highly regulated cytokinin genes, and all have been shown to be induced by cytokinin, with the exception of ARR 17, which usually shows very low expression levels regardless of treatment (40, 43).

Type-A RRs appear to negatively regulate cytokinin signaling through a few modes. One mode is a direct interaction with HPts (42), so they could compete with type-B RRs in the receipt of a phosphate group being relayed though the signaling pathway (Fig. 4). The reduction of a positive type-B signal output in this case would result in negative regulation. Additionally, the type-A RRs may work in a manner independent of phosphorylation and could interact with other cellular components based on the structure and function of the protein itself as is sometimes seen in bacterial two-component signaling (17, 44).

Cytokinin Response Factors

One additional set of proteins that have been linked to cytokinin signaling are CRFs or Cytokinin Response Factors. CRFs do not appear to be part of the direct phosphorelay in two component cytokinin signaling, but may function as a side branch to this pathway as they are known to directly interact with the HPts. CRFs are a subset of the Apetela2/Ethylene Response Factor or AP2/ERF transcription factor superfamily (45, 46). Interestingly, they do not appear to be involved in ethylene function and are completely unrelated transcription factors to the type-B

RRs. CRFs have no overlapping domains with other cytokinin signaling proteins; instead they contain a CRF protein-protein interaction domain that defines this group as independent of other AP2/ERF proteins, as well as a putative MAP kinase phosphorylation motif (47). Only some CRFs are transcriptionally induced by cytokinin. From the 12 CRFs in Arabidopsis there are four: CRF1, CRF2, CRF5, and CRF6 (43). The CRF2 and CRF5 promoters both contain type-B binding sites, show increased expression in wild type plants with the addition of cytokinin, and attenuated expression in type-B *arr1,12* mutants (45). These CRFs are clearly downstream of the cytokinin receptors as they are not induced by cytokinin in receptor mutant backgrounds (Fig. 3). Examinations of CRF cellular localization in protoplasts using 35S:GFP:CRF constructs indicated that CRFs were ubiquitously expressed throughout the cell and then seen to accumulate in the nucleus upon addition of exogenous cytokinin (45). A more distinct placement of CRFs in relation to the cytokinin signaling pathway is not simple, although it appears clear that they are downstream of the receptors and HPts, their relation to the RRs is less clear (45). Some CRFs have been shown to be targets of type-B RRs and have associated binding sites in their promoters, which could put them as simply downstream targets of type-Bs. However, CRFs have been shown to directly interact at the protein level with HPts using BiFC (bimolecular fluorescence complementation) and Y2H (yeast two-hybrid) which would put them as an independent side branch stemming from the HPts (47). As some type-B RRs are also predicted to be transcription factor targets of CRFs, a side branch status seems to be currently the best placement (Fig. 3).

Regardless, CRFs have been shown to be cytokinin regulated transcription factors that are involved in controlling a number of important cytokinin regulated features, similar to the type-B RRs. In particular, CRF6 has been linked to cytokinin regulated senescence and a number of different stresses, including osmotic, oxidative, salt, and UV-B (48). CRF2 has also been linked to chloroplast division, which is a cytokinin connected process (49). In a broader examination of CRFs in tomato (SlCRFs), most of these genes show transcriptional regulation in response to stresses that have also been connected to cytokinin signaling (46). Overall it is clear that CRFs are major players in regulating cytokinin response outputs as part of a cytokinin signaling pathway.

Conclusion

Cytokinin signaling is a process involved in nearly every stage of life, from embryo organ patterning, to regulation of the SAM throughout development, and finally during the negative regulation of senescence (5, 33). This involves the coordination of three main types of proteins through a multi-step phosphorelay pathway: histidine kinase receptors to perceive the signal, histidine phosphotransfer proteins to relay the signal into the nucleus, and response regulators to carry out the response. In addition to this positive phosphorelay signal there is a robust negative feedback loop. The positive signal that activates type-B RR response, strongly induces the negative regulating type-A RRs, which interfere with and shut down the signal that activates the type-B RRs and all positive cytokinin output. This complex co-ordination occurs through a highly redundant series of proteins that in turn elegantly regulate cytokinin growth and developmental processes.

- **Cytokinin is an essential plant hormone involved in many plant growth and developmental processes**
- **Cytokinin is not a single molecule but a class of molecules that are N^6-Substituted adenine derivatives**
- **Cytokinin signaling is a modified bacterial two-component pathway that functions via multi-step phosphorelay**
- **The cytokinin signaling pathway contains histidine kinase receptors to perceive the signal, histidine phosphotransfer proteins to relay the signal, and response regulators involved in signal output**

Three Immediate Future Goals and Directions for Cytokinin Research

1. Decoding specificity among redundancy. One of the consistencies seen throughout the cytokinin signaling components is a large amount of redundancy. Each step of the cytokinin signaling pathway has multiple proteins: in Arabidopsis there are 3 CHKs, 5 HPts, 10 type-A RRs, 12 type-B RRs, and 12 CRFs (Fig. 4) with comparable protein numbers identified in different angiosperm species (28, 44, 47). Nearly every CHK, RR, and CRF, for example, has been shown to have the capability of interacting with each of the 5 HPt members (18, 47). This is a huge amount of signaling overlap, which seems unnecessary as simply a failsafe system. Localization of some signaling components to specific organs, such as root, shoots, or leaves does cut down on the overlap, but not dramatically. One hypothesis is that there are unique

member proteins in the cytokinin signaling pathway that relay signaling for specific cytokinin-related responses. In essence there might be a cytokinin signaling module for each response. There have been some findings that support this idea of cytokinin signaling modules for senescence and cold response (48, 50, 51). It is unclear if this will hold true for all responses or how these modules might differ. At present this is an interesting theory and wide open avenue for investigation.

2. Intracellular cytokinin transport. How does cytokinin move within a cell and particularly to the receptor in the ER? There has been little is known about how cytokinin is generally transported within the plant. However, the identification of a new cytokinin transporter protein (ABCG14) within the last year appears to help explain how cytokinin moves in and out of a cell (52). Interestingly this new cytokinin transporter is not localized to the ER membrane, and as such it is currently unknown how cytokinin enters the ER (52). This has become an extremely important point now that the CHK receptors are facing inside towards the ER lumen. While cytokinin could merely enter the ER lumen by simple diffusion, for all the process that cytokinin regulates this seems rather unlikely. Transporter proteins (PINs) that load auxin into ER lumen were identified after the auxin receptor face was identified as being there (53). So a concerted effort to examine cytokinin movement into the ER or detailed study of related ABC transport proteins may shed light on this unknown.

3. Downstream to upstream connections. Although it has been about 60 years since the discovery of cytokinin, there is still much to be determined about this hormone. While a great deal of effort has been placed over the last 20 years on the immediate upstream components of the modified two-component signaling pathway connected to the cytokinin receptor, very little is known about how cytokinin works mechanistically. How does cytokinin regulate senescence, vascular patterning, sink/source relationships and other responses that this hormone is known to influence? The cytokinin signal relay from the CHK receptor perception to the RRs may be understood, but what happens after that is yet to be explored. This is particularly true in trying to connect the signal to the final cytokinin output response. Studies looking at the RR or CRF transcription factor target genes may provide the first step in unraveling what comes next and how cytokinin really functions to control plant growth and developmental processes.

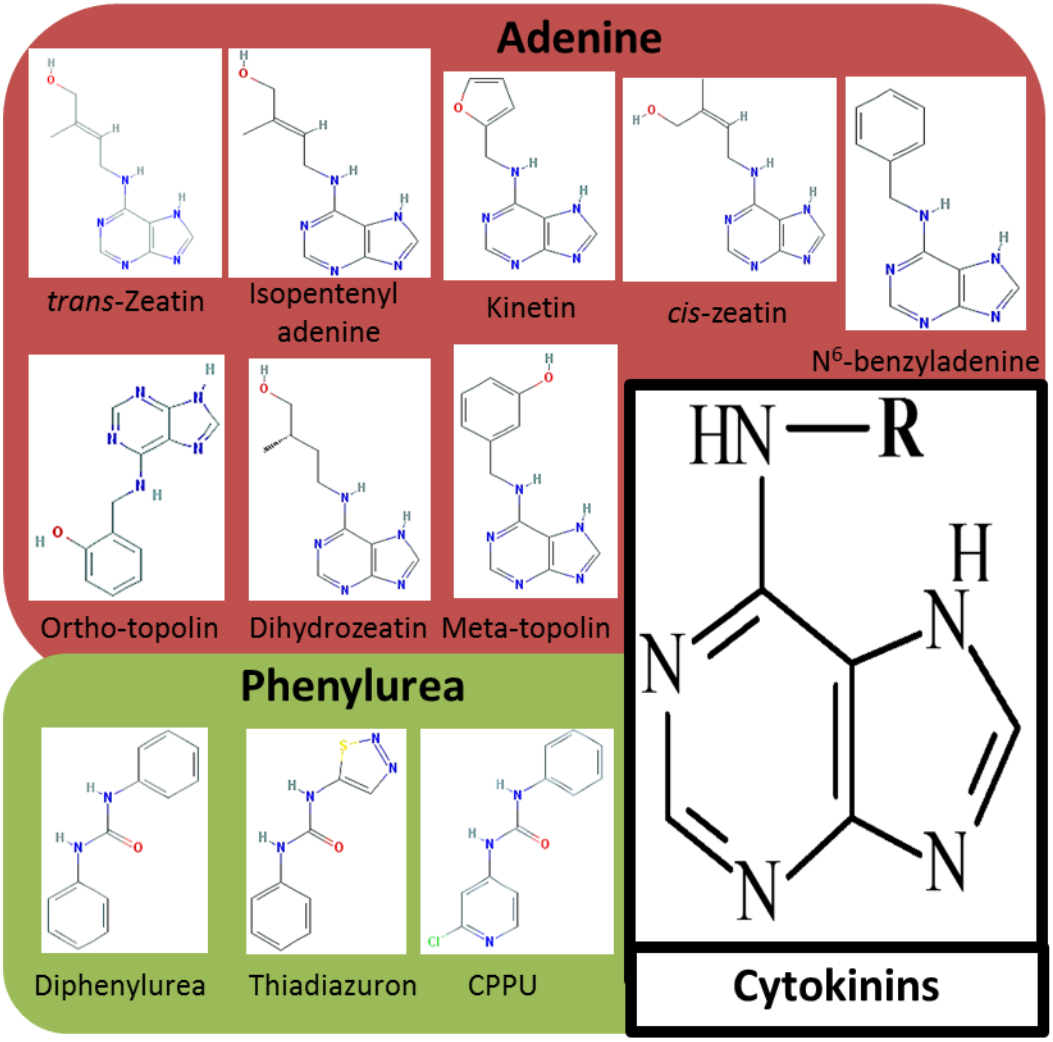

Figure 1. Structures and Forms of Cytokinins. The two major chemical classes of cytokinins: adenine-based (surrounded in red) and phenylurea-based (surrounded in green) are shown. The lower right corner shows a general form of cytokinin with an "R" group designating where different substituent groups can be added.

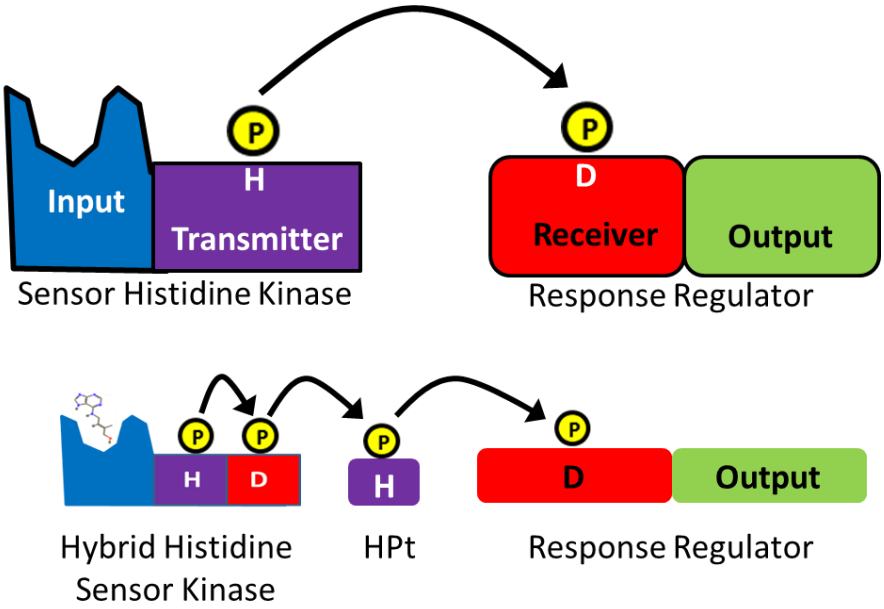

Figure 2. Generalized Two Component Phosphorelay Systems. (Top) Shows a general bacterial two-component system where input is perceived by a sensor histidine kinase. A histidine (H) in the transmitter domain is phosphorylated, then passes the phosphate via phosphorelay to an aspartic acid (D) in the receiver domain of a response regulator protein. This response regulator protein also contains an output domain to response to the initial perceived input. (Bottom) Diagrams the cytokinin multi-step phosphorelay system. The cytokinin input is perceived by a hybrid histidine sensor kinase that contains both a transmitter and receiver domain with (H) and (D) that are phosphorylated and then the phosphate is passed to a histidine phosphotransfer protein (HPt) and finally a response regulator, also containing an output domain.

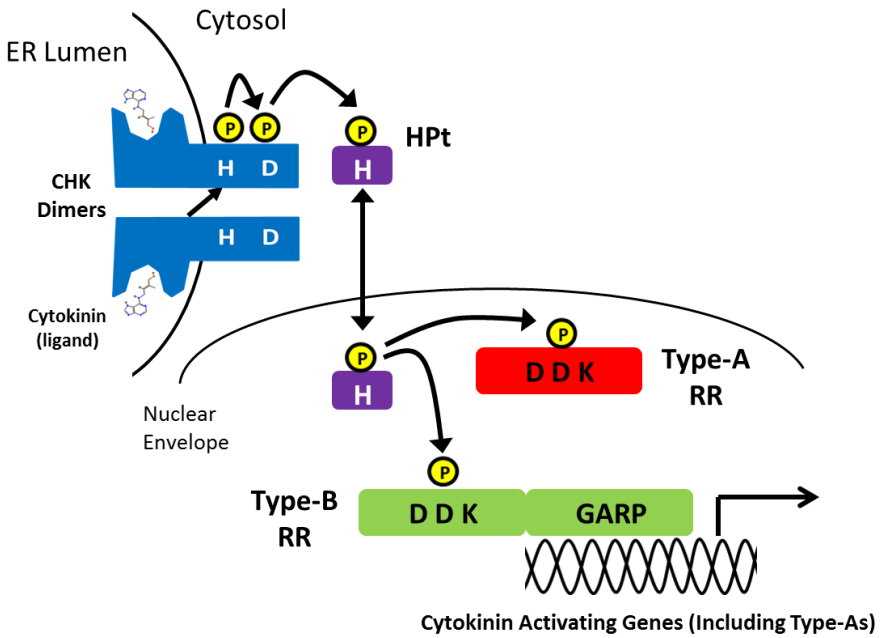

Figure 3. Cytokinin Two Component Phosphorelay. Diagram of the two component cytokinin signaling (TCS) phosphorelay pathway within the cell is shown. Cytokinin binds in the lumen of the ER to the dimerized receptor (CHK) at the ER membrane beginning the signaling pathway. The CHK is then autophosphorylated (P) at a conserved Histidine residue (H), which is then relayed to a conserved aspartic acid residue (D). The signal then moves in the cytosol to a histidine phosphotransfer protein (HPt) that then moves to the nucleus, transferring the (P) to a conserved (D) on one of two types of response regulators (RRs). Both RRs have conserved DDK amino acid motifs, with the type-B RR also containing a GARP transcription factor (output) domain that binds to and activates cytokinin regulated genes.

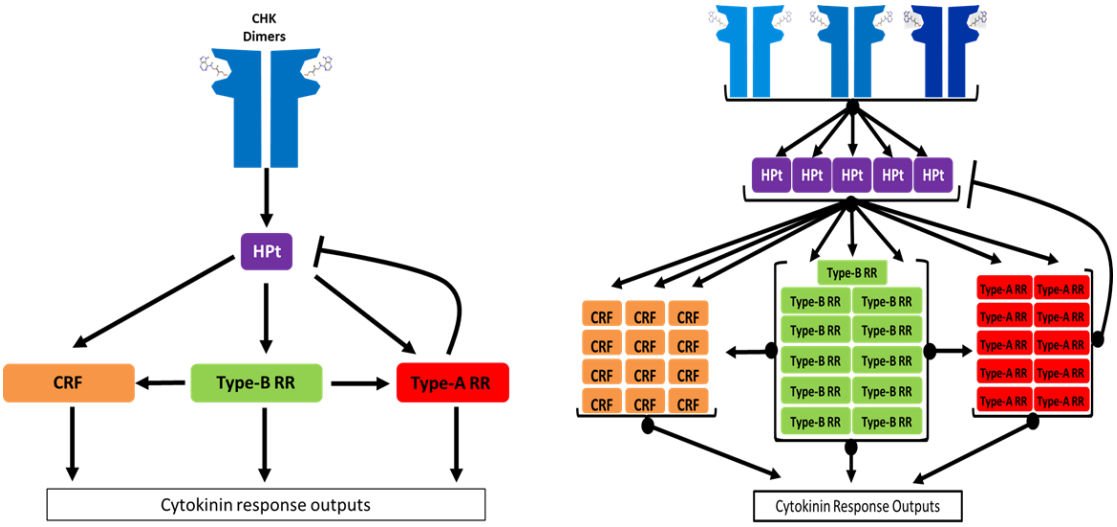

Figure 4. Plant Cytokinin Signaling Pathway. The relay of a cytokinin signaling through the two component pathway is shown with positive and negative feedback indicated. (Left) Shown is a simplified version where all pathway components of a particular class are indicated as a general group of proteins: Receptors (CHK), Histidine phosphotransfer proteins (HPt), Type-A and Type-B Response regulators (RR) and Cytokinin response factors (CRF). (Right) Shown is the number of protein at each step of this signaling pathway for the model system Arabidopsis, indicating that there is a lot of potential interactors and redundancy between different signaling protein classes: 3 AHKs, 5 AHPs, 10 RRAs, 11 RRBs, and 12 CRFs. Arrows in this pathway indicate positive signals and a block indicates a negative signal or regulation.

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Chapter 3: CRF2 alters native cytokinin levels under salt stress

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Author contributions

E.A.K. and A.M.R. designed research; E.A.K., L.P, O.N., and A.M.R performed research and analyzed data; E.A.K. and A.M.R. wrote the paper.

Keywords: Cytokinin, Cytokinin Response Factors, CRF2, Abiotic Stress, Salt, sodium, *Arabidopsis thalilana*, Arabidopsis, RNAseq, Transcriptome

One Sentence Summary: Upon exposure to salt (NaCl) as an abiotic stress, WT Arabidopsis plant show an increase in native cytokinin levels while *crf2* mutants show decreased levels.

Abstract

Cytokinins, known for involvement in processes relating to growth and development, have a growing body of work that examines their role in stress response. Here, we examine 10-day-old Arabidopsis seedlings after a 6-hour exposure to 150mM NaCl. Four types of cytokinins, cis-zeatin, trans-zeatin, isopentyl adenine, and dihydrozeatin, were measured in precursor, transportable, active, and conjugated forms. In addition to cytokinin measurement, RNA-seq was performed, measuring transcript levels in stressed and unstressed plants. Wild-type plants were used, as well as *crf1* and *crf2* T-DNA insertion mutants. The Cytokinin Response Factors comprise a small monophyletic group of transcription factors within the AP2/ERF family. In Arabidopsis, CRF1 and CRF2 form a clade and are upregulated in response to cytokinin. Without treatment, both mutants had relatively similar levels of cytokinin expression, however; with exposure to NaCl, wild-type and *crf1* total cytokinin levels increased, while those of *crf2* decreased. Stored tissue from wild-type and *crf2* were sent for RNA-seq analysis to examine differential mRNA transcript expression. Results showed 33 genes differentially expressed (DE) between *crf2* and WT without NaCl and 8014 with exposure. WT showed 12,407 genes DE with salt vs control and *crf2* showed 7,040. Between the two, 4385 genes were DE in common. Morphological measurements with the mutants reveal that *crf2* has larger seeds than WT. Physiologically, Fv/Fm measurements reveal that both *crf1* and *crf2* have greater photosynthetic capacity after 150 mM salt stress, and Promoter::GUS fusion lines indicate induction after salt exposure.

Introduction

In modern agriculture, the world's arable land is limited and it is important to understand the mechanisms underlying a plant's physiological response when conditions are suboptimal. Enabling a plant to survive to produce fruit, seed, and other consumable parts in poor conditions could drastically increase the amount of land used for farming. It is estimated that worldwide around 800 million hectares of arable land are affected by saline soils (Acosta-Motos et al, 2017). Salt stress has a variety of effects on the plant, including decreased growth, photosynthesis, and even death of the plant. Salt stress causes multiple problems that the plant must cope with, including osmotic stress and the inability to take up water, ion toxicity and imbalance, and subsequent oxidative stress arising from ion toxicity (Liang et al 2018, Acosta-Motos et al, 2017). Understanding how plants adjust to these problems can aid in the development of ameliorative strategies.

Cytokinins (CKs) are hormones that are known to be involved in cell division, acting in plant development, growth and senescence (Dello Ioio et al, 2007, Werner and Schmulling, 2006, Gan and Amasino, 1995). In more recent literature, they have found a role in plant stress, functioning in both biotic and abiotic response (Albrecht & Argueso, 2017). Many have shown that cytokinin application or increased expression in CK synthesis genes, increase a plant's tolerance to various abiotic stresses. For example, rice plants with inducible isopentenyl transferase, IPT, genes, allow increase in plant-manufactured cytokinins. Upon induction they showed higher grain yields and delayed onset of senescence in drought conditions (Peleg et al, 2011). In heat-stressed creeping bentgrass, the cytokinin zeatin riboside was injected into the soil near roots. As compared to untreated controls, plants exposed to the hormone showed increased chlorophyll content, less electrolyte leakage, fewer markers of oxidative stress, and greater

activity of ROS scavengers (Liu and Huang, 2002). These studies establish a clear link to abiotic stress and cytokinin, but there is not a clear picture of native cytokinin activity under stress response particularly in relation to specific genes involved, although recent methodologies are aiding in this respect (Simura et al, 2018; Keshishian et al, 2018).

Cytokinin Response Factors (CRFs) are a small family of genes within the AP2/ERF family of transcription factors, containing conserved CRF amino acid sequences at their N-terminal domain. CRFs are conserved across all land plants and can be divided into five distinct evolutionarily conserved clades in Angiosperms. In Arabidopsis clade I CRFs, CRF1 and CRF2, were shown to be upregulated by cytokinin application (Rashotte and Goertzen, 2010, Rashotte et al, 2006). The CRFs are likely a side branch of the cytokinin signaling pathway; they have been shown to interact with AHPs and ARRs (Cutcliffe et al, 2011). It is apparent that they function in ways outside of cytokinin response as well, as two of the five clades do not show any strong transcriptional response to cytokinin (Zwack et al, 2012). Previous research has shown some CRFs to have a role in cold and oxidative stresses (Zwack et al, 2016a, Zwack at al, 2016b).

Here we examine clade I CRFs in response to salt stress. This includes examination of growth and photosystem efficiency, changes in native cytokinin levels, and transcriptomic alterations in response to salt stress using wild-type (WT) and T-DNA insertion knockout mutants *crf1* and *crf2*. While mutant plants show little variation in morphology, cytokinin levels, or transcriptome effects under standard growth conditions, numerous changes, especially for *crf2* were found in response to salt stress, indicating that CRF2 is involved in cytokinin-regulated changes under this stress.

Results

Cytokinin levels increase in wild-type and crf1 plants under salt stress while they decrease in crf2 mutants

Measurements of four types of cytokinin (isopentyl adenine (iP), trans-zeatin (tZ), dihydrozeatin (DHZ), and cis-zeatin(cZ)) were taken using GC-MS/MS of 10 day old soil grown plants (Figure 1). For each of these cytokinin types, seven forms were measured, one precursor, one transported, one active, and four different glucose conjugated storage forms. The most prevalent form in all genotypes was the conjugated trans-zeatin-7-glucoside (WT has 21.3 pmol/g fresh weight), while trans-zeatin, which shows the greatest activity in Arabidopsis has less than 1/100 of that amount (WT has 0.16 pmol/g FW). Both mutants have relatively similar cytokinin levels compared to WT, with a few small decreases under untreated control conditions (Fig. 1).

When salt is applied (150 mM for 6h) however, cytokinin levels in *crf1* largely parallel the wildtype (WT), which increases in total cytokinins (CKs) by almost 42% (Figure 1, 2), and shows significant increase in all four native CK forms. In WT there is a significant increase in iP conjugates, tZ conjugates, DHZ riboside, and cZ precursor, riboside, and conjugated forms. *crf1* parallels this without significant difference except for one small decrease in a tZ conjugate. In contrast, for *crf2*, on the whole there are large decreases in CK levels, with a few increases (Figure 1). There are decreases in iP precursor and conjugates, tZ precursor and conjugates, DHZ conjugates, and cZ precursor and conjugates for a 19% overall decrease compared to either untreated *crf2* or untreated WT. These changes would suggest a strong role for CRF2 in the regulation of cytokinin levels being changed in response to salt stress, with CRF1 playing little to no role.

Differentially expressed transcripts between wild type and crf2 vary greatly with salt treatment but not with control

Due to the larger potential role of CRF2 in salt stress regulation, we examined genome-wide changes in transcript levels of *crf2* and WT by RNA-sequencing in response to salt stress following the treatment as conducted for examination of cytokinin measurements. The total number of differentially expressed (DE) transcripts (adjusted p-value of <0.05) between WT and *crf2* is 33. With salt exposure it is 8015. The total number of DE transcripts between WT treated and untreated was 12408, and with *crf2* was 7041. In both, relatively equal proportional amounts were up and down regulated (see Figure 3). There are around 500 transcripts that are DE in both WT and *crf2* but they are down-regulated in one genotype and up in the other (these are excluded from the Venns). In order to compare WT and *crf2* with salt treatment further, significantly up- and down-regulated lists were examined for GO classification (Table 1). WT up-regulated transcripts were enriched for endocytosis, exocytosis, catabolic processes, and regulation of nucleobase-containing compound metabolic process. *Crf2* up-regulated transcripts were enriched for response to abiotic stimulus, cellular amino acid metabolic process, porphyrin-containing compound metabolic process, generation of precursor metabolites and energy, and sulfur compound metabolic process. Both genotypes showed down-regulation for things such as translation and transcription from RNA polymerase II promoter.

Within the GO lists, nothing specific was mentioned about CK, so through a manual search, significantly-regulated transcripts relating to CK biogenesis and signaling were found and put into Table 2.

Mutant CRFs show greater photosynthetic capability when exposed to salt.

Fv/Fm saturates dark-adapted plants with light to measure the maximum absorption of photosystem II. This is a measure of a plant's ability to photosynthesize. After treatment with 150mM NaCl, *crf1* and *crf2* both had significantly higher Fv/Fm ratios than WT (Figure 4), indicating more remaining ability to photosynthesize after stress treatment.

crf1 and crf2 seeds are larger than WT

Measurements reveal that both *crf1* and *crf2* seeds are larger than WT. The *crf1* seeds have around 10% and *crf2* 21% greater seed volume as compared to WT (Figure 3). *Crf2* seeds were also shown to be significantly larger than *crf1*. *Crf2ox* seeds were measured, but their size variability was greater and although similar in size to *crf1*, this was not significant.

PROCRF2::GUS plants show induction with salt and cytokinin

Promoter::GUS fusion seedlings floated in 150 mM NaCl solution and 2 µM benzyl adenine, a synthetic cytokinin, showed slight, but distinct induction with both treatments at 1 and 4 hours (Figure 6).

Discussion

When examining the cytokinin measurement data, it is clear that CRF2 plays a pivotal role in mediating cytokinin production after salt stress. One key element that may play a role in this is the regulation of CYP735A2 hydroxylase (Table 2), which catalyzes the addition of an –OH group, converting an isopentenyl riboside to a trans-zeatin riboside (Takei et al, 2004). In Figure 2, this would be the precursor form, and CYP735A2 converts iPRMP to tZRMP. In a *crf2* background this is significantly down-regulated, but in wild type, it is significantly up. This lack of conversion in the *crf2* mutants may account for the increase in iP ribotide seen when almost all other changes are decreases. Conversely, the increase in WT CYP735A2 may account for the reason iP riboside monophosphates are decreased although everything else is unchanged or

increased. Looking broadly at the CK list, WT shows more gene transcripts which are more highly up-regulated, 7 total, which are responsible for CK synthesis and conjugation, including IPTs, CYP735s, LOG1, and UGTs, while in the *crf2* list, there are only 2 up-regulated (LOG1 and UGT) and to a lesser degree. Both lists show some variability, for example in WT there are some IPTs up- and some down-regulated. Both lists also show up-regulation of Cytokinin Oxidases, CKX, involved in CK breakdown and type-A Response Regulators (RRs), which negatively regulate CK signaling. Interestingly, both WT and *crf2* show down-regulation of CK receptor and phosphotransfer genes, AHKs and AHPs.

In the GO lists (Table 1), *crf2* is significantly up-regulated for "abiotic stress" processes while wild type is not. It is also up-regulated for "porphyrin-containing compound metabolic processes," which may imply roles in photosynthesis, as chlorophyll contains a porphyrin ring coordinated by Mg. *Crf2* has been implicated in chloroplast division previously (Okazaki et al, 2009). These findings, taken with the measurements indicating greater photosynthetic capability after salt exposure and induction in promoter::GUS fusions, imply a strong connection between CRF2 and response to salt. The additional connection to cytokinin clearly establishes CRF2 as a mediator of cytokinin production after salt stress exposure. Defining how this process occurs will be useful in further determining how cytokinin is connected to abiotic stress.

Materials and Methods

Seedling growth and stress exposure

Arabidopsis WT and *crf2* seeds were planted in damp sunshine #8 soil mix, grown in 16h, 26 °C light and 8h, 22 °C dark periods with light supplemented at 150 μE m−2 s−1. Once germinated, seedlings grew for ten days before treatment. At ten DAG (days after germination), seedlings

were excised from soil with roots intact. Plants were exposed to either MES + 150 mM NaCl or MES buffer as a control. (MES buffer = 2-(N-morpholino)ethanesulfonic acid +20% sucrose) for 6h under gentle shaking conditions at room temperature. After treatment, plants were carefully patted dry then fresh weight was measured. Seedlings were pooled to get samples of approximately 25 mg tissue. Immediately after samples were flash frozen in liquid nitrogen, ground by mortar and pestle into a fine powder, and then split into samples for RNA extraction and sequencing, as well as measurement of cytokinin levels, described below.

RNA extraction, Library preparation, and Illumina GAIIX sequencing

Three independent biological replicates were used to isolate total RNA using the Qiagen RNeasy Plant Mini-kit according to the manufacturer's instructions. Total RNA then used for messenger RNA isolation with polyA selection and subsequent library construction with the TruSeq RNA sample preparation protocol from Illumina (San Diego, CA). Three biological replicates were sequenced and analyzed for each of the 2 treatment and 2 genotype combinations. Single-end sequencing was performed on the 12 samples by the Illumina GAIIX platform, generating 334,571,961 1x54bp reads. Raw sequence data is available for download at NCBI Sequence Read Archive under the BioProject ID: XXX.

Illumina mRNA Sequence Data Analysis

High quality sequence data generated for each of the 12 samples were aligned to *Arabidopsis thaliana* TAIR10 reference genome. The associated annotation file, GFF format, was used to obtain genic information for downstream analysis. BAM alignments were generated using GSNAP (Genomic Short-read Nucleotide Alignment Program) (version released on 2013_05_09) (Wu and Watanabe, 2005) with the following parameters; indel penalty = 2, maximum mismatches = 0.06, terminal threshold = 1,000, novel splicing = 1, local splice

distance = 10000, distant splice penalty = 1,000 and everything else set to default. Read counts were generated using an in house pipeline of ALPHEUS (Miller et al., 2008) as previously performed in Gupta et al., 2013. Gene expression for each of the 12 samples was computed as a measure of the total number of reads uniquely aligning to the reference genome, binned by genic coordinates (information acquired from the annotation GFF3 file). Differential gene expression analysis was performed using the R (R core Team 2013) (http://www.R-project.org/) Bioconductor package DESEQ (Anders and Huber, 2010). Raw read counts obtained were normalized to account for differences in sequencing depth and composition using methods implemented within DESEQ package. Differential expression of pairwise comparisons (combinations of the different conditions) was assessed using the negative binomial test with the Benjamani–Hochberg false discovery rate (FDR) adjustment applied for multiple testing corrections. For this study, an FDR of 0.05 was applied and any candidate that had a p-adjusted value of ≤ 0.05 was considered to be significantly regulated.

Measurements of Cytokinin Levels

Extraction and quantification of cytokinins were performed as described previously (Svačinová et al., 2012) using the LC-MS/MS system consisting of an ACQUITY UPLC System and Xevo TQ-S triple quadrupole mass spectrometer (Waters). Results are presented as the average of five biological replicates ± standard deviation in pmol/ g FW. Statistical examinations were made between untreated and treated experiments using an ANOVA analysis.

Seed size measurements

WT, *crf1*, and *crf2*, *2ox* seeds were placed on filter paper and photographed at the same scale. The length and width at the widest part were measured using the ImageJ program, and area was calculated using a spheroid approximation as in Reifler et al (2006)

(V=4/3π*length*width*height, where width and height use the same width measurement).Three photos were taken of each genotype and 50 seeds were measured per photo.

Promoter expression analysis

CRF1 and CRF2 promoter GUS fusion lines, originally described in Zwack et al., 2012 were further examined under hormone and stress treatments as described in the text. GUS staining of lines was then performed as in Zwack et al., 2012.

Examination of Photosystem II efficiency

Plants grown under standard conditions in soil had mature leaves cut and placed in six well cell culture plates containing 3mM MES either with or without 150mM NaCl for 48h. After which plants were dark adapted for 30m and Fv/Fm measurements were performed in a manner similar to as in Zwack et al., 2016 with experimental treatments conducted in triplicate biological replicates with analysis using Student's t-Test.

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Figure Legends

WT	iP	tZ	DHZ	cZ
RMP	3.95±0.45	0.81±0.05	ND	10.00±2.15
R	0.63±0.17	0.14±0.03	0.009±0.002	0.57±0.09
-	0.85±0.19	0.16±0.02	ND	0.28±0.06
7G	15.75±0.54	21.33±1.53	1.38±0.12	
9G	2.14±0.04	11.94±0.98	0.14±0.02	0.32±0.05
OG		2.83±0.54	ND	0.73±0.13
ROG		0.58±0.03	ND	1.43±0.25
Total	23.30±1.03	37.80±2.86	1.53±0.11	13.34±1.89

WT+NaCl	iP	tZ	DHZ	cZ
RMP	2.10±0.14	0.95±0.14	ND	18.93±4.93
R	0.54±0.17	0.20±0.06	0.021±0.006	1.29±0.26
-	0.61±0.09	0.20±0.05	ND	0.31±0.08
7G	22.10±1.59	31.32±4.96	1.81±0.32	
9G	3.08±0.34	15.04±3.18	0.18±0.04	0.34±0.09
OG		4.65±0.67	ND	1.04±0.11
ROG		0.80±0.09	ND	2.21±0.38
Total	28.42±2.03	53.16±8.68	2.01±0.36	24.12±5.33

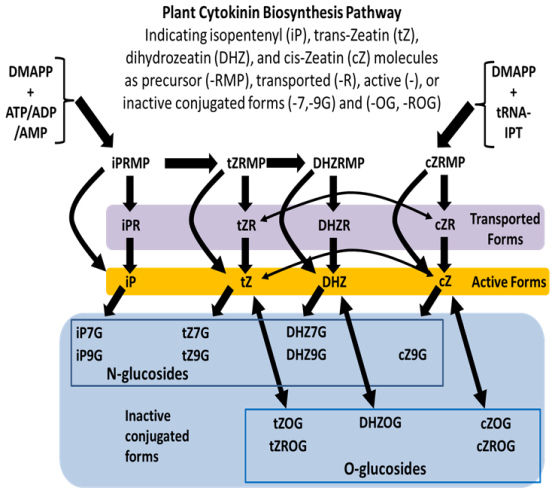
crf1	iP	tZ	DHZ	cZ
RMP	2.04±0.40	0.76±0.08	ND	7.95±1.00
R	0.60±0.20	0.19±0.06	0.010±0.003	0.87±0.23
-	0.86±0.11	0.18±0.06	ND	0.29±0.07
7G	13.75±2.19	18.18±3.37	1.05±0.20	
9G	1.60±0.23	9.86±1.60	0.12±0.03	0.34±0.04
OG		1.60±0.34	ND	0.71±0.16
ROG		0.54±0.10	ND	1.32±0.30
Total	19.21±2.24	31.32±5.43	1.18±0.23	11.47±1.58

crf1+NaCl	iP	tZ	DHZ	cZ
RMP	2.13±0.63	1.16±0.32	ND	13.41±3.85
R	0.59±0.16	0.24±0.07	0.022±0.005	1.08±0.26
-	0.82±0.13	0.19±0.06	ND	0.29±0.07
7G	18.71±5.27	27.85±6.74	1.62±0.45	
9G	2.43±0.61	11.99±2.99	0.15±0.03	0.29±0.08
OG		2.45±0.76	ND	1.02±0.23
ROG		0.77±0.16	ND	2.13±0.57
Total	24.69±5.38	44.65±10.32	1.79±0.48	18.22±4.44

crf2	iP	tZ	DHZ	cZ
RMP	3.55±0.71	0.89±0.14	ND	8.35±1.14
R	0.81±0.17	0.18±0.03	0.002±0.000	0.66±0.19
-	0.74±0.21	0.17±0.02	ND	0.25±0.05
7G	16.51±1.29	21.99±1.39	1.52±0.09	
9G	2.42±0.30	11.49±0.66	0.16±0.02	0.36±0.04
OG		2.96±0.23	ND	0.79±0.13
ROG		0.70±0.03	ND	1.45±0.21
Total	24.04±1.79	38.38±1.78	1.67±0.09	11.85±1.52

crf2+NaCl	iP	tZ	DHZ	cZ
RMP	1.58±0.22	0.57±0.18	ND	8.42±1.67
R	1.81±0.55	0.27±0.05	0.082±0.010	3.24±0.68
-	0.88±0.19	0.23±0.04	ND	0.30±0.07
7G	13.28±1.37	15.46±2.75	1.11±0.20	
9G	1.67±0.30	8.70±1.19	0.11±0.01	0.30±0.05
OG		2.18±0.55	ND	0.59±0.07
ROG		0.37±0.07	ND	0.92±0.19
Total	18.33±1.63	27.78±4.53	1.30±0.21	13.77±1.94

Figure 1. CRF2 is involved in regulating cytokinin levels with salt stress. Specific amounts of cytokinin compounds measured by LC/MS in pmol/g FW from 10d WT, *crf1*, *crf2* seedlings either MES-Buffer treated (Control) and Salt (150mM NaCl) treatment for 6h (n=5). Pastel colors indicate cytokinin form shown as detailed in Figure 1. Green/Red colors indicate significant ($p<0.05$) increase/decrease, respectively, as compared to WT of the same treatment group.



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Total	28.42±2.03	53.16±8.68	2.01±0.36	24.12±5.33

Figure 2. Cytokinin levels are altered by salt stress. (Left) Biosynthetic pathway for cytokinins in plants. (Right) Specific amounts of cytokinin compounds measured by LC/MS in pmol/g FW from 10d seedlings MES-Buffer treated (Control) and Salt (150mM NaCl) treatment for 6h (n=5). Pastel colors indicate cytokinin form shown as in the biosynthetic pathway. Green/Red colors indicate significant ($p<0.05$) increase/decrease vs untreated control. Results show general increases in cytokinin levels in response to salt stress.

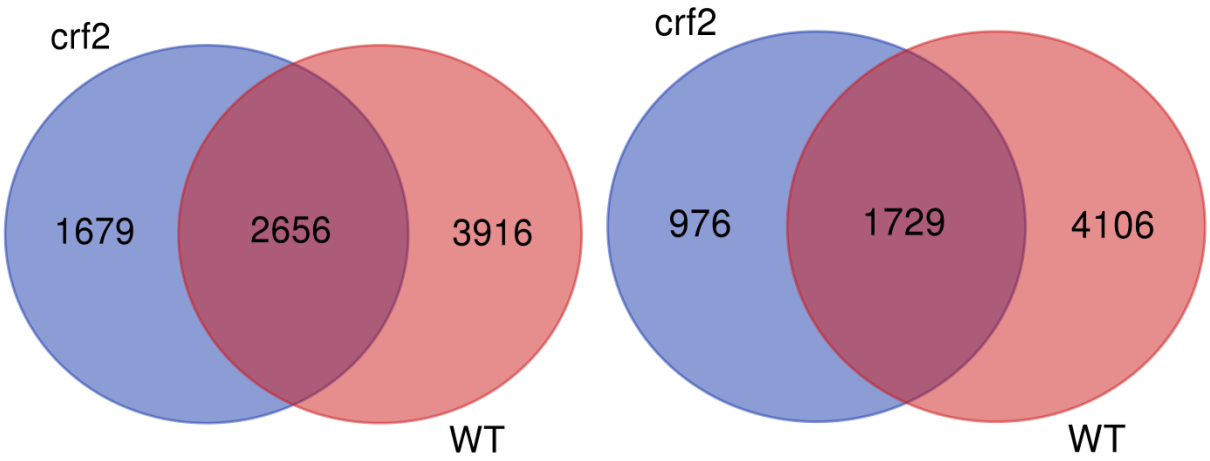


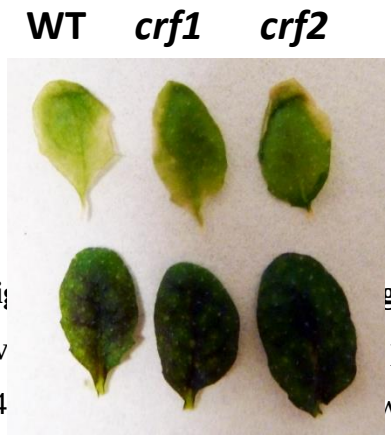
Figure 3. Venn diagrams showing overlap in DE transcripts. The left Venn shows the amount of overlap in up-regulated transcripts between *crf2* and WT when exposed to salt treatment. The right Venn shows overlap of down-regulated transcripts. In general, about 1/3 of DE transcripts in WT overlap with *crf2*. In the *crf2* background, around 2/3 overlap with WT.

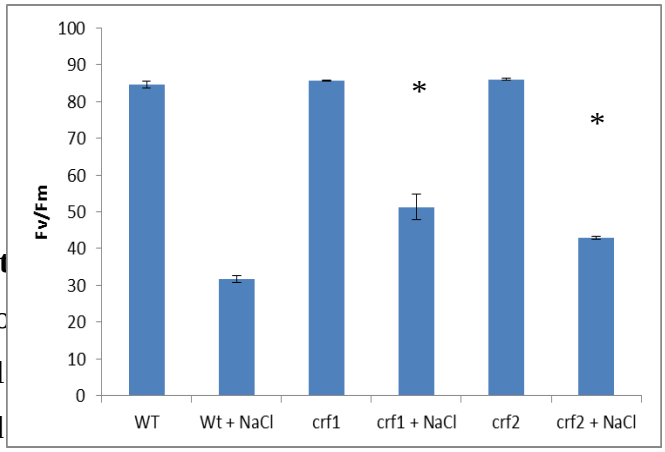
WT			***crf2***		
Up-regulated with NaCl	Fold Enrichment	FDR	**Up-regulated with NaCl**	Fold Enrichment	FDR
Catabolic Process	1.21	1.65E-02	Porphyrin-containing compound metabolic process	2.73	2.30E-02
Regulation of nucleobase-containing compound metabolic process	1.37	2.15E-02	Response to abiotic stimulus	1.79	2.42E-02
Endocytosis	1.62	1.33E-02	Sulfur compound metabolic process	1.78	3.39E-02
Excytosis	1.59	3.80E-02	Generation of precursor metabolites and energy	1.58	3.91E-02
Response to endogenous stimulus	1.54	1.81E-03	Cellular amino acid metabolic process	1.54	1.35E-02
Lysosomal transport	1.26	6.77E-04			
Down-regulated with NaCl			**Down-regulated with NaCl**		
Transcription from RNA polymerase II promoter	0.63	2.69E-02	Transcription from RNA polymerase II promoter	0.5	1.63E-02
rRNA metabolic process	1.76	2.98E-02	Translation	0.38	9.05E-03
Translation	1.53	4.23E-02	Lipid metabolic process	1.49	3.70E-02
			Chromatin organization	0.22	6.31E-03

Table 1. Gene Ontology Statistical Overrepresentation of DE genes. Selected results from Gene Ontology (GO) analysis for overrepresentation was examined for WT and *crf2* with exposure to salt at pantherdb.org using default settings. Up- and down-regulation for each genotype are shown. False Discovery Rate (FDR<0.05)

WT vs WT+NaCl					*crf2* vs *crf2*+NaCl			
Gene ID	**Function**	**log2 FC**	**padj**		**Gene ID**	**Function**	**log2 FC**	**padj**
CYP735A1	Biosynthesis	2.94	2.39E-05		CKX4	Metabolism - Cytokinin Oxidase	2.59	0.000131
CYP735A2	Biosynthesis	2.58	5.93E-06		LOG1	Biosynthesis	2.02	0.000348
UGT76C2	Conjugation	2.49	1.73E-41		ARR15	Signaling – Type A-RR	1.30	0.039326
ARR15	Signaling – Type A-RR	2.13	7.73E-05		UGT85A1	Conjugation	0.59	0.015452
ARR7	Signaling – Type A-RR	2.06	1.79E-14					
UGT85A1	Conjugation	1.68	5.45E-17		AHP3	Signaling - Phosphotransfer	-0.49	0.037417
CKX4	Metabolism - Cytokinin Oxidase	1.64	9.32E-07		ARR3	Signaling – Type A-RR	-0.86	0.034841
ARR9	Signaling – Type A-RR	1.49	1.48E-13		AHK4	Signaling - Receptor	-0.90	1.76E-05
UGT76C1	Conjugation	1.41	9.73E-16		IPT5	Biosynthesis	-1.47	0.009509
ARR4	Signaling – Type A-RR	1.29	6.13E-23		ARR11	Signaling – Type B-RR	-1.49	0.002035
ARR6	Signaling – Type A-RR	1.25	2.03E-05		CYP735A2	Biosynthesis	-1.58	0.029482
IPT2	Biosynthesis	1.21	1.20E-08		AHP1	Signaling - Phosphotransfer	-2.26	0.00348
CKX7	Metabolism - Cytokinin Oxidase	1.11	2.67E-06					
LOG1	Biosynthesis	0.74	0.001414					
CKX6	Metabolism - Cytokinin Oxidase	0.64	0.000421					
AHP5	Signaling - Phosphotransfer	-0.38	0.032798					
IPT9	Biosynthesis	-0.48	0.003939					
AHK4	Signaling - Receptor	-0.65	0.007775					
AHP3	Signaling - Phosphotransfer	-0.69	0.000165					
IPT3	Biosynthesis	-1.70	0.003976					
AHP1	Signaling - Phosphotransfer	-2.13	4.29E-12					

Table 2. Cytokinin-related transcripts differentially-regulated by NaCl stress. Manually curated list of CK-related genes involved in synthesis and signaling. Known gene names were searched to procure a list of genes including synthesis (IPTs, LOG1, CYP735As, and UGTs), breakdown (CKXs) and signaling (AHKs, AHPs, Type-A RRs, and Type-B RRs).





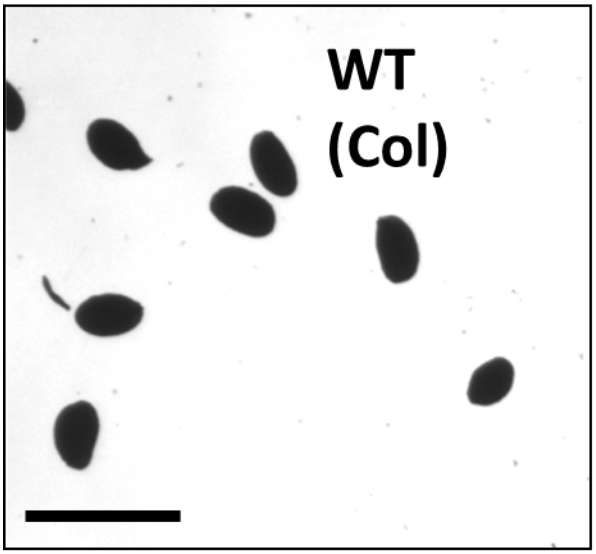
Fi greater photosynt

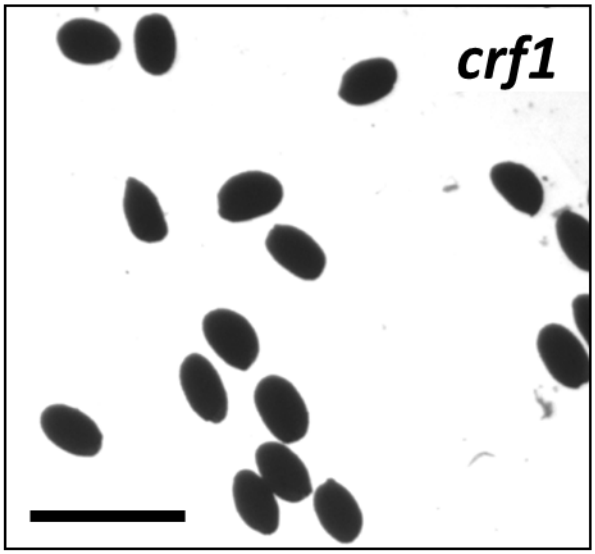
Fv possible amount c

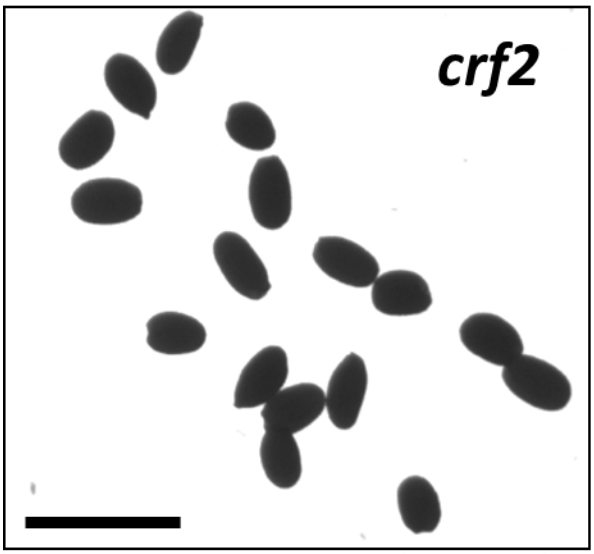
14 with 150mM NaCl

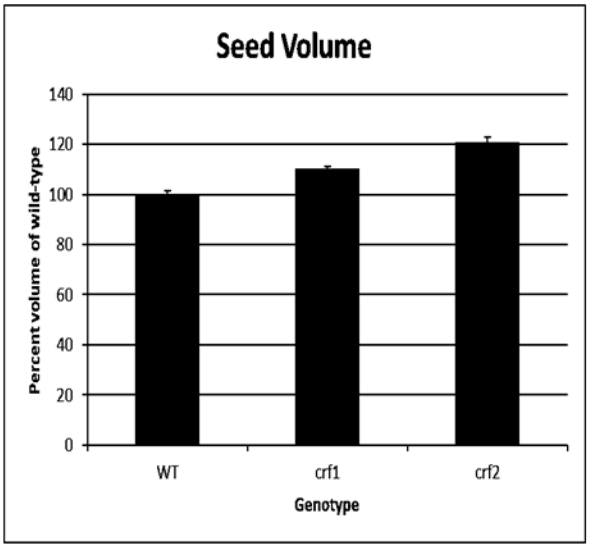
Fv/Fm measurements were taken. (Photo) Treated

Graph shows that *crf1* and *crf2* have significantly higher Fv/Fm ratios than WT after NaCl treatment indicating greater photosynthetic capability.









Measurements	WT	crf1	crf2	2ox
Length (mm)	0.445	0.462	0.471	0.461
Width (mm)	0.257	0.265	0.275	0.263
Volume (mm^3)	0.123	0.135	0.149	0.134
Std Error (vol)	0.0016	0.0013	0.0030	0.0076
p-value (vol)		0.00338	0.00160	0.229

Figure 5 (with accompanying table). *Crf1 and crf2* seeds are larger than WT. Seeds from WT, *crf1*, and *crf2* were photographed and measured using ImageJ. *Crf 1* and *crf2* showed significantly greater length, width, and volume as compared to WT. *Crf2* is also significantly larger than *crf1* (p-val=.0153). Although *2ox* has similar values compared to *crf1*, there was larger deviation in the measurements. Photos show representative seed pictures. The graph shows WT normalized to 100% with *crf1* and *crf2* sizes shown in relation to that. Bars show standard error. Length, width, and volume measurements in table represent average values.

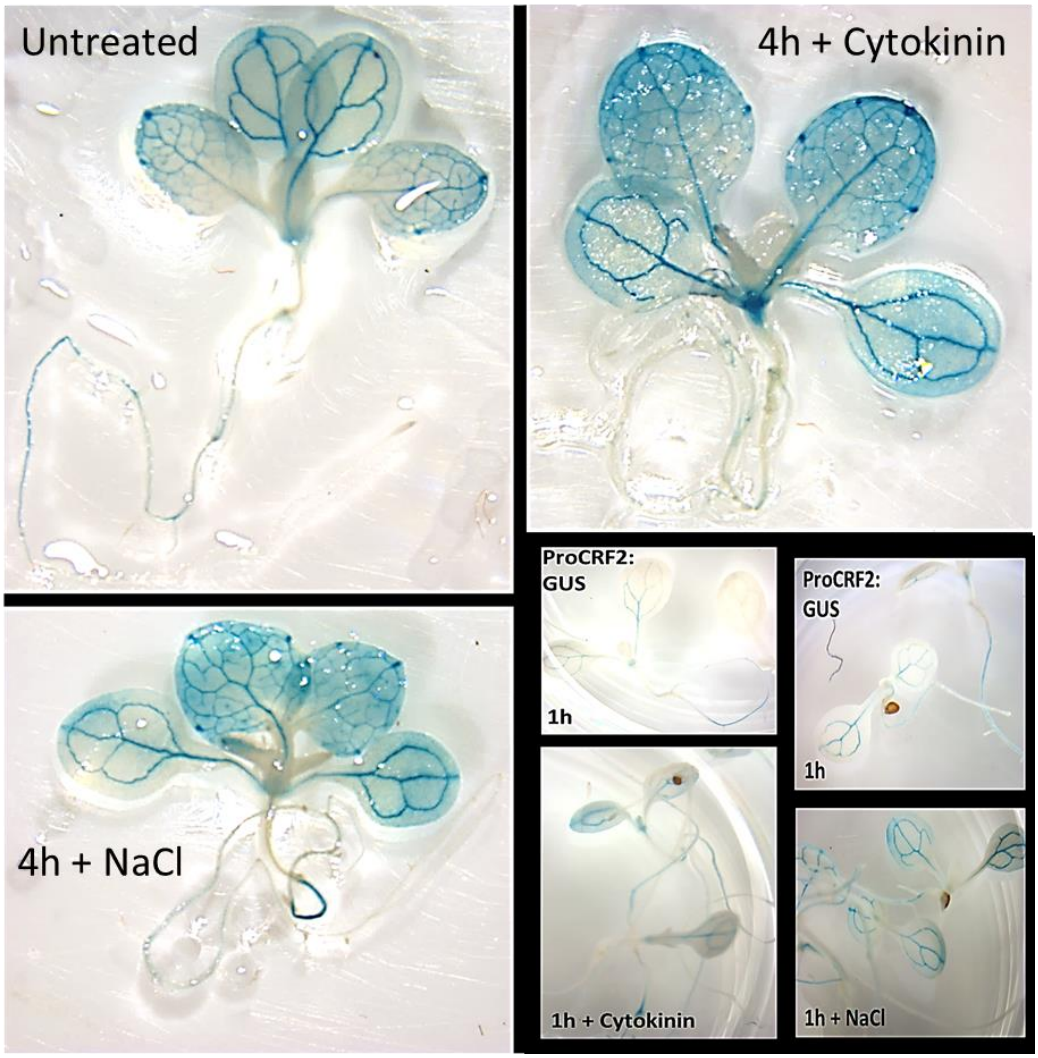


Figure 6. CRF2 is induced by Cytokinin and Salt. Promoter CRF2:GUS lines were examined for change in response to cytokinin (5µM BA) or salt (150mM NaCl) for 1h and 4h. Although slight, increases in CRF2 expression can be seen for both treatments as shown for representative plants.

Chapter 4: Salt and oxidative stresses uniquely regulate tomato cytokinin levels and transcriptomic response

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Author contributions

E.A.K. and A.M.R. designed research; E.A.K., H.T.H., T.R., L.P, A.S., F.S., O.N., and A.M.R performed research and analyzed data; E.A.K. and A.M.R. wrote the paper.

Keywords: Cytokinin, Abiotic Stress, Salt, Oxidative Stress, *Solanum lycopersicum*, Tomato, RNA-sequencing, Transcriptome

One Sentence Summary: Upon exposure to salt (NaCl) and oxidative (H_2O_2) stress, tomato cytokinin levels and transcriptomes are differentially regulated.

Abstract

Cytokinins are well-known to be involved in processes responsible for plant growth and development. More recently these hormones have begun to be associated with stress responses as well. However, it is unclear how changes in cytokinin biosynthesis, signaling, or transport relate to stress effects. This study examines in parallel how two different stresses, salt and oxidative stress, affect changes in both cytokinin levels and whole plant transcriptome response. *Solanum lycopersicum* seedlings were given a short term (6h) exposure to either salt (150mM NaCl) or oxidative (20mM H_2O_2) stress and then examined to determine both changes in cytokinin levels and transcriptome. LC-MS/MS was used to determine the levels of 22 different types of cytokinins in tomato plants including precursors, active, transported, and conjugated forms. When examining cytokinin levels we found that salt treatment caused an increase in both active and inactive cytokinin levels and oxidative stress caused a decrease in these levels. RNA-sequencing analyses of these same stress treated tissues revealed 6643 significantly differentially expressed genes (DEGs). Though many DEGs are similar between the two stresses, approximately one-third of the DEGs in each treatment were unique to that stress. Several cytokinin-related genes were among the DEGs. Examination of photosystem II efficiency revealed that cytokinins affect physiological response to stress in tomato, further validating the changes in cytokinin levels seen *in planta*.

Introduction

Abiotic stress results in billions of dollars of crop losses every year, including in the important crop plant tomato (Pineda et al., 2012; Qin et al., 2011; Acosta-Motos et al., 2017). Tomato growth and production is highly dependent on healthy functioning and photosynthesizing plants that are resistant to or tolerant of abiotic environmental stresses. Two primary stresses affecting tomato are salt stress and the broad stress of oxidative damage (Pineda et al., 2012; Acosta-Motos et al., 2017). These stresses are often thought of as overlapping in their effects, yet are also thought to be distinct in mode of action. In tomato, salt stress has been examined by a number of researchers, while oxidative stress is virtually unstudied (Acosta-Motos et al., 2017).

Salt stress related crop losses are a problem growing in magnitude as salt levels in farm lands are increasing due to abundant irrigation that continues to add residual salt. In tomatoes, salt stress is well known to reduce plant size, fresh weight, and photosynthesis (as recently reviewed in Acosta-Motos et al., 2017). As such attempts to understand how plants respond to salt stress in order to generate resistant or tolerant lines is of great importance. Salt stress is known to affect plants in two basic ways: either through osmotic complications or through ion toxicity (Acosta-Motos et al., 2017; Ismail and Horie, 2017). However, what remains unclear are mechanistic actions that occur as a plant responds to salt stress from either or both of these effects, particularly in regards to the genes involved in salt tolerance and resistance. Although there has been a great deal of progress made in understanding some of the basic components, signaling pathways, and initial genes involved in plant salt stress response, there is still much that is unknown (Acosta-Motos et al., 2017; Ismail and Horie, 2017; Singh et al., 2017).

Oxidative stress produced during abiotic stresses is highly detrimental to growth at all stages of development and can greatly reduce crop yields in plants (as reviewed in Pandey et al., 2011; Qin et al., 2011; Duque et al., 2013). In tomato, oxidative stress is a major abiotic factor (Pandey et al., 2011), which causes damage at both tissue and cellular levels that can rupture membranes, breakdown photosynthetic machinery, and cause cell death (Zwack and Rashotte, 2015). Plants utilize the redox potential of molecular oxygen (O_2) and water (H_2O) to drive both reductive (photosynthesis) and oxidative (respiration) energy production. During each processes partially reduced/oxidized O_2/H_2O can form. Such redox intermediates readily oxidize other molecules and are known as reactive oxygen species (ROS) (Apel and Hirt, 2004; Gill and Tuteja, 2010). ROS or oxidative stress damage can occur during various abiotic stresses, such as extreme temperatures that alter membrane fluidity disrupting electron transport chain reactions (Mittler, Finka, and Goloubinoff, 2012) and high light levels that overwhelm photosystem II and its light harvesting complexes with excitation energy (Gill and Tuteja, 2010). ROS can additionally be produced directly through O_2 interactions with heavy metals, UV radiation and pollutants such as ozone and sulfur dioxide (Mittler, 2002; Gill and Tuteja, 2010). Since many stresses stimulate a response that includes production of ROS, it is often involved in general stress responses (Apel and Hirt, 2004; Mittler et al., 2004; Saxena, Srikanth, and Chen, 2016). Yet for some crop species such as tomato only a handful of genes have been linked to oxidative stress and require additional study.

An underexplored avenue for regulation of both salt and oxidative stress responses is the plant hormone cytokinin. Cytokinins are essential plant hormones involved in numerous plant growth and developmental processes of great agronomic importance (Mok and Mok, 2001; Davies, 2004; To and Kieber, 2008; Muller, 2011). Notably, while both salt and oxidative

stresses are detrimental to plant health and linked to abiotic stress senescence, cytokinins are connected to delaying plant senescence. Cytokinins have also been strongly linked to plant abiotic stress responses in studies examining cytokinin biosynthesis, metabolism, and signaling, where changes in cytokinin levels, responsive genes, or receptor mutants have dramatically altered growth under stress conditions (as seen or reviewed in Peleg and Blumwald, 2011; Wilkinson et al., 2012; Nishiyama et al., 2011; Ramireddy et al., 2014; Zwack and Rashotte, 2015; Veselov et al., 2017). This has also been shown in tomato for salt stress, albeit to a lesser degree (Ghanem et al., 2008, 2011; Pandey et al., 2011; Pineda et al., 2012; Žižková et al., 2015). Oxidative stress remains largely unstudied in tomato (Mittova et al., 2003; Bose, Rodrigo-Moreno, and Shabala, 2014). As such, little is known in tomato of the mechanisms behind or pertinent genes involved in cytokinin-based regulation of salt or oxidative stress.

This study examines connections between salt stress, oxidative stress, and cytokinins. Distinct changes in cytokinin levels after salt and oxidative stress along with distinct transcriptome changes were found. Specific alterations were identified in cytokinin-related genes under each stress condition. These distinctive changes could be further linked to efficiency of photosystem II (Fv/Fm) under salt and oxidative stress conditions. Furthermore, cytokinins have effects on Fv/Fm under these stress conditions, possibly connecting the changes in cytokinin levels *in planta* to the observed physiological stress responses.

Results

Examination of cytokinin regulation by salt and oxidative stress reveal distinct responses

Cytokinins have been previously connected to various stress responses in plants. To further examine this several approaches were taken. A simple examination of the cytokinin-responsive

reporter line pTCS::VENUS that is well-known to indirectly indicate cytokinin levels in tomato roots (Zucher et al., 2013; Bar et al. 2016) was examined under salt and oxidative stress treatments. We found as previously shown that cytokinin (5µM *trans*-zeatin) strongly induces YFP (Yellow Florescence Protein) fluorescence over a buffer only control treatment in young seedlings after 24h (Figure 1). There was also a smaller qualitative change in YFP fluorescence in response to stress versus buffer control: fluorescence appeared to be induced with salt (150mM NaCl) and repressed with oxidative stress (20mM H_2O_2) (Fig. 1). Although these stress-induced changes are not as dramatic as the change seen with cytokinin treatment, they do suggest that cytokinin levels might be affected by stress.

In order to directly examine cytokinin level changes in response to these stress treatments, LC-MS/MS measurements of salt and oxidative stress treated seedlings (10d) were performed. Soil grown seedlings were gently washed and placed in MES buffer with light shaking, then exposed to either salt (150mM NaCl), oxidative stress (20mM H_2O_2), or the buffer control for 6h. Twenty-two different cytokinin forms were detected in this study including precursors, active, transported, and conjugated forms of isopentenyladenine (iP), *trans*-zeatin (*tZ*), dihydrozeatin (DHZ), and *cis*-zeatin (*c*Z). Cytokinin levels in buffer treated control plants show general similarities to those previously found in tomato (Žižková et al., 2015) and other eudicot plants with some variation (Sakakibara, 2006). The most abundant components are the *N*-glucoside forms (iP7G, tZ7G, and DHZ7G), with the active bases iP, *t*Z, and *cZ* making up a small part of the total measured cytokinins (Figure 2). Salt treated plants showed a significant increase in 11 individual cytokinins, including the important active form *t*Z, as well as the total amount of cytokinin bases, ribosides, and nucleotide forms (Fig. 2). Although there is more than a 25% increase in overall cytokinin levels compared to control, this was not significant, likely

due to the lack of a significant increase in total levels of cytokinin conjugates. Oxidative stress treated plants showed the opposite result with significantly decreased levels of seven different cytokinin forms, including *t*Z. There is also an opposing overall 20% decrease in cytokinin levels, although not significant, again likely due to non-significant changes in the high levels of conjugate cytokinin forms. However, a comparison of overall cytokinin levels in plants between salt and oxidative stress treatment is significantly different ($p<0.01$) indicating a clear difference in cytokinin levels between these two stresses. This result is similarly true when comparing of all general classes of cytokinins to each other (bases, ribosides, nucleotides, *O*-glucosides, and *N*-glucosides) at $p<0.05$ level (Supplemental Table 1). Interestingly, the only significantly regulated cytokinin under both stresses was *cZ*, which has previously been suggested to play a role in environmental stress response (Schäfer et al., 2015)

Transcriptomic effects of salt and oxidative stresses

As part of the split experimental design, plants from the same stress treatments described above were examined using RNA-sequencing. RNA was extracted from these seedlings to produce cDNA libraries from which single-end Illumina GAIIX RNA-sequencing was performed. This generated a total of 186,460,653 1×54bp reads from all samples, which were aligned to the *S. lycopersicum* reference genome. Further analysis resulted on average in 14.3 million uniquely aligning reads per sample from which gene expression was quantified, using the total number of reads per sample that uniquely aligned to the reference binned by gene (Table 1). Genes used for differential expression (DE) analysis were restricted to those found to be significantly regulated based on a $P_{adj} < 0.05$ (Supplemental Table 2) as compared between stress treated and untreated control.

qPCR was performed in order to confirm the results of RNA-sequencing on 5 DE genes affected by salt and oxidative stress treatments (Table 2). This comparison yielded similar expression directionality and level of regulation for genes examined indicating that the $\log_2$ fold-change values obtained from RNA-sequencing were accurate. In addition to confirming the overall validity of the RNA-sequencing data, these qPCR data suggest that a number of hormone related genes are affected under stress conditions, including ACC synthase (ethylene), IAA-Amido Synthetase (auxin), and His-containing phosphotransmitter Hpt4-like (cytokinins). Universal Stress Protein A and a sodium-proton antiporter were also altered by stress.

Overall transcriptomic changes under each stress condition were compared using the lists of significantly differentially expressed genes (DEGs) in a Venn diagram, 3950 DEGs were identified under oxidative stress and 4617 DEG were found under salt stress. A similar number of DEGs has been found to be regulated by other stresses in other systems (Nishiyama, et al., 2011). While abiotic stresses are sometimes thought of as triggering similar gene expression changes in plants, here we see strong differences between the overall set of stress-regulated DEGs where 71.0% of all DEGs show unique stress regulation and only a 29% overlap. A similar trend is found when the stress induced and repressed gene lists are separately compared to each other: 67.6% of induced and 75.6% of repressed DEGs show unique stress regulation (Figure 3A). In contrast almost no induced genes from one stress are repressed by the other list: only 38 oxidative stress induced and salt repressed, and 74 salt induced and oxidative stress repressed. Additionally, a Principal component analysis (PCA) and variance decomposition (both as implemented in SAS JMP Genomics 5.1) of the overall, full transcriptome dataset ($n = 9$) showed distinct differences between each set of treatment samples and the buffer control (Fig. 3B). Approximately 35% of the variation appears to be from treatment, with at least 20% of the

variation appears as a difference between salt and oxidative stress treated samples (Fig. 3B). Together this suggests a distinct transcript pattern of gene regulation from salt versus oxidative stress.

In order to additionally compare patterns of gene regulation between salt and oxidative stress, GO (Gene Ontology) enrichment analysis was performed on the lists of DEGs for each stress. Since each gene list contained a large number of DEGs, lists were further divided into induced and repressed lists for each stress. Importantly, in each of these lists, response to abiotic stress was found as significantly enriched 2.0-2.5 fold (Table 3). Several other stress or stimulus response GO terms were also found as enriched (Table 3). Distinct GO terms were found as significantly enriched for each stress (Supplemental Table 4). One such example is for ethylene related GO categories (biosynthetic, metabolic, and response to ethylene) that were significantly enriched for oxidative yet not for salt stress. There are also a number of GO terms that are enriched for both stresses only when comparing the induced lists (calcium ion/calmodulin binding, glucosyltransferase activity, phosphatase activity) and similarity only when comparing the repressed lists (chlorophyll binding, structural constituent of cytoskeleton). Based on changes that were seen from examination of direct and indirect measurements of cytokinins in Figures 1 and 2, it would be predicted that some cytokinin related category would be found as enriched from this GO analysis, although that was not the case. We believe that this is due in part to poor annotation of GO terms in descriptions of cytokinin-related genes in tomato.

In order to more thoroughly examine the regulation of cytokinins under stress conditions, a manual examination of DEG lists was made, using search terms such as “cytokinin” and “zeatin” as well as searching by Solyc# based on previous publications of cytokinin-related gene lists and phylogenetic comparisons to between tomato and Arabidopsis (Sun et al., 2017; Capua

and Eshed, 2017). From that examination 35 cytokinin-related DEGs could be identified as significantly regulated by stress (Table 4). These 35 genes are involved in cytokinin biosynthesis, transport, and signaling, yet only 3 were found with GO identifiers relating to cytokinin. Twenty-five of these genes show a unique regulation between salt and oxidative stresses: 10 oxidative stress only, 15 salt only, and 10 both oxidative and salt regulated.

Examination of cytokinin treatment on photosystem II efficiency under oxidative and salt stress

Based on the findings that cytokinin levels can be affected under specific stress conditions as seen from both cytokinin measurements and transcriptomic results (Fig 1-3, Table 3), we further examined the effects of cytokinin on abiotic stress response. The classic cytokinin senescence bioassay, normally used to examine developmental senescence, was modified to examine abiotic stress responses of tomato leaf disks (Mok and Mok, 2001; Zwack et al. 2013). Previous work, again verified here, shows that cytokinin treatment of leaf disks reduces leaf senescence as measured using a chlorophyll fluorimeter to determine efficiency of photosystem II or Fv/Fm (Zwack et al., 2016; Fig 4, 5). Oxidative stress (20mM H_2O_2) reduces Fv/Fm by 10-30% over simple buffer treatment. However, application of exogenous cytokinin (5μM BA) either before (pre – initial stress treatment 0h) or after (post 48h) this oxidative stress treatment significantly increases Fv/Fm levels over stress treatment alone (Fig. 4). This is consistent with findings here that cytokinin levels are reduced in oxidative stress treated plants (Fig 1-2), such that the exogenous application to raise that level could potentially restore plants to a normal functioning level.

Salt stress (150mM) treatments show a similar reduction in Fv/Fm to 30-40% of buffer treated leaf disks (Fig. 5). A cytokinin pre-treatment is able to significantly reduce the drop in Fv/Fm levels after a salt treatment, although salt still has an effect compared to buffer or

cytokinin treatment alone (Fig. 5). While a cytokinin application post-salt stress treatment was unable to increase Fv/Fm levels. Since these results show cytokinin levels were increased after salt stress treatments (Fig. 2), it seems that further increasing cytokinin levels after stress has begun has no remediation effect and a cytokinin application has only a minor effect once salt stress is given.

Discussion

Tomato production and yield is highly dependent on healthy photosynthesizing and fully functioning plants that can resist or tolerate abiotic stress. A greater understanding of the genes that regulate salt and oxidative stress resistance and tolerance is highly valuable towards the improvement of healthy tomato plants and fruits grown under field conditions. Whereas many studies of tomato have focused on older reproductively active plants or on fruits, here we examined stress response in young seedlings that are sensitive to both salt and oxidative effects. Although fruit production is the ultimate goal of tomato cultivation, abiotic stress in young tomato seedlings can lead to highly reduced plant growth, fruit production, and death (reviewed in Acosta-Motos et al., 2017). As cytokinins are well known to be involved in growth and development with links to delaying senescence, this study examined connection between cytokinins and abiotic stress in tomato seedlings.

The first indication we identified of a connection between cytokinins and both salt and oxidative stress was seen the cytokinin-responsive transgenic reporter line in tomato: pTCS::VENUS (Capua and Eshed, 2017). Here we found changes in YFP fluorescence in response to each stress in the roots of this stable tomato pTCS::VENUS line, specifically designed to show cytokinin responsiveness (Fig. 1). Salt (NaCl) treatment resulted in some

increase in YFP fluorescence over buffer control treatment. Although this induction is not nearly as strong as with cytokinins, it does indicate there is an interaction between salt stress and cytokinin response. In contrast, a H_2O_2 oxidative stress treatment results in reduced levels of cytokinin responsive YFP fluorescence, suggesting that cytokinin activity is negatively regulated by oxidative stress treatment. While these findings indicate indirect cytokinin connections to each stress through activation/repression of this type-B response regulator binding site linked to YFP, it provided a basis to conduct direct and quantifiable measurements of cytokinin levels under these same stress conditions.

Direct measurements of cytokinin levels using LC-MS/MS of stress treated tomato seedlings found similar evidence of distinct regulation for each stress (Fig. 2) as seen with the pTCS::VENUS reporter line over a longer exposure. Seedlings exposed to salt stress (150mM NaCl for 6h) showed a significant increase of several different cytokinin forms over buffer treated samples, including precursors, transported, active and conjugated forms (Fig. 2). Importantly the highly active form *tZ* is significantly increased to nearly 200% the level of untreated plants and levels of *cZ* are even more highly increased. Although *cZ* is known to be a less active cytokinin form it has been previously connected to salt and other stress responses (Prerestova et al., 2017; Schäfer et al., 2015). Together this shows that cytokinin levels are increased in young tomato seedlings in response to salt stress. Similar increases in cytokinin levels have been previously found in connection to salt stress (Žižková et al., 2015; Joshi et al., 2017; Veselov et al., 2017; Prerestova et al., 2017; Šimura et al., 2018). However, there are also a number of studies where decreased levels of cytokinins are seen after salt stress. Interestingly many of those studies were of developmentally older plants or specific tissues, such as fruits exposed to stress treatments of greater length, sometimes weeks in duration (Ghanem et al.,

2008, 2011; Nishiyama et al., 2011). This may suggest developmental differences in response to salt or possibly a more pronounced effect of general oxidative stress effects after a lengthy salt exposure. Additionally, it has been found that plants with reduced cytokinin levels, due to decreased cytokinin synthesis or increased degradation, can have increased tolerance to abiotic stresses including salt stress (Ghanem et al., 2008, 2011; Nishiyama, et al., 2011; Mackova et al., 2013; Žižková et al., 2015; Veselov et al., 2017). Taken together the results of our examinations of salt stress and cytokinins indicate a clear interaction, which may vary depending on the experimental parameters being addressed.

Oxidative stress treatment (20mM H_2O_2 for 6h) resulted in an overall reduction in the levels several different cytokinin forms (active, transported, and conjugated) in contrast to the increases seen under salt stress (Fig. 2). The active *t*Z form was found to be significantly reduced 25% after oxidative stress compared to buffer treated and a full 2.5 fold lower when compared to levels after salt stress treatment (Fig. 2). It is interesting to note that the one cytokinin form found to be at significantly increased levels was *cZ*, which was also induced by salt, although to a much higher level. It is possible that *cZ* functions in a general stress role as previously noted (Schafer et al., 2015). Since this is the first study specifically examining effects of oxidative stress treatment on cytokinin levels it is difficult to find comparable measurement data, however, a general reduction in cytokinin levels is often found in response to different stresses, as seen in Nishiyama et al., 2011. Our finding of reduced cytokinin levels after oxidative stress treatment is in line with this and we believe consistent with its role as a general stressor.

Total levels of cytokinin in plants showed an increase of about 25% after salt treatment and a reduction of about 20% after oxidative stress compared to buffer treatment (Supplemental Table 1). Although, each of these levels are not significantly different from buffer treatment, a

comparison of total cytokinin levels between salt treated and oxidative stress treated plant is significantly different (p=0.011, T-test). This supports a general finding of distinct changes in cytokinin levels for salt versus oxidative stress. While several cytokinin compounds that were measured are not affected in this snapshot of cytokinin levels after stress treatment, the prominent active cytokinin, *t*Z follows a differentially regulated pattern: significantly induced by salt and significantly reduced by oxidative stress. It is additionally important to note that stress responses to both salt and oxidative stress are known to have different dynamics in particular in relation to cytokinin levels, as reviewed in Zwack and Rashotte, 2015. Examinations of cytokinin levels and transcriptomic alterations in this study were performed at 6 hours after stress exposure to ensure initial changes in response had occurred. The generally similar findings of the pTCS reporter line at 24h and Fv/Fm over longer periods needed to see alterations due to stress overall support the early findings, although additional work is needed to fully understand changes in cytokinin dynamics during stress response.

Transcriptomic findings similarly indicate distinct profiles of gene regulation in salt and oxidative stress treated seedlings. This can most easily be seen in Figure 3 from the Venn diagrams, where about 70% of DEGs have a unique salt or oxidative stress regulation pattern. A principal components analysis (PCA) of all transcriptome data also revealed three distinct groupings of results, with at least 30% of the differences explained by stress specific treatment (Fig. 3B).

It is difficult to compare the transcriptome findings here with previous results in tomato since there have been relatively few genome-wide transcriptomic studies in tomato and fewer under abiotic stress conditions. This is the first RNA-sequencing based experiment that has been conducted in tomato on salt and oxidative stress. Previous large-scale transcript experiments

performed in tomato were often focused on older plants, aspects related to fruit, and most were conducted using early tomato microarrays, which allowed for only partial (~1/3) genome analysis (Ouyang et al., 2007; Sun et al., 2010). As such, expression of nearly 2/3 of tomato genes remained previously unexamined. Findings here do appear to generally parallel those for other plant species, with large numbers of genes being found to be stress regulated. Core sets of stress-related genes that are affected under the salt and oxidative stress conditions are examined here, as evidenced from GO enrichment analysis (Supplemental Table 4). One set of genes that we specifically looked for to be stress regulated were cytokinin-related genes, although they were not identified as overrepresented from GO enrichment analysis. We believe this is largely due to incomplete annotation of the tomato genome as well as incomplete identification of cytokinin-related genes in tomato. Despite these barriers, a manual search revealed 35 cytokinin-related genes involved in biosynthesis/metabolism, transport, and signaling, which showed various levels of regulation under stress conditions (Table 4). Interestingly the pattern of gene regulation mirrored that found in the full DEG list, with about 70% showing distinct, non-overlapping regulation between stresses. In all, 113 cytokinin-related genes were identified, with 35 (31%) being found as DEG under stress conditions. A similar examination of Arabidopsis seedlings exposed to short term salt stress revealed 25 of 92 cytokinin-related genes were affected (27%), compared here to 21% for salt alone in this study (Nishiyama et al., 2011). This suggests 20-30% of cytokinin-related genes appear to be stress regulated.

To examine this problem from another direction, we looked at how many cytokinin-regulated genes in tomato could be connected to stress response. To do this we generated a combined list of DEGs after treatment by cytokinin in tomato, from the only two cytokinin-based transcriptome experiments previously performed in tomato (Gupta et al., 2013; Shi et al, 2013)

as shown in Supplemental Table 5. From this list of nearly 1100 DEGs just over a third overlap with the stress DEGs found in this study. Additionally, this combined tomato cytokinin-regulated list was also found to be significantly over enriched for many different stress related terms, such as 'response to abiotic stress' from a GO analysis (Supplemental Table 5). While this is in many ways is an imperfect comparison based on a number of experimental sampling differences, it does suggest again the connection between cytokinins and abiotic stress regulation. Interesting most (70%) of the overlap between these lists is unique to salt or oxidative stress treated lists. Further supporting the finding that cytokinin-stress interactions appear to occur in a stress-specific manner.

We additionally examined if these cytokinin salt/oxidative stress specific patterns could be tested with a physiological parameter. Both salt and oxidative stresses are well known to be detrimental to plant growth and general photosynthetic processes, as previously detailed. One simple, non-destructive examination of photosystem II efficiency is the measurement of chlorophyll fluorescence or Fv/Fm using a fluorimeter. The classic cytokinin senescence bioassay that was modified to study abiotic stress responses was used in this study (Letham, 1971; Zwack et al., 2013; 2016). Tomato leaf disks were floated in buffer in the presence or absence of cytokinin, either before (pre) or after (post) salt (NaCl), or oxidative (H_2O_2) stress treatment. Found here, and as previously shown, photosystem II efficiency (Fv/Fm) is correlated with leaf senescence in response to the addition of cytokinin, which delays it, and stress, which promotes senescence (Fig. 4, 5; Zwack et al., 2016). Figure 4 shows this for oxidative stress and cytokinin interactions (visual images of leaf disks used for Fv/Fm measurements are shown). Figure 5 shows this specifically for salt and cytokinin interactions (false colored images of leaf disks generated by the fluorimeter used for Fv/Fm measurements are shown). Under salt stress

there is a rapid decrease in Fv/Fm levels or an increase in senescence (Fig. 5). Importantly a cytokinin pre-treatment lessens the effect of salt-induced reduction of Fv/Fm levels, indicating there is an interaction between cytokinins and salt stress, potentially involved in tolerance (Fig. 5). However, addition of cytokinin post-salt treatment shows no positive effect to lessen senescence. Oxidative stress causes a similar increase in senescence that can be lessened by the addition of a cytokinin pre-treatment (Fig. 4). Interestingly, after oxidative stress treatment has begun a cytokinin post-treatment is able to reduce senescence, which does not occur with salt treatment (Fig. 4).

Overall we present the first examination of both cytokinin levels and changes in whole genome transcript levels to salt and oxidative stress in tomato. While we find some overlap between these two stresses, there are many distinct, stress specific effects. Levels of several different cytokinin forms, including the active form *t*Z are increased after salt treatment and reduced after oxidative stress treatment. Quite distinct patterns of DEG regulation was similarly found after the same salt and oxidative stress treatments. Interestingly, a number of cytokinin-related genes were also found to be regulated in salt versus oxidative stress-dependent manners. Additional examination of cytokinin treatments during leaf abiotic stress senescence assays measuring photosystem II efficiency further supports the finding that cytokinins are connected to stress responses in a distinct manner for salt and oxidative stresses.

Materials and Methods

Tomato seedling growth and stress exposure

Solanum lycopersicum cv. MicroTom seeds were planted in damp sunshine #8 soil mix, grown in 16h, 26°C light and 8h, 22°C dark periods with light supplemented at 150μE m^{-2} s^{-1}. For stress

treatments seedlings, ten days after germination were excised from soil with roots intact and soil was carefully removed. Initial examinations of tomato pTCS::VENUS lines, a generous gift from Dr. Yuval Eshed, were made after seedlings were exposed to one of four treatments for 24h under gentle shaking conditions at room temperature: MES + 5μM *trans*-zeatin, MES + 150mM NaCl, MES + 20mM H_2O_2, or MES buffer (3mM MES buffer, pH 5.7) as a control. Roots from three plants in three biological treated replicates were viewed using a Nikon Eclipse 80i microscope epifluorescence microscope with a UV source and a yellow fluorescent protein (YFP) filter. A representative photo of a root from each treatment was taken with a Qimaging Fast 1394 digital camera and cropped using Adobe Photoshop CS3 without altering the original photo integrity.

Further examination of plants for LC-MS/MS and RNA-sequencing were grown as above then exposed to one of three treatments for 6h under gentle shaking conditions at room temperature: MES + 150mM NaCl, MES + 20mM H_2O_2, or MES buffer (3mM MES buffer, pH 5.7) as a control. After treatment, plants were carefully patted dry to determine fresh weight, then flash frozen in liquid nitrogen and ground by mortar and pestle into a fine powder. Samples were then split to allow RNA extraction/sequencing and as well as measurement of cytokinin levels from the same samples as described below.

RNA extraction, Library preparation, and Illumina GAIIX sequencing

Three independent biological replicates were used to isolate total RNA using the Qiagen RNeasy Plant Mini-kit according to the manufacturer's instructions. Total RNA then used for messenger RNA isolation with polyA selection and subsequent library construction with the TruSeq RNA sample preparation protocol from Illumina (San Diego, CA). Three biological replicates were sequenced and analyzed for each of the 3 treatment combinations. Single-end sequencing was

performed on the 9 samples by the Illumina GAIIX platform, generating 186,460,653 1x54bp reads. Raw sequence data is available for download at NCBI Sequence Read Archive under the BioProject ID: PRJNA476376, SRA accession: SRP150651.

Illumina mRNA Sequence Data Analysis

High quality sequence data generated for each of the 9 samples were aligned to Solanum lycopersicum genome downloaded from NCBI as performed in Gupta et al., 2013 (ftp://ftp.ncbi.nlm.nih.gov/genomes/Solanum_lycopersicum/). The associated annotation file, GFF format, was used to obtain genic information for downstream analysis. BAM alignments were generated using GSNAP (Genomic Short-read Nucleotide Alignment Program) (version released on 2013_05_09) (Wu and Watanabe, 2005) with the following parameters; indel penalty = 2, maximum mismatches = 0.06, terminal threshold = 1,000, novel splicing = 1, local splice distance = 10000, distant splice penalty = 1,000 and everything else set to default. Read counts were generated using NCGR's in house pipeline, ALPHEUS (Miller et al., 2008) as previously performed in Gupta et al., 2013. Gene expression for each of the 9 samples was computed as a measure of the total number of reads uniquely aligning to the reference genome, binned by genic coordinates (information acquired from the annotation GFF3 file). Differential gene expression analysis was performed using the R (R core Team 2013) (http://www.R-project.org/) Bioconductor package DESEQ (Anders and Huber, 2010). Raw read counts obtained were normalized to account for differences in sequencing depth and composition using methods implemented within DESEQ package. Differential expression of pairwise comparisons (combinations of the different conditions) was assessed using the negative binomial test with the Benjamani–Hochberg false discovery rate (FDR) adjustment applied for multiple testing corrections. For this study, an FDR of 0.05 was applied and any candidate that had a p-adjusted

value of ≤0.05 was considered to be significantly regulated. Full lists of gene expression are given in Supplemental Table 2.

Generation of a tomato cytokinin-regulated list was generated by combined lists of all RNA-sequenced DEGs after cytokinin treatment previously performed in tomato (Gupta et al., 2013; Shi et al, 2013) and removing duplicates as shown in Supplemental Table 5. This list of nearly 1100 DEGs was directly compared to the lists of stress DEGs found in this study.

Gene ontology GO analysis for overrepresentation was examined using the Overrepresentation Test at the PantherDB.org webpage using default settings. GO complete categories (Molecular Function, Biological Process, and Cellular Component) were examined using a False Discovery Rate (FDR<0.05) for each set of DEGs for salt and oxidative stress separated using distinct induced and repressed lists (Supplemental Table 4). Additional examination of the combined cytokinin-regulated list was performed in the same manner (Supplemental Table 5).

Confirmation of RNA-sequencing by qPCR

Five genes identified as DEG under stress conditions vs buffer control were selected to validate the RNA sequencing results using quantitative real-time PCR analysis (qPCR) following a modified protocol from Shi et al., 2013. Total RNA for each treatment was extracted as described above then used to generate cDNA for qPCR by reverse transcription using Quanta qScript cDNA supermix. qPCR was performed using PerfeCTa SYBR Green Supermix (QuantaBio, Beverly, MA) in 20µL reactions on an Eppendorf realplex2 (Hamburg, Germany). The qPCR conditions were as follows: 15sec 95°C, 20sec 58°C, 30sec 68°C (40 cycles), followed by melt curve analysis. All qPCR reactions were performed using two biological replicates and three technical replicates. For these replicates plants were grown and treated under

identical conditions as for transcriptome analysis. Fold change was calculated using the delta CT method with TIP41 as a control gene. Analysis for change was analyzed using a one-tailed t-Test and $p<0.05$ as a cut off for significance. Primer sequences for the genes which were verified through qPCR are presented in Supplemental Table 3.

Measurements of Cytokinin Level

Extraction and quantification of cytokinins were performed as described previously (Svačinová et al., 2012) using the LC-MS/MS system consisting of an ACQUITY UPLC System and Xevo TQ-S triple quadrupole mass spectrometer (Waters). Results are presented as the average of five biological replicates ± standard deviation in pmol/ g FW for samples treated and described above. Statistical examinations were made between buffer control treated and stress treatments using an ANOVA analysis with $p<0.05$.

Cytokinin-abiotic stress senescence assay

For salt stress priming and recovery, 50-60 d old plants (MicroTom) were used to make leaf disks (1mm diameter) and then floated in 3mM MES buffer in cell culture plates. Full treatments were made either in buffer as noted, + salt (150mM NaCl) or + cytokinin (5μM BA) for the entire experiment. Pre-treatment included treatment at the initiation of the experiment followed by an addition of another treatment or buffer at 48h indicated as a post-treatment. Pre and post-treatments were done using the same levels of salt and cytokinin as in full treatments. Oxidative stress priming and recovery was conducted in a similar manner as for salt stress, except 15 d old plants (M82) were used and 20mM H_2O_2 was used as an oxidative stress treatment. Leaf disks were grown under standard conditions. Fv/Fm measurements were performed in a manner similar to as in Zwack et al., 2016 with 9-18 leaf disks were used for all experimental treatments examined and conducted in triplicate biological replicates with analysis

using Student's t-Test. Additional experiments performed under each stress were performed in both M82 and MicroTom cultivars with similar findings as presented in the results section (data not shown).

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Tables

Table 1. Total reads from RNA-sequencing runs. Below is the total number of RNA sequencing reads from each biological replicate of as well as the number of those reads that were then uniquely aligned to the Heinz tomato reference genome. Samples are of the *Solanum lycopersicum* cv. Microtom (MT) either buffer treated (Control), or stress treated (+20mM H_2O_2), (+150mM NaCl) for 6h.

Sample	Total RNA Reads	Uniquely Aligned Reads
MT-Control rep1	**20,105,395**	**12,102,357**
MT-Control rep2	**8,608,669**	**5,648,071**
MT-Control rep3	**29,611,517**	**17,630,048**
MT+H_20_2 rep1	**18,838,675**	**14,791,987**
MT+H_20_2 rep2	**22,658,651**	**17,233,910**
MT+H_20_2 rep3	**15,577,188**	**12,031,139**
MT+NaCl rep1	**18,086,167**	**11,616,237**
MT+NaCl rep2	**21,937,106**	**15,517,603**
MT+NaCl rep3	**31,037,285**	**22,555,867**

Table 2. qPCR Confirmation of RNA-sequencing Transcriptomic results. Five genes found to be DE for at least one stress comparison to buffer treated samples by RNA-sequencing were selected for further verification of expression change by qPCR. qPCR was performed using 2 biological replicates and 3 technical replicates treated in the same manner as for RNA-sequencing experiments and normalized to TIP41 gene expression as a control. All qPCR expression changes follow RNA-sequencing expression changes in a significant manner ($p<0.05$, T-test) as indicated by font color (green-induced, red-repressed, grey-unchanged).

Solyc #	Gene	Description	MT vs MT+NaCl	MT vs MT+H2O2
Solyc01g107400	**IAA-AS**	**Indole-3-Acetic Acid-Amido Synthetase**	**3.29 ± 0.05**	**2.04 ± 0.02**
Solyc08g081550	**ACS**	**1-Aminocyclopropane-1-Carboxylate Synthase-like**	**1.96 ± 0.10**	**1.21 ± 0.02**
Solyc06g008820	**NHX1**	**Sodium Hydrogen Exchanger-like**	**1.23 ± 0.01**	**1.30 ± 0.01**
Solyc09g011670	**USPA**	**Universal Stress Protein A**	**0.82 ± 0.01**	**1.28 ± 0.00**
Solyc08g066350	**HPT4-like**	**Histidine-Containing Phosphotransfer 4-like**	**0.72 ± 0.00**	**1.18 ± 0.04**

Table 3. Gene Ontology Enrichment Analysis of Salt and Oxidative Stress DE genes. Gene ontology GO analysis for overrepresentation was examined using an Overrepresentation Test at the PantherDB.org webpage using default settings. Stress and oxidative categories with a False Discovery Rate (FDR<0.05) for each stress separated by induced and repressed DEGs are shown.

Salt Stress Induced	Fold Enrichment	FDR		**Oxidative Stress Induced**	Fold Enrichment	FDR
response to abiotic stimulus	2.07	5.29E-03		oxidation-reduction process	1.68	1.94E-09
response to stress	1.53	2.80E-04		regulation of response to stimulus	2.25	3.03E-02
regulation of response to stress	3.27	1.81E-02		response to stimulus	1.77	2.88E-17
regulation of response to stimulus	2.99	2.14E-05		response to abiotic stimulus	2.37	4.26E-04
response to stimulus	1.64	1.80E-14		response to oxygen-containing compound	2.45	1.38E-03
cellular response to stimulus	1.43	1.25E-04		cellular response to oxidative stress	3.03	4.25E-02
				cellular response to stimulus	1.35	8.88E-03
Salt Stress Repressed	Fold Enrichment	FDR		response to oxidative stress	2.15	1.41E-02
response to abiotic stimulus	2.25	8.95E-03				
response to oxidative stress	2.2	4.60E-02		**Oxidative Stress Repressed**	Fold Enrichment	FDR
response to stimulus	1.38	1.02E-03		response to abiotic stimulus	2.42	7.66E-03

Table 4. Cytokinin-related Genes are Altered by Salt and Oxidative Stress Treatment.

Genes with cytokinin related functions (biosynthesis (biosynthesis/metabolism), signaling, or transport) show regulation after stress treatment. Results are presented in average Fold Change (FC) compared to a buffer treated control of transcriptome (RNAseq) analyses of 10d old Salt (150mM NaCl) or Oxidative (20mM H_2O_2) stress treatment for 6 hours, n=3 biological replicates, similar to plants with cytokinin levels measured.

Salt Induced					**Oxidative Stress Induced**			
Gene ID	**Name/Description**	**FC**	**Function**		**Gene ID**	**Name/Description**	**FC**	**Function**
Solyc01g105360	UGT85A1-like glucosyltransferase	22.94	Biosynthesis		Solyc04g016230	Zeatin O-xylosyltransferase	18.30	Biosynthesis
Solyc10g079320	Zeatin O-glucosyltransferase	15.50	Biosynthesis		Solyc12g057080	UGT85A1-like glucosyltransferase	17.38	Biosynthesis
Solyc04g016230	Zeatin O-xylosyltransferase	13.74	Biosynthesis		Solyc07g006800	Zeatin O-glucosyltransferase	5.11	Biosynthesis
Solyc08g061930	Cytokinin Oxidase	6.75	Biosynthesis		Solyc04g080820	Cytokinin Oxidase (SlCKX4)	4.24	Biosynthesis
Solyc03g078490	UGT85A1-like glucosyltransferase	6.16	Biosynthesis		Solyc10g079930	Zeatin O-glucosyltransferase	3.82	Biosynthesis
Solyc07g006800	Zeatin O-glucosyltransferase	5.96	Biosynthesis		Solyc12g057060	UGT85A1-like glucosyltransferase	3.77	Biosynthesis
Solyc10g079930	Zeatin O-glucosyltransferase	5.80	Biosynthesis		Solyc11g066670	Zeatin O-glucosyltransferase	3.47	Biosynthesis
Solyc03g120320	F-box kelch-repeat KMD-like	5.11	Signaling		Solyc03g078490	UGT85A1-like glucosyltransferase	2.09	Biosynthesis
Solyc02g071220	Type-A Response regulator ARR8-like	3.45	Signaling		Solyc10g079600	Type-A Response regulator ARR9-like	2.00	Signaling
Solyc04g081290	LOG1-like	3.24	Biosynthesis		Solyc01g088550	PUP11-like Transporter	1.92	Transport
Solyc10g079600	Type-A Response regulator ARR9-like	2.72	Signaling		Solyc08g062820	LOG8-Like	1.66	Biosynthesis
Solyc02g090400	Type-B Response regulator ARR13-like	2.64	Signaling		Solyc05g054390	Type-B Response regulator ARR1-like	1.49	Signaling
Solyc11g066670	Zeatin O-glucosyltransferase	2.55	Biosynthesis					
Solyc07g005660	PUP5-like Cytokinin Transporter	2.42	Transport					
Solyc04g074870	PUP3-like Cytokinin Transporter	2.30	Transport					
Solyc08g062820	LOG8-Like	1.94	Biosynthesis					
Solyc05g054390	Type-B Response regulator ARR1-like	1.90	Signaling					
Solyc12g057060	UGT85A1-like glucosyltransferase	1.64	Biosynthesis					
Solyc05g015610	Cytokinin Receptor HK3	1.55	Signaling					
Salt Repressed					**Oxidative Stress Reduced**			
Gene ID	**Name/Description**	**FC**	**Function**		**Gene ID**	**Name/Description**	**FC**	**Function**
Solyc08g066350	Histophosphotransfer Protein Hpt4-like	Neg Inf	Signaling		Solyc10g079700	Type-A Response regulator ARR9-like	-4.41	Signaling
Solyc06g048600	Type-A Response regulator ARR17-like	-5.89	Signaling		Solyc12g087870	PUP3-like Cytokinin Transporter	-2.51	Transport

Solyc01g098400	Histophosphotransfer Protein Hpt1-like	-2.89	Signaling		Solyc05g006420	Type-A Response regulator ARR5-like	-2.51	Signaling
Solyc02g079330	ENT3-like Cytokinin Transporter	-2.60	Transport		Solyc08g081960	Cytokinin Response Factor SlCRF2	-2.42	Signaling
Solyc04g016190	Zeatin O-glucosyltransferase	-2.24	Biosynthesis		Solyc04g008110	Cytokinin Receptor HK4	-1.77	Signaling
					Solyc07g047770	Cytokinin Receptor HK2	-1.69	Signaling
					Solyc04g016190	Zeatin O-glucosyltransferase	-1.68	Biosynthesis

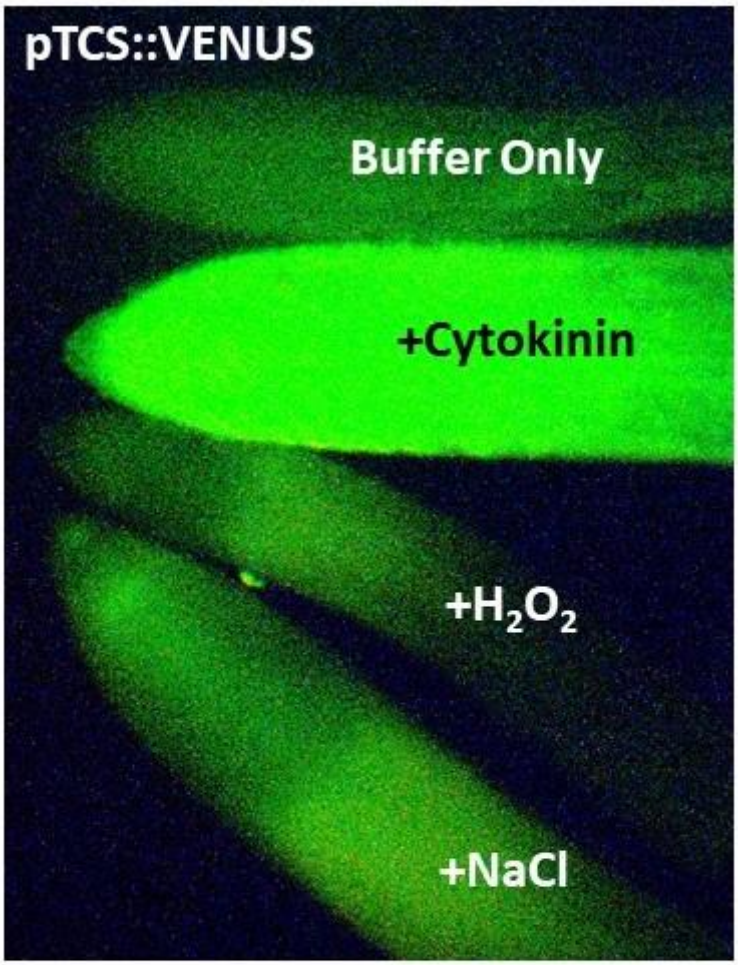


Figure 1. Stress Interactions with a Cytokinin Responsive YFP Reporter line (TCS::VENUS).

Visualization of the stable cytokinin responsive YFP reporter line (pTCS::VENUS) in tomato roots. pTCS::VENUS was treated for 24h in MES buffer, Cytokinin (5µM *trans*-zeatin), Oxidative (20mM H_2O_2) or Salt (150mM NaCl) stress. Representative fluorescing root tips are shown. Results show a strong increase in YFP expression after cytokinin treatment as expected. Oxidative stress show a reduction, while Salt stress shows a moderate increase in expression compared to Buffer only, indicating stress interaction with cytokinin.

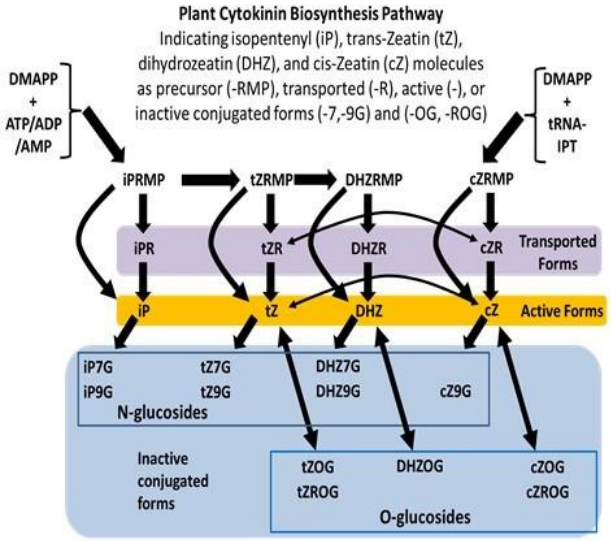


Figure 2. Cytokinin Levels are Altered by Abiotic Stress. (Left) Biosynthetic pathway for cytokinins in plants. Abbreviations in text. (Right) Specific amounts of cytokinin compounds measured by LC/MS in pmol/g FW from 10d tomato seedlings without (Control), Salt (150mM NaCl) or Oxidative Stress (20mM H202) treatment for 6h (n=5). Pastel colors indicate cytokinin form shown as in the biosynthetic pathway. Green/Red colors indicate significant ($p<0.05$) increase/decrease vs untreated control. Results show general increases in various cytokinin levels in response to salt stress, in contrast to decreasing levels seen for oxidative stresses.

MT - Control	iP	tZ	DHZ	cZ
RMP	0.69±0.21	0.32±0.08	ND	0.98±0.17
R	1.27±0.42	0.27±0.08	0.033±0.008	0.31±0.07
-	0.50±0.08	0.16±0.02	ND	0.09±0.01
7G	26.82±5.27	15.69±3.38	4.03±1.13	
9G	0.15±0.04	0.42±0.10	1.36±0.48	0.12±0.04
OG		0.13±0.03	0.137±0.031	0.21±0.02
ROG		0.32±0.09	ND	0.04±0.01

MT + NaCl	iP	tZ	DHZ	cZ
RMP	1.18±0.28	0.42±0.13	ND	2.46±0.64
R	2.70±0.72	0.39±0.12	0.050±0.009	0.87±0.19
-	0.58±0.11	0.30±0.09	ND	0.41±0.06
7G	35.07±6.83	18.53±4.76	2.60±0.76	
9G	0.22±0.03	0.52±0.11	0.74±0.23	0.13±0.02
OG		0.20±0.02	0.103±0.023	0.30±0.06
ROG		0.28±0.08	ND	0.08±0.02

MT + H_2O_2	iP	tZ	DHZ	cZ
RMP	0.52±0.14	0.22±0.07	ND	1.44±0.37
R	0.65±0.19	0.11±0.03	0.046±0.010	0.44±0.12
-	0.49±0.13	0.12±0.02	ND	0.13±0.01
7G	23.47±4.85	12.54±3.37	1.46±0.42	
9G	0.14±0.03	0.33±0.07	0.34±0.07	0.10±0.02
OG		0.12±0.02	0.076±0.019	0.20±0.04
ROG		0.18±0.06	ND	0.04±0.01

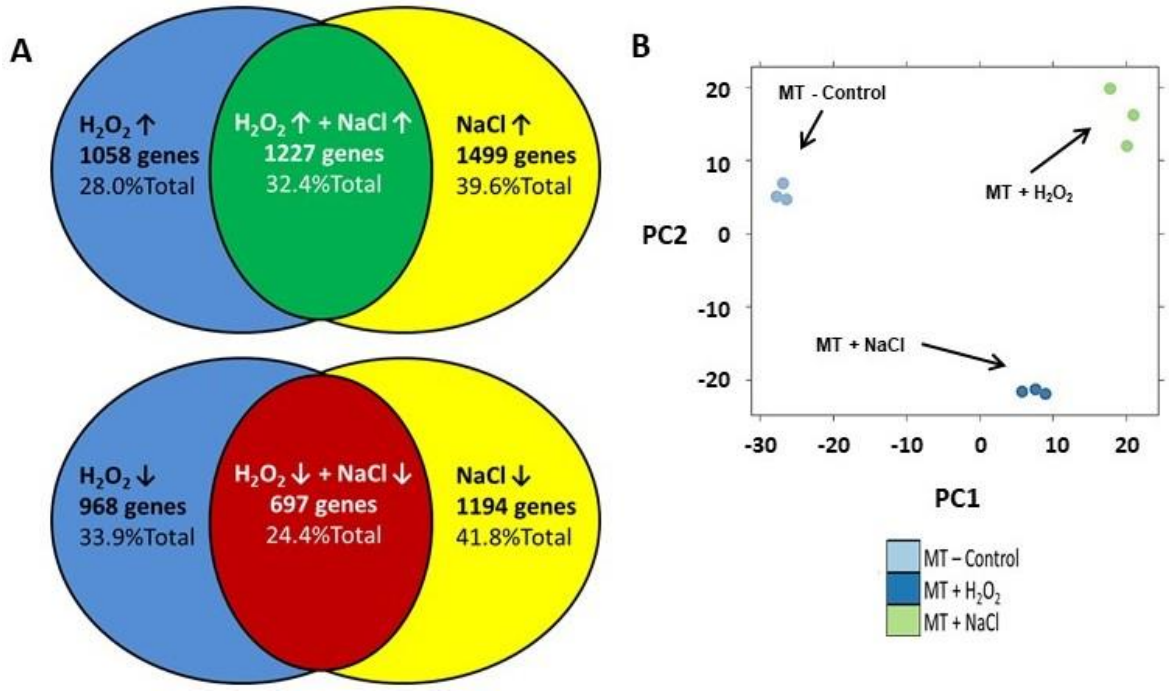
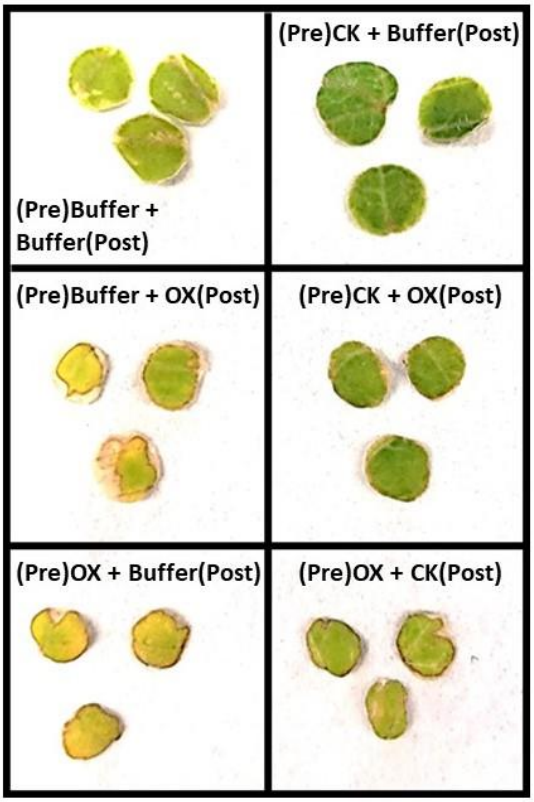


Figure 3. Transcriptome Analysis Reveals Distinct Patterns Abiotic Stress Gene Regulation. (A) Venn diagrams indicating the numbers of tomato genes significantly regulated by H_2O_2 and NaCl stress treatments: Top – induced genes, Bottom – repressed genes. While 32.4% of the induced and 24.4% of the repressed genes overlap between these stresses, the majority 67.6% of the induced and 75.6% of the repressed genes show unique stress regulation. (B) PCA analysis of each transcript replicate indicates a strong and distinct clustering of due to treatment (PC1) as well as a major component of transcript differences due to type of stress (PC2).



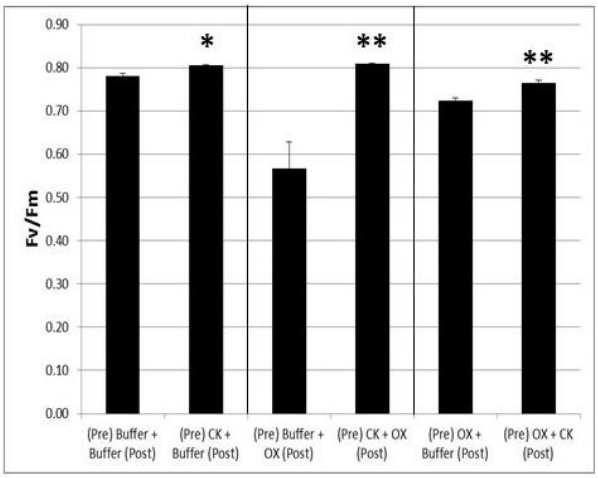


Figure 4. Cytokinin and Oxidative Stress Treatment Interactions. Visualization and Ave±SE of Fv/Fm of Tomato leaf disks treated with cytokinin (CK, 5µM *trans*-zeatin) or oxidative stress (OX, 20mM H_2O_2). Initial treatment (Pre) was followed, after 48h by an additional treatment (Post). * indicates significance at $p<0.05$, ** $p<0.01$. Results show CK improves Fv/Fm, while oxidative stress decreases it. Both pre- and post-treatments with CK significantly lessen oxidative stress reduction in Fv/Fm levels.

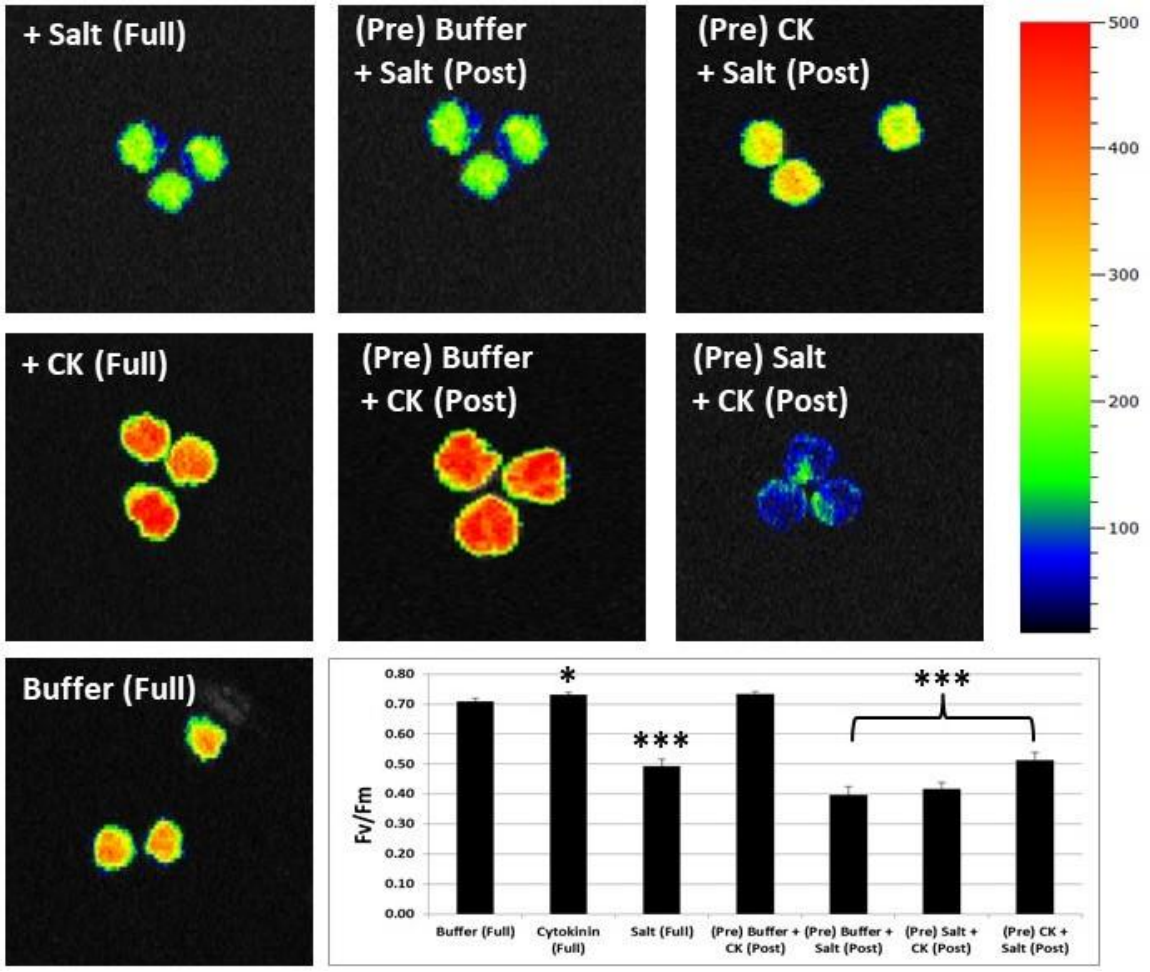


Figure 5. Cytokinin and Salt Treatment Interactions. Visualization and Ave±SE of Fv/Fm of tomato leaf disks treated with cytokinin (5µM BA) or salt (150mM NaCl) on a relative color scale. Total treatment was for 96h: (Pre) pretreatments for the first 48h, followed by additional 48h treatment (Post). * indicates significance at $p<0.05$, *** $p<0.001$. Results show CK treatment improves Fv/Fm, while salt decreases it. Buffer pretreatments are similar to full treatment times. CK pretreatment lessens salt reduction in Fv/Fm, but post-treatment cannot rescue salt treatment.

Supplemental Information

Supplemental Tables

Supplemental Table 1. Cytokinin levels of stress treated plants. Specific amounts of cytokinin compounds measured by LC/MS in pmol/g FW from 10d tomato seedlings without (Control), Salt (150mM NaCl) or Oxidative Stress (20mM H_20_2) treatment for 6h (n=5).

Supplemental Table 2. Transcriptome results of stress treated plants. Complete RNA sequencing reads from each biological replicate of as well as the number of those reads that were then uniquely aligned to the Heinz tomato reference genome. Samples are of the *Solanum lycopersicum* cv. Microtom (MT) either buffer treated (Control), or stress treated (20mM H_2O_2), (150mM NaCl) for 6h.

Supplemental Table 3. Primers used for qPCR verification of transcriptome analysis. Primer pair sequences that were used for verification of fold change of DEGs from the stress treated transcriptome analysis are given.

Supplemental Table 4. Complete gene ontology enrichment analysis of salt and oxidative stress DEGs. Gene ontology GO analysis for overrepresentation was examined using an Overrepresentation Test at the PantherDB.org webpage using default settings. All categories with a False Discovery Rate (FDR<0.05) for each stress separated by induced and repressed DEGs are shown for Molecular Function, Biological Process, and Cellular Component.