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Extracellular ATP Signaling: Induction of Superoxide Accumulation and Possible Regulation by Ectoapyrases in *Arabidopsis thaliana*

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Extracellular ATP Signaling: Induction of Superoxide Accumulation and Possible Regulation by Ectoapyrases in *Arabidopsis thaliana*

by

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For my family

Dad, Mom, Helen and Yul Eui

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Extracellular ATP Signaling: Induction of Superoxide Accumulation and Possible Regulation by Ectoapyrases in *Arabidopsis thaliana*

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Plants are constantly assaulted with abiotic and biotic stresses. They have developed mechanisms to transduce those stresses into adaptive physiological responses, including the production of reactive oxygen species as an intermediate signal, a mechanism they share in common with animal responses to stress. In animals, extracellular ATP (xATP) is a signal that induces the production of reactive oxygen species. In this dissertation, we document that xATP can serve as a signal also in plants by inducing superoxide production via NADPH oxidases. We also characterize intermediate elements in the ATP signaling pathway, downstream gene expression changes and the possible regulation of this signal by apyrases in *Arabidopsis thaliana*.

ATP- treated *Arabidopsis* leaves had increased superoxide accumulation. Inhibitors of the P2 receptors that initiate xATP responses in animals were able to block ATP-induced superoxide production in *Arabidopsis*. Mutants missing NADPH oxidase subunits did not show ATP-induced superoxide accumulation, indicating that NADPH oxidase activity is responsible for the ATP-induced superoxide production. A cation channel blocker, a calcium chelator, and a calmodulin antagonist also blocked this ATP response, implicating increases in $[Ca^{2+}]_{cyt}$ and the activation of calmodulin as intermediate signaling steps between xATP and superoxide production. Genes that are induced by various stresses were up-regulated by xATP, including genes involved in the biosynthesis of jasmonates and ethylene.

Ectoapyrases are enzymes that hydrolyze extracellular nucleotides. *Arabidopsis* apyrases were assessed as possible regulators of the xATP signal, a role they could carry out directly by quenching the signal and indirectly by increasing the AMP product that can readily be converted to adenosine, a negative feedback suppressor of ATP effects in animals, although this has not been demonstrated in plants to date. Adenosine decreased ATP-induced superoxide production, and three independent lines overexpressing the apyrase gene, Atapy2 (*Arabidopsis thaliana* apyrase 2) had reduced superoxide production in response to ATP treatment. The level of two mRNAs encoding apyrases increased in response to wounding, but only Atapy2 had increased gene expression when

treated with oligalacturonic acid (OGA). These differences suggest differential regulation of these similar apyrases. This dissertation provides evidence for a novel signal transduction pathway in *Arabidopsis* leading to the induction of superoxide production and the possible regulation of this pathway by apyrases.

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List of Abbreviations

ABA	Abscisic acid
ABC	ATP- binding cassette
ACR	Apyrase conserved region
ACS6	ACC synthase 6
Atapy	*Arabidopsis thaliana* apyrase
Atrboh	*Arabidopsis thaliana* respiratory burst oxidase homolog
BAPTA	Bis(2-amino-5-bromophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid
$[Ca^{2+}]_{cyt}$	Cytosolic calcium concentration
CaM	Calmodulin
DMSO	Dimethyl sulfoxide
DPI	Diphenyleneiodonium
ECM	Extracellular matrix
H_2O_2	Hydrogen peroxide
$LaCl_3$	Lanthanum chloride
LOX2	Lipoxygenase 2
MDR	Multi-drug resistance
MES	2-[Morpholino]ethanesulfonic acid
NBT	Nitroblue tetrazolium
O_2^-	Superoxide
PAL1	Phenylalanine ammonia lyase 1

phox	Phagocyte oxidase
PPADS	Pyridoxalphosphate-6-azophenyl-2’,4’-disulphonic acid
OGA	Oligogalacturonic acid
RB	Reactive blue 2
ROS	Reactive oxygen species
W5	N-(6-Aminohexyl)-1-naphthalenesulfonamide
W7	N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide
xATP	Extracellular ATP
xADP	Extracellular ADP

CHAPTER 1

Introduction

Extracellular nucleotide signaling is well-characterized in animals. There is recent evidence that extracellular nucleotides may be acting as signals in plants as well (Lew and Dearnaley 2000, Demidchik et al. 2003, Steinebrunner et al. 2003, Tang et al. 2003). The effects of extracellular purine (ATP and ADP) and pyrimidine (UTP and UDP) nucleotides are mediated through two classes of receptors called P2 receptors and adenosine receptors (for reviews see Dubyak and El-Moatassim 1993, Di Virgilio et al. 2001, Ralevic and Burnstock 1998). P2 receptors recognize primarily ATP, ADP, UTP and UDP, but not adenosine or AMP. Adenosine receptors, also known as P1 receptors, bind to adenosine (Ralevic and Burnstock 1998).

Purine and pyrimidine receptors

P2 receptors are divided into two subfamilies: P2Y receptors and P2X receptors (Ralevic and Burnstock 1998, Di Virgilio et al. 2001). P2Y receptors have seven membrane-spanning domains. The amino terminal (N-terminal) domain faces the extracellular matrix and the carboxy terminal (C-terminal) domain faces the cytoplasmic side of the plasma membrane. P2Y receptors are coupled to G proteins and act through activation of phospholipase C, metabolism of inositol 1,4,5-triphosphate (IP_3), and the release of Ca^{2+} into the cytoplasm.

P2X receptors are ligand-gated ion channels. They are activated by extracellular ATP (xATP) and mediate fast permeability changes to monovalent and divalent cations: Na^+, K^+, and Ca^{2+}. P2 receptors mediate many diverse responses in animals including platelet aggregation, inflammatory response, neurotransmission, smooth muscle contraction, apoptosis and the immune response, including the respiratory burst in blood cells (Gordon 1986, Dubyak and El-Moatassim 1993, Di Virgilio 1998, Di Virgilio et al 2001).

The adenosine/P1 receptor family is comprised of A_1, A_{2A}, A_{2B} and A_3 subtypes. All adenosine receptors are coupled to G proteins. Like other G protein-coupled receptors including P2Y receptors, adenosine receptors have seven transmembrane domains of hydrophobic amino acids. The N-terminal domain faces the extracellular side and the C-terminal domain faces the cytoplasmic side of the plasma membrane (Ralevic and Burnstock 1998). Adenosine receptors mediate activity of adenylate cyclase and are known to be involved in vasodilatation, inhibition of neurotransmission, cardiac depression, and inhibition of platelet aggregation (Ralevic and Burnstock 1998)

Extracellular ATP signaling in plants

Recently, the role of xATP as a signaling molecule has been studied in the plant, *Arabidopsis thaliana* (Lew and Dearnaley 2000, Demidchik et al. 2003, Steinebrunner et al. 2003, Tang et al. 2003). Demidchik et al. (2003) found that in

Arabidopsis roots expressing aequorin, cytosolic Ca^{2+} concentrations increased in a dose-dependent manner in response to ATP at concentrations between 300 nM to 1 mM ATP with a peak at 1 mM ATP. Lew and Dearnaley (2000) found that when ATP and ADP were applied to root hairs in *Arabidopsis*, they caused a membrane depolarization not associated with phosphate release. xADP was the most effective at depolarizing the root hairs with the half-maximal concentration of 10 µM, and xATP was less effective, having a 400 µM ATP half-maximal concentration (Lew and Dearnaley 2000). Tang et al. (2003) showed that extracellular ATP at concentrations approaching the intracellular level, blocked root gravitropism and stimulated lateral root growth in *Arabidopsis*. Consistent with these results, they also showed that auxin distribution in the roots at these high concentrations (mM) of ATP may be altered using DR5-ß-glucuronidase transgenic plants (Tang et al 2003). Steinebrunner et al. (2003) found that exogenously applied ATP to pollen prevented germination. The authors suggest that this block in pollen germination may be due to xATP signaling. These recent findings in *Arabidopsis thaliana* show that, like in animals, extracellular nucleotides may be acting as signaling molecules in plants.

Sources of extracellular nucleotides

ATP is a ubiquitous intracellular component present in mM concentrations, in the cytoplasm, so any cell potentially could be a source for extracellular ATP

(Gordon 1986, Dubyak and El-Moatassim 1993). There are numerous cell types that release xATP, and various mechanisms for that release have been described (Gordon 1986). The most obvious occasion of release would be a sudden breakage of intact cells so that ATP is released extracellularly (Gordon 1986, Dubyak and El-Moatassim 1993). Other sources of ATP release are packaging of ATP in exocytotic granules or vesicles and transport of ATP from the cytosol extracellularly by plasma membrane proteins (Abraham et al. 1993, Roman et al. 1997).

ATP is co-packaged into granules containing adrenergic and cholinergic neurotransmitters and is released into synaptic spaces during neurotransmission (Gordon 1986, Dubyak 1991). Platelet dense granules contain a high concentration of ATP and ADP (100 mM) and the exocytotic release of ATP has been recognized as an indicator of platelet activation (Dubyak and El-Moatassim 1993). Intact human platelets contain ~4.5 µM of releasable ATP and ADP. Given a normal platelet count, the concentration of ATP and ADP would rise to ~50 µM range when the platelets are activated (Dubyak and El-Moatassim 1993). Activated cytolytic T-lymphoctyes can release pore-forming perforins, which induce the formation of nonselective pores in target cells (Dubyak and El-Moatassim 1993) and could be a source of xATP. Activated cytolytic T-lymphocytes and helper T-lymphocytes accumulate xATP (Filippini et al 1990).

Release of xATP can also occur through intrinsic, plasma membrane transporter proteins (Abraham et al. 1993, Roman et al. 1997). The multidrug resistance (*mdr1*) gene product, P-glycoprotein, released ATP into the extracellular medium in proportion to the number of its molecules that were in the plasma membrane (Abraham et al. 1993). Patch clamp studies indicate that P-glycoprotein serves as an ATP- conducting channel in the plasma membrane (Abraham et al. 1993). Roman et al. (1997) showed that other proteins in the ABC transporter superfamily may also release ATP into the extracellular medium.

Mechanical stimulation has been shown to induce the release of extracellular ATP (Lazarowski et al. 2000, Ostrom et al. 2000). Mitchell et al. (1998) showed that hypotonic stimulation of ocular ciliary epithelial cells results in a three-fold increase in xATP concentration. McNamara et al. (2001) demonstrated that when a bacterial flagellum binds to a host protein, that in turn transduces the host's defenses and promotes the release of ATP from the host cell (McNamara et al. 2001).

In plants, less is known about ATP release and ATP signaling compared to animals. Thomas et al. (2000) showed that plants overexpressing *MDR1* did in fact have increased extracellular ATP. These results are similar to the animal results showing that P-glycoprotein is an ATP transporter (Abraham et al. 1993). In plants, mechanisms for ATP release may be similar to animal cells such as secretion of ATP into the extracellular matrix, as well as breakage of intact cells

releasing ATP. There may also be a release of ATP in response to pathogens, other activators of defense signaling, or mechanical stimulation. This area requires further study in plants.

Metabolism of extracellular nucleotides by ectoenzymes

Many cell types and presumably all tissue types have the capacity to metabolize extracellular nucleotides and nucleosides (Zimmerman 1996). A key function of the ectoenzymes is to inactivate nucleotides as signaling molecules or for purine salvage (Zimmerman 1996). Ectoenzymes involved in hydrolysis of extracellular nucleotides include ATP-diphosphohydrolases (also known as apyrases or NTPDases), ecto-nucleotide pyrophosphatases, specific ATPases and ADPases, alkaline phosphatases, and 5'- nucleotidases (Zimmerman 1996). Hydrolysis of ATP to AMP can be catalyzed by apyrases or ecto-nucleotide pyrophosphatases. 5'- nucleotidase converts AMP to adenosine (Komoszynski and Wojctzak 1996, Zimmerman 1996). ATP, ADP and AMP can also be dephosphorylated by alkaline phosphatases (Zimmerman 1996). Apyrase, 5'-nucleotidase, and alkaline phosphatase can metabolize a broad variety of purine and pyrimidine nucleotides (Zimmerman 1996).

Apyrases are ubiquitous in eukaryotic cells (Plesner 1995). They hydrolyze several different nucleoside di- and triphosphates. Their activity is dependent on divalent cations, Mg^{2+} or Ca^{2+}, and they are insensitive to inhibitors

of ATPases and phosphatases (Komoszynski and Wojctzak 1996). In many tissues, nucleoside di- and triphosphates appear to be hydrolyzed by only apyrase (Zimmerman 1996). Km values for ATP and ADP are in the range of about 10 – 20 µM (Zimmerman 1996). Apyrases' high enzymatic activity and vast abundance suggest that they play an important role in metabolism.

Apyrases have been characterized in many different plant species including pea (Chen and Roux 1986, Hsieh et al. 2000), *Arabidopsis thaliana* (Steinebrunner et al. 2000, Steinebrunner et al. 2003), potato (Handa and Guidotti 1996), *Dolichos biflorus* (Etzler et al. 1999), and *Medicago truncatula* (Cohn et al. 2001). In plants, apyrases have been proposed to function in toxin resistance (Thomas et al. 2000), phosphate transport (Thomas et al. 1999), pollen germination (Steinebrunner et al. 2003), and Nod signaling (Eztler et al. 1999).

Respiratory burst in animal cells

xATP either directly induces the production of ROS in different animal cells, including rat alveolar macrophages (Murphy et al. 1993), human eosinophils (Dichmann et al. 2000), human neutrophils (Kuroki and Minakami 1989, Seifert et al. 1989a), porcine thyroid cells (Nakamura and Ohtaki 1989), and guinea pig peritoneal macrophages (Nakanishi et al. 1991), or "primes" many of these cell types (Kuhns et al. 1988, Ward et al. 1988, 1989, Seifert et al. 1989b, Walker et al. 1989, 1991, Zalavary et al. 1996, Schmid-Antomarchi et al. 1997), ()

for an enhanced response when they are stimulated with inflammatory stimuli such as N-formyl methionyl leucyl phenylalanine (fMLP) and phorbol esters. Adenosine acts through A_2 adenosine receptors to block the fMLP-stimulated induction of superoxide radicals (Cronstein et al. 1985, Bengtsson et al. 1996, Zalavary et al. 1996).

The respiratory burst is characterized by a marked increase in oxygen consumption in phagocytic cells, which is due to the activation of NADPH oxidase (Vignais 2002). These cells include neutrophils, eosinophils and macrophages (Vignais 2002). Phagocytes participate in the immune response by engulfing, digesting, and killing bacterial or parasitic invaders. These phagocytes, after engulfing the invader, deliver secretory granules filled with proteolytic enzymes, cytotoxic proteins and chelators to kill and digest the invader (Henderson and Chappell 1996). At the same time, they induce a large respiratory burst of reactive oxygen species (ROS) to aid in the killing (Henderson and Chappell 1996). Macrophages arrive later than neutrophils and eosinophils and clean up remaining debris (Henderson and Chappell 1996). NADPH oxidase is responsible for the NADPH-dependent reduction of oxygen (O_2) to superoxide (O_2^-). Superoxide is highly reactive and quickly dismutates to hydrogen peroxide (H_2O_2) which can lead to other ROS including hydroxyl radical (OH^-), singlet oxygen and hypochlorous acid (Henderson and Chappell 1996).

The active NAPDH oxidase is a complex made up of a core heterodimer, flavocytochrome b_{558}, in the plasma membrane and cytosolic subunits. Flavocytochrome b_{558} is made up of a large ß subunit, gp91phox (phox is for phagocyte oxidase), and a small a subunit, p22phox (Vignais 2002). The cytosolic subunits are p67phox, p47phox, and p40phox (Babior 1999, Vignais 2002). Upon stimulation, the cytosolic subunits are heavily phosphorylated and move to the membrane where they associate with flavocytochrome b_{558} to make the active NADPH oxidase (Babior 1999, Vignais 2002). Activation of NADPH oxidase also requires the participation of two small G-proteins, Rac2 and Rap1A (Babior 1999, Vignais 2002).

Role of reactive oxygen species in plants

ROS have been studied in response to abiotic and biotic stresses. During a defense response, host-derived or pathogen-derived elicitors are recognized by binding to a receptor in the plant and leading to the production of ROS called the oxidative burst (Lamb and Dixon 1997). Wounding and herbivory can also lead to the production of ROS (Felton et al. 1994, Orozco-Cardenas and Ryan 1999, Orozco-Cardenas et al. 2001). Abiotic stress such as cold, salt, and drought also induce an accumulation of ROS (Xiong et al. 2002). ROS have also been implicated in growth and development (Foreman et al. 2003, Sagi et al. 2004).

In the plant defense response, ROS may function in the direct killing of microbes, cross-linking the cell wall, initiating the hypersensitive response (HR) and the induction of defense genes (Mehdy et al. 1996). Transgenic plants that have a reduced capability to detoxify ROS are hypersensitive to pathogen attack and initiate the hypersensitive response (HR) at levels of pathogen that do not induce HR in wild-type plants (Mittler et al. 1999).

Biphasic accumulation of ROS is characteristic of the response of plants to an avirulent pathogen or ozone (Lamb and Dixon 1997, Mahalingam and Federoff 2003). Wounding and mechanical stress also can result in multiple phases of superoxide accumulation in potatoes (Johnson et al. 2003, Razem and Bernards 2003). The oxidative burst has two distinct phases in response to an avirulent pathogen (Lamb and Dixon 1997). Both avirulent and virulent pathogens evoke a first peak (Phase I), which is weak and transient. Phase I occurs quickly usually within an hour of inoculation. Phase I has been detected as early as two minutes (Lamb and Dixon 1997). The second peak (Phase II) is more massive and prolonged and is evoked by an avirulent pathogen. Phase I is due to a nonspecific reaction, but the second peak is caused by the recognition of the plant to the avirulent pathogen (Lamb and Dixon 1997).

In response to wounding, potato tubers generate superoxide (Razem and Bernards 2003). There is an initial oxidative burst after wounding between 30 and 60 min and three other subsequent bursts that the authors attribute to wound

healing (Razem and Bernards 2003). They suggest that the ROS are involved in the cross-linking of suberin polyphenolics. The initial burst does not appear to require new oxidase proteins, but the subsequent bursts result in an increase in oxidase components, related to the mammalian NADPH oxidase (Razem and Bernards 2003).

Johnson and colleagues (2003) describe a biphasic production of superoxide in response to mechanical stress. An increase of superoxide production was observed with increasing applied pressure. The superoxide production was inhibited by DPI, suggesting a possible role for NADPH oxidase in the production of superoxide.

Several sources for ROS exist including NADPH and NADH oxidases, apoplastic peroxidases, amine oxidases, oxalate oxidases and from the electron transport chain in chloroplast and mitochondria, and peroxisomes (Allan and Fluhr 1997, Bolwell et al. 1998, Vranova et al. 2002, Delannoy et al. 2003, Do et al. 2003). Environmental stress conditions that limit CO_2 fixation can lead to the the overreduction of the photosynthetic electron transport chain, resulting in the formation of superoxide and singlet oxygen radicals (Vranova et al 2002). Also, when energy-requiring processes are reduced or terminated as a result of stress, the electron transport chain in mitochondria may become overreduced resulting in the formation of superoxide (Vranova et al. 2002).

Various enzymes may also catalyze the formation of ROS. Catabolism of purines results in the generation of superoxide by xanthine oxidase in peroxisomes (Vranova et al. 2002). Allan and Fluhr (1997) found evidence that in response to fungal elicitor, crytogein, ROS are generated by peroxidases or amine oxidases or flavin-containing oxidases, indicating that there are multiple sources for ROS in response to a pathogen in tobacco cells. Bolwell et al. (1998) suggest that rose and French bean cells have different sources of ROS in response to elicitors. Rose cells appear to be producing ROS by an NADPH oxidase. In contrast, French beans have no superoxide detected although H_2O_2 accumulates, suggesting the role of cell wall peroxidases in the production of H_2O_2.

In addition, an NADPH oxidase, similar to the one in phagocytes, has been implicated in the production of superoxide in the plant defense response (Grant and Loake 2000). Diphenyleneiodonium (DPI), a suicide substrate inhibitor of mammalian NADPH oxidases, blocked the oxidative burst in plants (Levine et al. 1994, Auh and Murphy 1995, Guo et al. 1998). Several NADPH oxidase subunit, gp91phox, homologs were identified in plants: six in *Arabidopsis thaliana*, called Atrboh A-F (Keller et al. 1998, Torres et al. 1998); one in rice, called rbohA (Groom et al. 1996); two in tomato, called LeRBOH1 and Wfi1 (Amicucci et al. 1999, Sagi et al. 2004); three in tobacco, called NbrbohA and NbrbohB from *Nicotiana benthamiana* (Yoshioka et al. 2003) and NbrbohD from *Nicotiana tabacum* (Simon-Plas et al. 2002); and two in potato, called StrbohA

and StrbohB (Yoshioka et al. 2001). In addition, Xing et al. (1997) found that cytosolic subunits of NADPH oxidase translocate to the plasma membrane upon treatment with race-specific elicitors from *Cladosporium fulvum* in tomato cells, and a Rac homolog in rice has been found to be involved in pathogen-induced cell death (Kawasaki et al. 1999). Others have found that subunit antibodies of cytosolic components from mammals cross-react to plant proteins (Tenhaken et al. 1995, Desikan et al. 1996, Dwyer et al. 1996).

Plants with a reduced or disrupted gp91phox homolog have less ROS accumulation and are compromised in their responses to biotic and abiotic stress. *Arabidopsis atrbohD/F* double mutants have reduced ROS production when treated with avirulent bacterium, *Pseudomonas syringae* pv. tomato DC3000 and have reduced cell death and electrolyte leakage (Torres et al. 2002). The *atrbohD/F* mutant was also compromised in response to ABA (Kwak et al. 2003). In tomato, antisense lines of Lerboh1 have reduced ROS accumulation and compromised wound response (Sagi et al. 2004). In *Nicotiana benthamiana*, virus induced silencing was used to suppress NbrbohA and NbrbohB genes (Yoshioka et al. 2003). Both genes were required for accumulation of ROS and resistance to *Phytophtora infestans* (Yoshioka et al. 2003). In *Nicotiana tabacum*, antisense lines of NtrbohD were not able to produce ROS (Simon-Plas et al. 2002).

In this dissertation, we address whether xATP may be acting as a signal for the accumulation of superoxide as in phagocytes and whether this

accumulation is due to NADPH oxidases. We found that at concentrations consistent with a signal, xATP increases superoxide accumulation, and that accumulation is dependent on NADPH oxidases. Additionally, we assessed whether apyrases may be regulating an xATP signal. Using overexpressing lines of apyrase, we were able to reduce xATP-induced superoxide accumulation.

CHAPTER 2

Extracellular ATP Induces Increased Superoxide Accumulation Via NADPH Oxidases in *Arabidopsis*

Introduction

Plants have adopted diverse ways to deal with biotic and abiotic stress. One of these ways is the production of reactive oxygen species (ROS). ROS have been implicated in stress signaling in response to both biotic and abiotic stresses. The oxidative burst, a large accumulation of ROS, is central to the defense of plants in response to pathogens (Lamb and Dixon 1997). ROS are generated in response to wounding and herbivory (Felton et al. 1994, Orozco-Cardenas et al. 1999, Orozco-Cardenas et al. 2001). Various abiotic stresses, including salt, drought, and cold, induce the accumulation of ROS (Xiong et al. 2002), and ROS are involved in the signal transduction of abscisic acid, which is a well-known hormone in response to abiotic stress (Kwak et al. 2003).

In the plant defense response, ROS are implicated in the direct killing of microbes, cross-linking the cell wall, initiating the hypersensitive response and the induction of defense genes (Mehdy et al. 1996). The two main ROS are superoxide (O_2^-) and hydrogen peroxide (H_2O_2), but also include hydroxyl radical ($^{\cdot}OH$), singlet oxygen and hypochlorous acid (Henderson and Chappell 1996). Pathogen- or host-derived elicitors bind to plant receptors to induce the

production of ROS in plants (Doke 1983, Levine et al. 1994, Hahlbrock et al. 1995, May et al. 1996, Jabs et al. 1997, Allan et al. 2001). Transgenic plants that have a reduced capability to detoxify ROS demonstrate programmed cell death (PCD) in response to pathogen levels that do not initiate PCD in wild-type plants (Mittler et al. 1999).

NADPH oxidase is responsible for the respiratory burst in mammalian phagocytic cells (Vignais 2002). The respiratory burst is characterized by a large consumption of oxygen which is due to the activation of NADPH oxidase. NADPH oxidase catalyzes the conversion of O_2 to superoxide (O_2^-) in a NADPH-dependent manner. This burst is necessary for the immune response to pathogens. Chronic granulomatous disease (CGD) is a disorder that is characterized by the failure of O_2^- production by phagocytes, which results in increased susceptibility to bacterial and fungal infections (Babior 1999).

NADPH oxidases of mammalian phagocytic cells have a core heterodimer, flavocytochrome b_{558}, in the plasma membrane made up of two plasma membrane subunits, gp91phox and p22phox (phox for phagocyte oxidase), and three cytosolic subunits, p67phox, p47phox, and p40phox. Activation of NADPH oxidase also requires the participation of two small G-proteins, Rac2 and Rap1A (Babior 1999, Vignais 2002). When phagocytic cells are activated, the cytosolic subunits are phosphorylated and relocate to the plasma membrane, where they

associate with flavocytochrome b_{558} to make the active NADPH oxidase (Babior 1999, Vignais 2002).

NADPH oxidase, homologous to that in animal phagocytic cells, has been suspected as a key player in the production of superoxide in plants., (Grant and Loake 2000). Diphenyleneiodonium (DPI), a suicide substrate inhibitor of mammalian NADPH oxidases, was able to block the oxidative burst in plants (Levine et al. 1994, Auh and Murphy 1995, Guo et al. 1998). NADPH oxidase subunit gp91phox homologs were identified in *Arabidopsis thaliana* (Keller et al. 1998, Torres et al. 1998), rice (Groom et al. 1996), tomato (Amicucci et al. 1999), tobacco (Simon-Plas et al. 2002, Yoshioka et al. 2003), and potato (Yoshioka et al. 2001). In addition, Xing et al. (1997) found that cytosolic subunits of NADPH oxidase translocate to the plasma membrane upon treatment with race-specific elicitors from *Cladosporium fulvum* in tomato cells, and a Rac homolog in rice has been found to be involved in pathogen-induced cell death (Kawasaki et al. 1999). Others have found that subunit antibodies of cytosolic components from mammals cross-react with plant proteins (Tenhaken et al. 1995, Desikan et al. 1996, Dwyer et al. 1996).

Plants with reduced or disrupted gp91phox homolog have compromised responses to biotic and abiotic stress and have a reduced capability to accumulate ROS. *Arabidopsis* plants disrupted in gp91phox homologs, AtrbohD and AtrbohF, have reduced ROS production when treated with avirulent bacterium,

Pseudomonas syringae pv. tomato DC3000 and reduced cell death and electrolyte leakage (Torres et al. 2002). The *atrbohD/F* mutant was also compromised in response to ABA (Kwak et al. 2003). Antisense lines of tomato Lerboh1 had a reduced ROS accumulation and compromised wound response (Sagi et al. 2004). In *Nicotiana benthamiana*, NbrbohA and NbrbohB, genes were suppressed using virus induced silencing (Yoshioka et al. 2003). They found that both genes were required for accumulation of ROS and resistance to *Phytophtora infestans* (Yoshioka et al. 2003). In *Nicotiana tabacum*, antisense lines of NtrbohD were not able to produce ROS (Simon-Plas et al. 2002). These data show that NADPH oxidase homologs in plants are important in the accumulation of ROS and subsequent plants responses to various stresses.

Extracellular purines and pyrimidines are well-characterized as signals in animals. Extracellular purines and pyrimidines are mediated by P2 receptors and adenosine receptors (Ralevic and Burnstock 1998). Nucleoside di- and triphosphates bind to P2 receptors and adenosine binds to adenosine receptors. P2 receptors are divided into subfamilies: P2X and P2Y. P2X receptors are ligand-gated cation channels. They are activated by extracellular ATP (xATP) and mediate fast permeability changes to monovalent and divalent cations (Ralevic and Burnstock 1998). P2Y receptors are G-protein coupled receptors with seven transmembrane domains. P2Y receptors activate phospholipase C, which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol 1,4,5-

triphosphate (IP_3), and leads to the increase of cytosolic Ca^{2+} (Ralevic and Burnstock 1998). P2 receptors are involved in many important responses in animals including neurotransmission, inflammation, platelet aggregation, apoptosis, and the immune response (Dubyak and El-Moatassim 1993, Di Virgilio 1998, Di Virgilio et al. 2001). Extracellular ATP is also known to induce the respiratory burst in phagocytes (Kuroki and Minakami 1989, Murphy et al. 1993, Dichmann et al. 2000), while adenosine inhibits the respiratory burst (Cronstein et al. 1985, Bengtsson et al. 1996, Zalavary et al. 1996).

Recently, there has been evidence that there may be xATP signaling in plants (Lew and Dearnaley 2000, Demidchik et al. 2003, Steinebrunner et al. 2003, Tang et al. 2003). Demidchik et al. (2003) found that xATP induces increases in cytosolic Ca^{2+}. Low concentrations of extracellular ADP (xADP) and xATP were shown to lead to membrane depolarizations in plant roots (Lew and Dearnaley 2000). xATP was also shown to inhibit root gravitropism and pollen germination in *Arabidopsis* (Steinebrunner et al. 2003, Tang et al. 2003).

ATP is ubiquitous inside of cells and is found in millimolar concentrations in the cytoplasm (Gordon 1986, Dubyak and El-Moatassim 1993). Potentially any cell could be a source of xATP. Breakage of an intact cell during a wound would release ATP into the extracellular matrix (Gordon 1986, Dubyak and El-Moatassim 1993). Other sources of xATP are packaging of ATP in exocytotic vesicles and transport of ATP from the cytosol extracellularly by plasma

membrane proteins (Abraham et al. 1993, Roman et al. 1997). In plants, less is known about the source of xATP, but Thomas et al. (2000) showed that plants overexpressing an ABC (ATP-binding cassette) transporter gene, MDR1, had increased amounts of xATP on the surface of leaves than wild-type plants. Plasma membrane transporters have been shown to transport ATP from the cell into the extracellular matrix (Abraham et al. 1993, Roman et al. 1997). This may be a source of xATP in plants also (Thomas et al. 2000). Ectoenzymes, including apyrases (also known as NTPDases and E-type ATPases), specific ATPases and ADPases and alkaline phosphatases, can hydrolyze xATP, quenching an xATP or xADP signal (Zimmerman 1996).

In the present study, we studied whether xATP could induce an accumulation of ROS in plants, similar to the animal phagocytic cells. We show that xATP induces a significant biphasic accumulation of superoxide in a dose-dependent fashion. This accumulation of superoxide is dependent on AtrbohD and AtrbohF, NADPH oxidase subunit gp91phox homologs.

Materials and Methods

Plant Growth Conditions

For superoxide assays, *Arabidopsis thaliana* ecotype Wassilewskija or *atrbohD/F* double mutants were grown on Metro Mix 350 (Hummert, Earth City, MO) under continuous light for 4-5 weeks. The seeds for the *atrbohD/F* double mutant were obtained from J. Kwak (Kwak et al. 2003). For gene expression analysis, *Arabidopsis* seeds were sown on 1.5 % agar plates containing ½ Murashige and Skoog basal salt mixture (Sigma), 1 % sucrose, and 1 X B5 vitamin mixture (1 mg/l nicotinic acid, 10 mg/l thiamine·HCl, 1 mg/l pyrodoxine·HCl, 100 mg/l m-inositol). The seedlings were grown for 10-14 days under continuous light. All experiments were performed on plants of the same age and grown under the same conditions.

Superoxide Detection

Superoxide accumulation was detected according to Jabs et al. (1996). Rosette leaves of full-grown plants were pressure-infiltrated on one side of the leaf with different solutions (described below) using a 1 ml syringe with no needle and incubated for the appropriate time. Leaves were then cut off with a razor blade and immersed in 10 mM potassium buffer, pH 7.8 (9.15 mM K_2HPO_4, 0.085 mM KH_2PO_4) and 10 mM sodium azide (NaN_3). To visualize superoxide accumulation, an equal volume of 0.2 % nitroblue tetrazolium (NBT) (Sigma or

Fisher) in 10 mM potassium buffer, pH 7.8 was added to the leaves in potassium buffer and NaN_3. The leaves were vacuum-infiltrated until all leaves were infiltrated (about 2 minutes), and the vacuum was broken quickly. All of the leaves were incubated simultaneously until staining was apparent (about 5-10 minutes). Immediately, the buffer was removed and 70 % ethanol was added to stop the reaction. Leaves were boiled in 96 % ethanol to clear the leaves (Sang et al. 2001) and stored in 70 % ethanol until further analysis.

Time Course

Arabidopsis leaves were infiltrated with 50 µM ATP dissolved in phosphate buffer (PB), pH 7.5 (0.16 mM KH_2PO_4, 1.1 mM K_2HPO_4), PB alone or xanthine (2mM) (Sigma)/xanthine oxidase (5 units) (Sigma) as the positive control. Leaves were cut from the plant after 3, 30, 60, 120, 240, and 480 minutes and immediately immersed in 10 mM potassium buffer and 10 mM NaN_3. Superoxide was detected as described in "Superoxide Detection."

ATP, ADP, and AMP Dose Treatments

Arabidopsis leaves were infiltrated with 1, 5, 10, 50, or 100 µM ATP, ADP, or AMP. PB alone was infiltrated as the negative control and OGA (10 µg/ml) as the positive control. All leaves were incubated for 1 h at room temperature. The 1 h incubation time was selected to insure uniformity of

treatment conditions. The leaves were cut off and immediately immersed in 10 mM potassium buffer and 10 mM NaN_3, and superoxide was detected as describe above in "Superoxide Detection." OGA was obtained from M. Mehdy.

Seedlings were submerged gently in 10 ml of 2-[Morpholino]ethanesulfonic acid (MES) pH 5.7 (0.5 g/l) alone, 1, 5, 50, or 100 µM ATP. ATP solutions were dissolved in MES pH 5.7. After submerging the seedlings, they were vacuum-infiltrated for 30 s, and the vacuum was broken quickly. The seedlings were treated for 30 minutes, and the solutions were poured out. The seedlings were collected at 30, 60, 90, and 180 minutes after the initial treatment and immediately frozen in liquid nitrogen and stored at -80 ºC for RNA isolation.

Inhibitor Treatments

Leaves were infiltrated with PB alone, 50 µM ATP, 50 µM ATP plus inhibitor, or inhibitor alone. The leaves were incubated at room temperature for 1 h, cut, and immersed in 10 mM potassium buffer and 10 mM NaN_3. Superoxide was detected as described in "Superoxide Detection." The inhibitors used were 250 µM Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), 30 µM reactive blue 2 (RB), 10 µM adenosine, 1 mM lanthanum chloride, 1mM 1,2-Bis(2-amino-5-bromophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA), 50 µM N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), 50 µM N-(6-

Aminohexyl)-1-naphthalenesulfonamide (W5), and 250 µM diphenyleneiodonium (DPI). All inhibitors were dissolved in dH_2O except for DPI which was dissolved in dimethyl sulfoxide (DMSO). A negative control with the same amount of DMSO was also infiltrated. All inhibitors were obtained from Sigma except for W5 (Calbiochem).

Seedlings were pretreated with inhibitor or MES. 10 ml solutions of MES or inhibitor (PPADS, RB, or adenosine) were poured gently into the Petri dishes containing seedlings. They were briefly vacuum-infiltrated (30s), and the vacuum was broken quickly. The seedlings incubated in the solutions for 10 minutes, and the solutions were poured out. Treatments of either 50 µM ATP or MES for the negative controls were added to the seedlings. The seedlings were again vacuum-infiltrated for a brief time (30s) and incubated in the solutions for 30 minutes. The solutions were poured out, and the seedlings were collected at 30, 60, 90, and 180 minutes. The seedlings were immediately frozen in liquid nitrogen and stored at -80 ºC for RNA isolation.

atrbohD/F Double Knockouts

Full-grown *atrbohD/F* double knockout leaves or wild-type leaves were infiltrated with either 50 µM ATP in PB or PB alone. Leaves were incubated at room temperature for 1 hr, cut off the plants and immersed in 10 mM potassium

buffer and 10 mM NaN_3. Superoxide was detected as described above in "Superoxide Detection."

RNA Isolation and Northern Analysis

RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. 10 µg RNA was denatured by incubation with a glyoxyl loading dye (Ambion) for 30 minutes at 50 °C. All of the RNA was loaded onto a 1.2 % agarose gel, separated by electrophoresis until the dye front reached the bottom of the gel, and transferred by downward capillary action onto a Bright star nylon membrane (Ambion). The RNA was cross-linked to the membrane using short UV light for 2 minutes. The Northern analysis was done using the Northern Max- Gly kit (Ambion) according to the manufacturer's instructions. Radioactivity was detected using a Phosphorimager (model 445SI; Molecular Dynamics). Specific cDNA probes for *Arabidopsis thaliana* respiratory burst oxidase homologue D (AtrbohD), Phenylalanine ammonia lyase (PAL1), Lipoxygenase 2 (LOX2), or ACC synthase 6 (ACS6) were hybridized to the membranes. Probes were randomly labeled with dCTP-a^{32}P (NEN-Perkin Elmer) using the DECAprime II kit (Ambion) according to the manufacturer's instructions. The primers used for AtrbohD (AF055357) were 5'-CACGCACTCAAAGGTCTCAAG-3' (forward) and 5'-CAGACGAAAGCTTTGATGCC-3' (reverse). The primers used for PAL1

(NM_129260) were 5'-GGAGCTCCCATTCCAATATG-3' (forward) and 5'-GAAGAAGGTATGATTCACAC-3' (reverse). The primers for LOX2 (L23968) were 5'-TATTGTAGAGAGTCCTTGTCG-3' (forward) and 5'-GACCAAGTTATGCCCTCCAG-3' (reverse). The primers for ACS6 (NM_117199) were 5'-GGTTAAAGGCCAAAGCCGGT-3' (forward) and 5'-GGCGAATGAGGCGAGAAGAA-3' (reverse). For ACS6, dCTP-a^{32}P was directly incorporated into the cDNA during PCR.

Computer Analysis

All leaves were analyzed with ImageJ downloaded from the website, http://rsb.info.nih.gov/ij/index.html. Stained areas were measured and divided by the total leaf area. This ratio was multiplied by 100 to obtain the percentage of stained leaf area.

The Northern analysis with inhibitor treatments were also analyzed using Image J. The areas of the bands from the Northern image were measured, as well as the bands from the equal loading gel. The expression levels were adjusted according to the equal loading bands. The resulting graphs show the relative expression levels.

Statistical analyses were done using the student's T-test in Microsoft Excel.

Results

Superoxide accumulation with ATP, ADP, and AMP treatments

To determine whether ATP-induced accumulation of superoxide is dose-dependent, we treated plants with a range of ATP concentrations from 1 µM to 100 µM (Fig. 2.1 A, C). We detected superoxide levels by incubating treated leaves with nitroblue tetrazolium (NBT; Jabs et al., 1996). When NBT is reduced by superoxide a dark black-purple precipitate forms, which can be easily visualized in *Arabidopsis* leaves. In response to varying concentrations of ATP, there were two distinct peaks of increased superoxide accumulation: one at 1 µM ATP and the other at 50 µM ATP (Fig. 2.1 A). All concentrations of ATP tested were significantly higher than the phosphate buffer control. The positive control of OGA also had a statistically significant increase over the buffer control. ATP treatments *in vivo* significantly increase the superoxide accumulation in a dose-dependent manner, but there appear to be two separate peaks at 1 µM and 50 µM ATP treatments.

ADP and AMP were also tested to see if they would generate the same increase in superoxide accumulation as ATP (Fig. 2.1 A, C). Across the concentrations tested, ATP-induced superoxide accumulation was generally greater than ADP-induced superoxide accumulation, while AMP-induced superoxide accumulation was less than that of either ATP or ADP (Fig. 2.1 A, C).

Time course for superoxide accumulation with xATP treatment

We next determined the time course of ATP-induced superoxide accumulation in response to infiltration of 50 μM ATP (Fig. 2.2 A, B). Superoxide accumulation peaked at two distinct time points, 3 min and 4 h (Fig. 2.2 A). At the first peak, the average leaf area that was stained was 42.3%, compared to 4.7% for phosphate buffer. After this initial peak, superoxide accumulation gradually increased, reaching a second peak of 31.4% staining 4 hours after infiltration (Fig. 2.2 A). At all of the time points measured, there was a significant accumulation of superoxide in response to xATP treatment as compared to the phosphate buffer control.

P2 receptor inhibitors block ATP-induced superoxide accumulation

In order to determine possible signaling components between xATP, superoxide accumulation and xATP induced gene expression changes, we co-treated plants with various inhibitors to see if we could alter the xATP responses characterized above. Because P2 receptors mediate ATP signaling in animals, two inhibitors of P2 receptors were used, PPADS and RB (Ralevic and Burnstock, 1998). ATP-induced superoxide was reduced to levels similar to control when co-treated with ATP and either 250 μM PPADS or 30 μM RB (Fig. 2.3 A, C). PPADS and RB alone were not significantly different from the phosphate buffer control (Appendix, Fig. 1).

Adenosine reduces ATP-induced superoxide accumulation

In neutrophils, adenosine, a product of ATP turnover, inhibits O_2^- production through activation of A_2 adenosine receptors (Cronstein et al., 1985; Bengtsson et al., 1996). To determine whether or not adenosine can inhibit xATP-induced superoxide accumulation, we co-treated plants with 10 µM adenosine and 50 µM ATP and measured superoxide accumulation (Fig. 2.3 A, C). Co-treatment with adenosine reduces xATP induced superoxide accumulation to levels similar to the buffer only control as measured by leaf area stained (Fig. 2.3 A). Adenosine treatment alone was not significantly different from the phosphate buffer control (Appendix, Fig. 1).

Inhibitors of Ca^{2+} signaling reduce ATP-induced superoxide accumulation

Because canonical P2 receptor signaling involves downstream Ca^{2+} signaling, we tested whether or not Ca^{2+} signaling is involved in the ROS response to xATP. We used BAPTA to chelate extracellular Ca^{2+} and lanthanum chloride ($LaCl_3$) to block Ca^{2+} channels and assayed for O_2^- accumulation (Fig. 2.3 B, C). Both BAPTA and $LaCl_3$, when applied in addition to ATP, reduced ATP-induced superoxide accumulation to levels similar to control. BAPTA and $LaCl_3$ alone were not significantly different from the phosphate buffer control (Appendix, Fig. 1). These data suggest extracellular Ca^{2+} may be involved in the signal transduction pathway linking xATP to superoxide accumulation.

In order to further study the involvement of Ca^{2+} signaling in the mediation of ATP-induced superoxide accumulation, we used a calmodulin (CaM) antagonist, W7, to block the action of CaM and assayed for superoxide accumulation (Fig. 2.3 B, C). Addition of W7 to the ATP treatment reduced superoxide accumulation compared to ATP treatment in the absence of W7. W5, which is a much less potent CaM antagonist, was ineffective in blocking ATP-induced superoxide accumulation (Appendix, Fig. 1). W5 and W7 alone also were not significantly different from the phosphate buffer control (Appendix, Fig. 1). These data suggest that CaM is involved in mediating the ATP-induced superoxide accumulation.

NADPH oxidase mutant, *atrbohD/F*, do not accumulate superoxide

Homologues of mammalian NADPH oxidase subunits have been implicated in production of superoxide in plants (Simon-Plas et al. 2002, Torres et al. 2002, Kwak et al. 2003, Yoshioka et al. 2003, Sagi et al. 2004). To determine whether ATP-induced ROS accumulation could be attributed to NADPH oxidase production of superoxide, we tested ATP effects on double knockouts of NADPH oxidase subunit homologs, *atrbohD* and *atrbohF*, in *Arabidopsis*. The double knockout plants fail to accumulate superoxide in response to ATP application (Fig. 2.4).

AtrbohD has increased transcript accumulation with xATP treatment

Given the increased accumulation of superoxide in response to ATP treatment, we examined the abundance of an *Arabidopsis* NADPH oxidase subunit homolog, AtrbohD, 1 h after xATP treatment (Fig. 2.5 A). Steady state levels of AtrbohD message increased in a dose-dependent manner when the plants were treated with different concentrations of xATP with maximal expression at occurring 50 μM ATP (Fig. 2.5 A).

Transcript accumulation of PAL1, LOX2, and ACS6 with xATP treatment

We next examined the effect of xATP treatment on the expression pattern of several genes reported to be induced by ROS or biotic or abiotic stress. PAL1 is involved in the defense response of plants, and expression of this gene is induced by ROS (Levine et al. 1994, Desikan et al. 1998, Desikan et al. 2001). PAL1 expression was induced by xATP with maximal expression at 100 μM xATP (Fig. 2.5 B). We also examined the effect of xATP on the abundance of two genes, LOX2 and ACS6, which encode biosynthetic enzymes for jasmonic acid and ethylene, two plant hormones involved in wound and defense responses (Penninckx et al. 1996, Turner et al. 2002, Wang et al. 2002). The level of LOX2 messages varied in a dose-dependent manner in plants treated with xATP with maximal expression occurring at 5 μM xATP (Fig. 2.6 A). ACS6 transcript abundance was increased by xATP at all concentrations tested (Fig. 2.6 B).

P2 receptor inhibitors reduce expression of AtrbohD and PAL1

We also examined the effect of P2 receptor inhibitors and adenosine on xATP induced changes in gene expression (Fig. 2.7). When seedlings were pretreated with 250 µM PPADS, 30 µM RB, or 10 µM adenosine, xATP failed to induce AtrbohD expression (Fig. 2.7 A, C). Similarly, pretreatment of seedlings with PPADS, RB, or adenosine, reduced the xATP-induced expression of PAL1 (Fig. 2.7 B, D). These data indicate that xATP responses, induction of accumulation of superoxide and changes in gene expression, are significantly reduced by P2 receptor inhibitors and adenosine.

Discussion

When they are activated by a pathogen, phagocytic blood cells in animals have a respiratory burst that results in the production of ROS (Vignais 2002). This respiratory burst can be induced by xATP in macrophages (Murphy et al. 1993), neutrophils (Kuroki and Minakami 1989), and eosinophils (Dichmann et al. 2000). NADPH oxidase activity in the plasma membrane is responsible for this respiratory burst (Vignais 2002). NADPH oxidase homologs to the phagocytic gp91phox subunit have been identified in plants and shown to be responsible for the production of ROS in response to infection (Torres et al. 2002, Yoshioka et al. 2003), wounding (Sagi et al. 2004), and the phytohormone ABA (Kwak et al. 2003). Many aspects of the plant defense response are analogous to the animal immune response (Bergey et al. 1996, Staskawicz et al. 2001). To test whether a similar burst of ROS production is occurring in response to xATP in plants and whether this accumulation is due to NADPH oxidase, we tested wild-type (Fig. 2.1) and *atrbohD/F* (Fig. 2.4) mutant *Arabidopsis* plants for superoxide accumulation when treated with xATP.

The superoxide induced by xATP accumulated in a dose-dependent manner (Fig 2.1 A, C). There were two peaks of superoxide at 1 µM and at 50 µM ATP, both statistically significant compared to the phosphate buffer control. The response to the low µM concentrations of ATP suggests that xATP is most likely

acting as a signal as opposed to disrupting an ATP gradient as proposed by Thomas et al. (2000).

Others have suggested that xATP is acting as a signal in plants (Lew and Dearnaley 2000, Demidchik et al. 2003, Steinebrunner et al. 2003, Tang et al. 2003). Demidchik et al. (2003) observed a two-fold increase in cytosolic Ca^{2+} concentrations at 300 nM. We have seen marked increases of xATP-induced superoxide accumulations as low as 500 nM ATP (Song and Roux, unpublished). These results make it seem likely that receptors like the animal P2 receptors are mediating an extracellular nucleotide signal.

Twelve mammalian P2 receptors have been identified and characterized (Di Virgilio et al. 2001). Multiple P2-like receptors may exist in plants and two different receptors may be mediating the two different peaks of superoxide accumulation (Fig. 2.1 A). In addition, multiple gp91phox homologs were found in *Arabidopsis* (Torres et al. 1998), and different subunits may be activated in response to different concentrations of xATP.

xADP did not induce any peaks of superoxide accumulation although all concentrations were significant compared to AMP and the phosphate buffer control (Fig. 2.1 A). P2 receptors in animals are known to have different potencies to various nucleotides, thus xADP being less potent than xATP is consistent with ligand specificity of P2 receptor signaling (Ralevic and Burnstock 1998).

In the absence of any pathogens or elicitors, two temporally distinct peaks of xATP-induced O_2^- accumulation were observed (Fig. 2.2 A). Biphasic peaks have been describe in reponse to avirulent pathogens, ozone, wounding and mechanical stress (Lamb and Dixon 1997, Mahalingam and Federoff 2003, Johnson et al. 2003, Razem and Bernards 2003). In response to OGA, virulent pathogens, and avirulent pathogens, plants produce a nonspecific, weak and transient burst of ROS fairly quickly after treatment, usually within one hour called Phase I (Lamb and Dixon 1997). However, in the case of an avirulent pathogen, plants have a more prolonged and massive accumulation hours after inoculation, usually between 3 and 6 hours, called Phase II (Lamb and Dixon 1997). Additionally, mechanical stress and wounding induce multiple peaks of superoxide production, possibly associated with wound healing (Johnson et al. 2003, Razem and Bernards 2003). The biphasic peak that we observed in response to ATP suggests a possible role for xATP in response to a stress, such as wounding, mechanical stress, or infection, although additional studies will have to be done to show a role for ATP in any of these responses.

To further study whether an xATP signal may be mediated by a P2-like receptor, we used two P2 inhibitors, PPADS and RB. We found that both P2 inhibitors blocked the xATP-induced superoxide accumulation (Fig. 2.3 A), suggesting that plants may have xATP signaling mediated by P2-like receptors, similar to animals. In order to definitively prove that an ATP signal is mediated

by P2-like receptors in plants, the receptor would have to be identified, cloned, and functionally characterized. That is, under conditions in which the receptor was disrupted the xATP-induced superoxide accumulation would have to be blocked. To date no P2-like receptors have been identified in plants.

To investigate whether adenosine (P1) receptors may mediate the opposite response, we co-treated plants with adenosine and found that adenosine significantly reduces superoxide accumulation. Adenosine, the breakdown product of ATP and ADP, is known to bind to A_2 receptors in neutrophils and block the respiratory burst (Cronstein et al. 1985, Bengtsson et al. 1996, Zalavary et al. 1996). It is not yet known whether adenosine may act as a signal in plant cells, but the data presented here are consistent with the possibility that adenosine may be binding adenosine receptors and may be responsible for a negative feedback to reduce superoxide production (Fig. 2.3 A).

Ectoenzymes, such as apyrase can hydrolyze ATP and ADP to AMP, and in animals 5'-nucleotidase hydrolyzes AMP to adenosine (Zimmerman 1996). In *Arabidopsis*, a search of the database found only one putative 5'-nucleotidase which was similar to a cytosolic animal 5'-nucleotidase. No ecto-nucleotidase was found that was highly similar to the one in animals, but we have not done a thorough search to exclude the possibility that a homolog with low sequence similarity to the animal ecto-5'-nucleotidase exists in *Arabidopsis.* Additionally,

no adenosine receptor has been identified, so much remains to be discovered in this area of extracellular nucleotide signaling.

Overexpression of apyrases in *Arabidopsis* reduces xATP-induced accumulation of superoxide (Chapter 3; Fig. 3.5). xATP-induced superoxide production may be tightly controlled by the ubiquitous ectoenzymes and the negative feedback through adenosine receptors.

xATP induces increases in cytosolic Ca^{2+} in *Arabidopsis* (Demidchik et al. 2003; Jeter and Roux, unpublished). P2 receptor signaling is mediated through cytosolic Ca^{2+} increases in animals, and Ca^{2+} is an intermediate signal leading to the xATP-induced respiratory burst (Kuroki and Minakami 1989, Murphy et al. 1993). We decided to test whether we could block superoxide accumulation by using a Ca^{2+} channel blocker, $LaCl_3$, and Ca^{2+} chelator, BAPTA. Treatments with either $LaCl_3$ or BAPTA significantly reduced xATP-induced superoxide accumulation (Fig. 2.3 B), suggesting an increase in cytosolic $[Ca^{2+}]$ is an intermediate signal in xATP-induced superoxide accumulation.

Calmodulin (CaM), which is a second messenger for the Ca^{2+} signal (Yang and Poovaiah 2003), has been implicated in the defense response of plants (Ali et al. 2003). We used a potent CaM antagonist, W7, to block the xATP-induced superoxide accumulation (Fig. 2.3 B). W5, a much less potent CaM antagonist, had no effect on xATP-induced superoxide accumulation (Appendix,

Fig. 1). These data suggest that CaM is a signal transduction component upstream of superoxide, but downstream of ATP.

In phagocytic cells, xATP induces the production of superoxide by stimulating the activity of NADPH oxidase (Kuroki and Minakami 1989, Murphy et al. 1993, Dichmann et al. 2000). Six homologs of the gp91phox subunit of NADPH oxidase were found in *Arabidopsis*, Atrboh A-F (Torres et al. 1998). AtrbohD and AtrbohF are the most highly expressed in *Arabidopsis* (Torres et al. 2002). *atrbohD/F* mutants are impaired in ROS accumulation in response to incompatible interaction to pathogens, *Pseudomonas syringae* pv. tomato DC3000 and *Peronospora parasitica*, and have reduced cell death after infection with *P. syringae* (Torres et al. 2002). *atrbohD/F* mutant plants also do not accumulate ROS in response to ABA (Kwak et al. 2003).

We treated the *atrbohD/F* mutant with xATP and found that the mutant had similar superoxide accumulation as the buffer control (Fig. 2.4 A) in response to xATP treatment, indicating that AtrbohD and AtrbohF are required for the production of most of the superoxide in xATP signaling. There was a low amount of staining, indicating that there might be a low level of superoxide accumulation due to other sources of superoxide such as from the electron transport chain in chloroplasts and mitochondria or from enzymes such as peroxidases or other NADPH oxidase homologs.

It is likely that superoxide accumulation is due to the production of superoxide rather than decreased dismutation to H_2O_2, because the half-life of superoxide is approximately 2-4 µs (Vranova et al. 2002). We measured the accumulation of superoxide an hour after treatment. The fact that inhibitor treatment with DPI (Appendix, Fig. 1) and the *atrbohD/F* mutant (Fig. 2.4) both reduced superoxide accumulation is also consistent with the conclusion that xATP affects superoxide production rather than a decrease in dismutation to H_2O_2.

Kwak et al. (2003) found that AtrbohD transcript levels increased in response to ABA. In order to further study the role of AtrbohD, we also looked at transcript accumulation with xATP treatments by Northern analysis (Fig. 2.5 A). AtrbohD message increases in a dose-dependent manner in response to xATP and peaks at 50 µM ATP (Fig. 2.5 A). The peak of AtrbohD transcript at 50 µM ATP is consistent with the second peak of superoxide accumulation at 50 µM ATP (Fig. 2.1 A), but AtrbohD does not have increased transcript level at 1 µM ATP. If increased gene expression leads to increased activity, which has not been measured, then it would be expected that only one of the superoxide peaks is a result of AtrbohD involvement. There are five other Atrboh genes in *Arabidopsis*, and one of those may be responsible for the peak of superoxide at 1 µM ATP. Alternatively, activity of the Atrboh proteins may be affected by xATP signaling.

PAL1 is a gene that functions in wound and defense signaling. PAL is the first enzyme in the phenylpropanoid pathway which is involved in the synthesis of

antimicrobial compounds, phytoalexins, and salicylic acid (Klessig et al. 2000). PAL1 expression is induced by ROS (Levine et al. 1994, Desikan et al. 1998, Desikan et al. 2001). We found that PAL1 had expression at 1 µM ATP (Fig. 2.5 B) which is consistent with the 1 µM ATP superoxide accumulation (Fig. 2.1 A). PAL1 expression is induced by H_2O_2, so it is likely that superoxide dismutated to H_2O_2 and induced the expression of PAL1. PAL1 also has increased expression at 50 µM ATP and 100 µM ATP (Fig. 2.5 A). The induction of PAL1 further suggests a possible role for xATP in defense signaling.

Jasmonic acid and ethylene are phytohormones that function in response to abiotic and biotic stresses, but they also regulate developmental processes (Turner et al. 2002, Wang et al. 2002). Jasmonic acid functions in fruit ripening, pollen maturation, root growth, wound response, abiotic stress responses, and defense responses to pathogens and insects (Turner et al. 2002). Ethylene functions in seed germination, root hair development, flower senescence, fruit ripening, abscission, response to pathogen attack, wounding, hypoxia, ozone, and freezing (Wang et al. 2002). We looked at the expression of two genes involved in the biosynthesis of these hormones: LOX2, which encodes a lipoxygenase critical for jasmonic acid synthesis, and ACS6, which encodes an ACC synthase that catalyzes the rate-limiting step in ethylene biosynthesis.

LOX2 expression is induced by wounding (Bell and Mullet 1993). An enhanced accumulation of LOX2 mRNA is induced by xATP in a dose-dependent

manner (Fig. 2.6 A), but this dose-response curve does not resemble the one for superoxide accumulation (Fig. 2.1 A). LOX2 expression peaks at 5 µM ATP and goes down at 50 µM and 100 µM (Fig. 2.6 A). The expression pattern suggests that LOX2 induction appears to be independent of superoxide accumulation.

An enhanced accumulation of ACS6 mRNA was induced by all concentrations of ATP tested (Fig. 2.6 B). These data suggest that xATP may function in the induction of these hormones and may be involved in signal transduction upstream of jasmonic acid and ethylene in response to various stresses, including wounding and defense responses, and possibly have a role in developmental processes of plants.

Two inhibitors of mammalian P2 receptors, PPADS and RB, both reduced the stimulatory effects of xATP on the message level of AtrbohD and PAL1 (Fig. 2.7). This result is consistent with the superoxide reduction by these inhibitors (Fig. 2.3 B). Although it suggests that P2-like receptors may exist in plants, its confident interpretation must await direct biochemical and functional evidence for or against this conclusion. Adenosine, the breakdown product of ATP and ADP, often negatively regulates responses that are turned on by P2 receptors in animals (Komoszynski and Wojctzak 1996), and it was also able to reduce the xATP-induced expression of AtrbohD and PAL1 (Fig. 2.7). This result is consistent with the hypothesis that adenosine may be a negative feedback inhibitor of xATP responses in plants, as it is in animals, but these results do not prove that

adenosine receptors exist in plants. It is possible that adenosine could reduce superoxide accumulation in plants by a mechanism not related to signaling,.

ATP is an important energy source to cells, so it seems difficult to imagine that cells would release ATP under any circumstance. Concentrations of intracellular ATP are in the mM range and in the nM range in the extracellular matrix (Dubyak 1991). Although it seems counterintuitive, animals have long been recognized to utilize xATP as a signal (Dubyak and El-Moatassim 1993). The most obvious situation in which xATP would reach high levels would be after a wound when intact cells break open and release cytoplasmic ATP into the surrounding extracellular matrix (Di Virgilio et al. 2001). Stephen Stout directly measured ATP at numerous wound sites and found that the concentrations of ATP there were typically in the range of 5 µM to 45 µM (Stout and Roux, unpublished). This concentration of ATP is within the range of signaling that induced superoxide accumulation (Fig. 2.1 B) and gene expression changes (Figs. 2.5 and 2.6).

Plasma membrane proteins from the ABC transporter family can also release ATP into the extracellular matrix. Thomas and colleagues (2000) found that when they overexpressed MDR1 in *Arabidopsis*, the leaves had increased ATP on the surface compared to wild-type plants. Additional mechanisms exist for the release of ATP such as secretion of vesicles containing ATP in animal

cells (Gordon 1986, Dubyak and El-Moatassim 1993), a process that could potentially operate in plants, too.

Human cells can release ATP in response to pathogens (McNamara et al. 2001) and mechanical stimulation (Ostrom et al. 2000, Lazarowski et al. 2000). Plant cells release ATP in response to various abiotic stresses, such as osmotic stress and cold stress, and mechanical stimulation (Jeter, Tang, and Roux, submitted), but much study still remains to fully understand the mechanisms and the circumstances in which plants are releasing ATP to the ECM and subsequent xATP signaling.

We propose a model for the induction of superoxide production by xATP and its tight control by NADPH oxidase homologs in *Arabidopsis.* We propose that this signaling is terminated by the breakdown of ATP by ectoenzymes, such as apyrases and 5'- nucleotidase, to adenosine which through an adenosine receptor pathway, mediates the reduction of superoxide levels (Fig. 2.8). Although the early signaling steps that occur in response to pathogens, wounding and abiotic stresses are still relatively unknown, superoxide production has been identified as a common early step mediating these responses. In accord with the results of this Chapter, we propose that following a wound or other stimulus that results in the disruption of the plasma membrane (Mehdy et al. 1996, Orozco-Cardenas et al. 2001, Xiong et al. 2002), the ATP released binds to P2-like receptors in *Arabidopsis*, which leads to the mobilization of Ca^{2+}, CaM activation,

increased activity of NADPH oxidase, and increased superoxide production. Based on well-established precedents in animals, we speculate that the role of xATP as a signaling molecule in plants may go beyond the mediation of diverse responses of plants to abiotic and biotic stresses to include functions in diverse developmental processes.

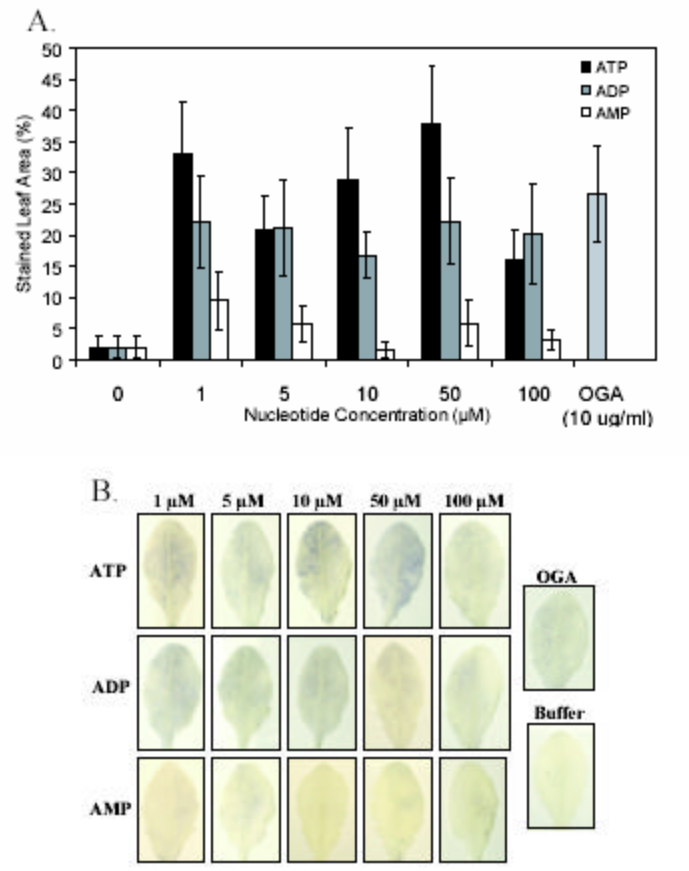

Figure 2.1 Superoxide accumulation after treatments with xATP, xADP, and xAMP treatments.
A. Arabidopsis leaves were treated with various concentrations of ATP, ADP and AMP and superoxide accumulation was detected by the NBT reduction assay after 1 h. Phosphate buffer was the negative control and OGA was the positive control. The percentage of the leaf area stained was determined using ImageJ. B. Images of representative leaves treated with various nucleotides, OGA, or buffer. For all values, n = 8. The error bars represent standard deviation. All ATP and ADP treatments are statistically significant compared to the buffer or AMP treatments (P = 0.001). This experiment was done in duplicate with similar results.

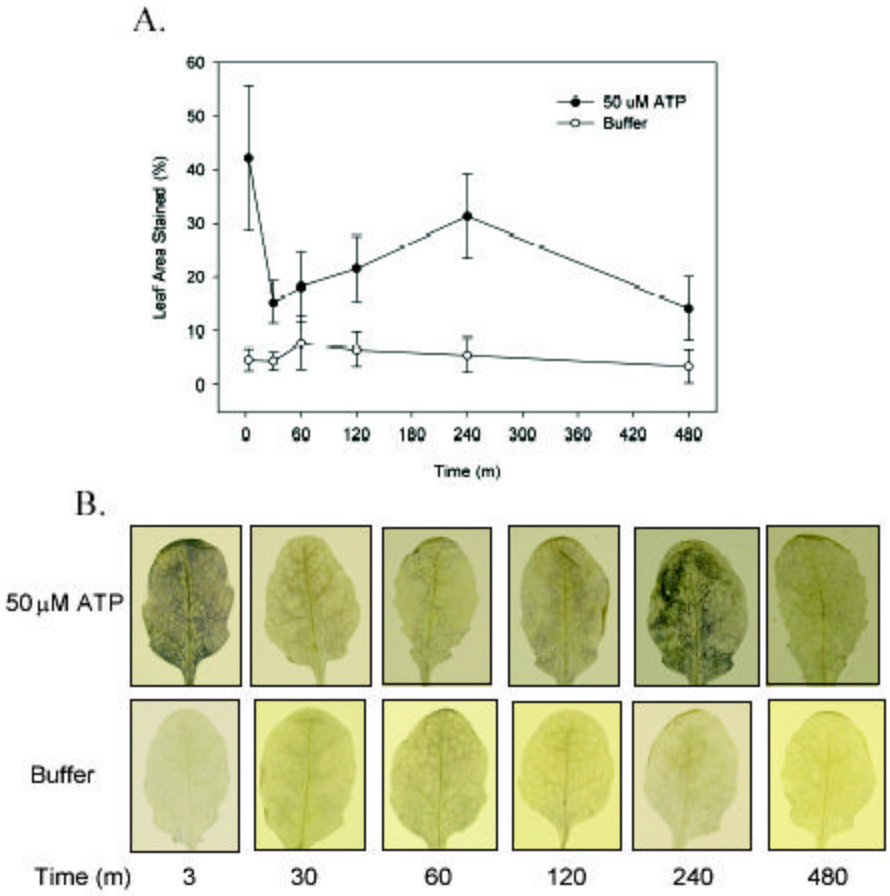

Figure 2.2 Time course for superoxide accumulation with xATP treatment.
A. Phosphate buffer or 50 µM ATP was infiltrated into leaves and superoxide accumulation was detected at various times by the NBT reduction assay. The percentage of the stained leaf area was measured using ImageJ. B. Images of representative leaves at each time point. For all average values shown, n = 10. The error bars represent the standard deviation. All ATP treatments induced statistically significant increases in superoxide accumulation compared to buffer control using the student's T-test with P = 0.0009. This experiment was done in duplicate with similar results.

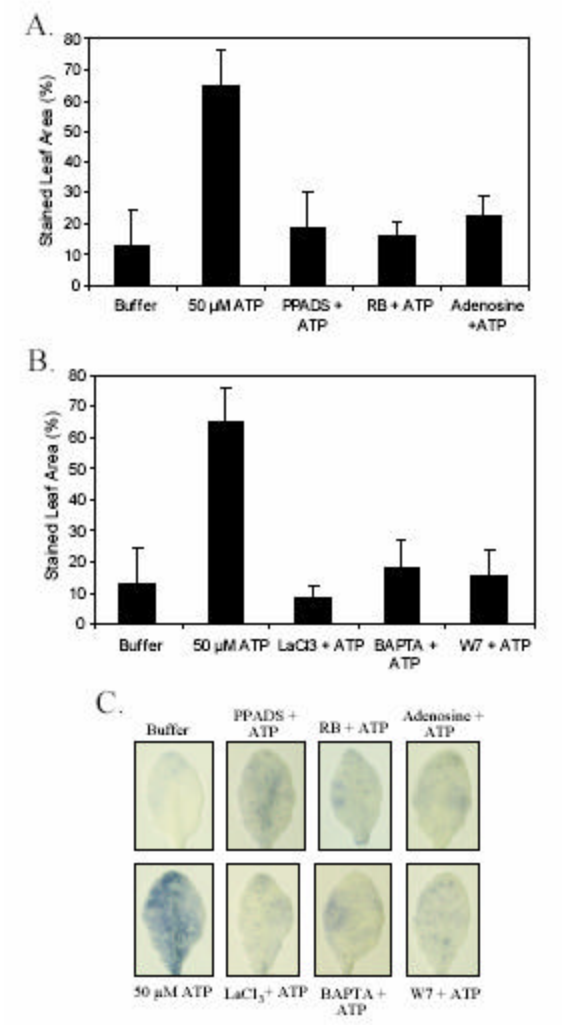

Figure 2.3 Inhibitors of P2 receptors and adenosine, and Ca^{2+} signaling reduce the superoxide accumulation induced by ATP.

A. Leaves were infiltrated with either phosphate buffer, 50 µM ATP, 250 µM PPADS plus 50 µM ATP, 30 µM reactive blue 2 plus 50 µM ATP, or 10 µM adenosine with 50 µM ATP and assayed for superoxide after 1 h. B. Leaves were infiltrated with either phosphate buffer, 50 µM ATP, 1 mM $LaCl_3$ plus 50 µM ATP, 1 mM BAPTA plus 50 µM ATP, or W7 plus 50 µM ATP and assayed for superoxide accumulation after 1 h. All leaves were analyzed for the percentage leaf area stained using ImageJ. C. Images of representative leaves treated with buffer, ATP, or ATP plus inhibitor. For all values, n = 8. The error bars represent standard deviation. For treatment with inhibitor plus ATP compared to ATP alone, $P = 1.1 \times 10^{-8}$. This experiment was done in duplicate with similar results.

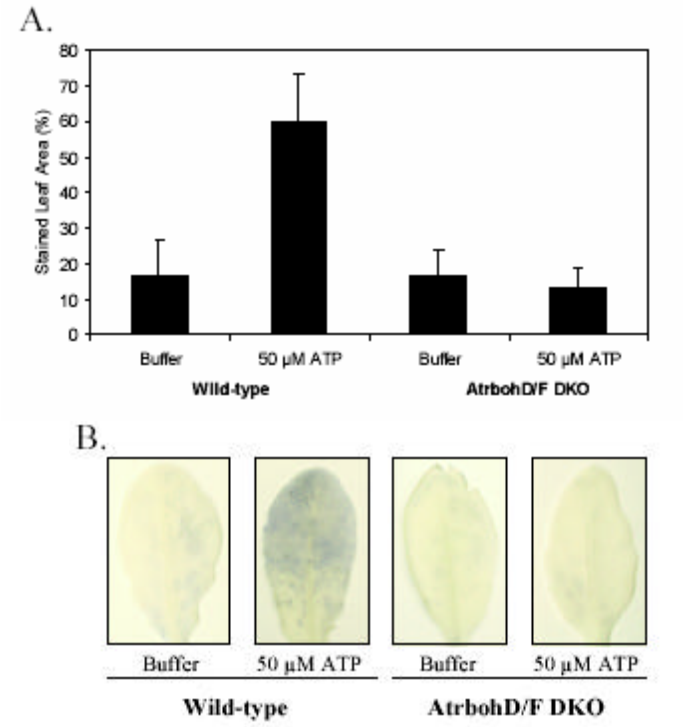

Figure 2.4 xATP does not induce superoxide accumulation in *atrbohD/F* double mutant plants.

A. Wild-type leaves and *AtrbohD/F* double mutant were treated with either phosphate buffer or 50 µM ATP. Superoxide was detected 1 h after treatment by NBT reduction. The percentage of stained leaf area was determined using ImageJ. B. Images of representative leaves treated with ATP or buffer. For all values, n = 11. The superoxide accumulation induced by ATP in both *atrbohD/F* mutant treatments was significantly less than in ATP-treated wild-type plants, $P = 1.5 \times 10^{-7}$. This experiment was done in duplicate with similar results.

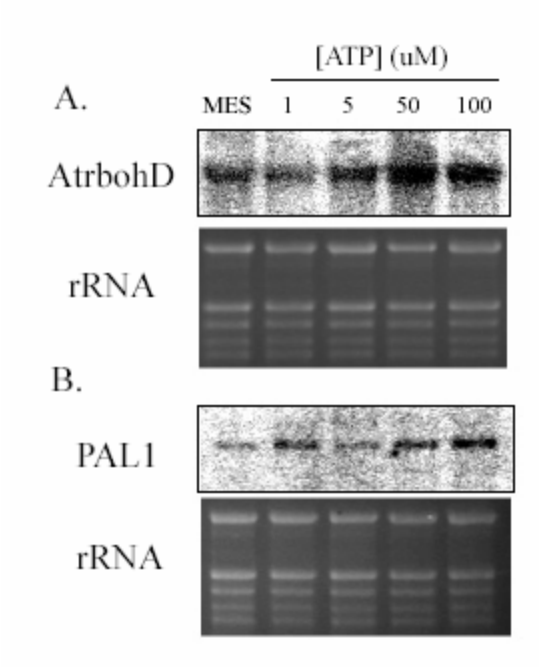

Figure 2.5 xATP induces gene expression changes for AtrbohD and PAL1.
Two week old seedlings were treated with various concentrations of ATP, or MES buffer, pH 5.7 as a negative control. Northern analyses were done to measure the transcript level of AtrbohD and PAL1 using specific cDNA probes. A. AtrbohD transcript level at 60 minutes increased in a dose-dependent manner with increasing concentrations of xATP and peaked at 50 µM ATP. B. PAL1 transcript level at 3 hours increased at 1 µM ATP, 50 µM ATP and 100 µM ATP, but not 5 µM ATP. rRNA shows the equal loading of the lanes. These experiments were done in duplicate with similar results.

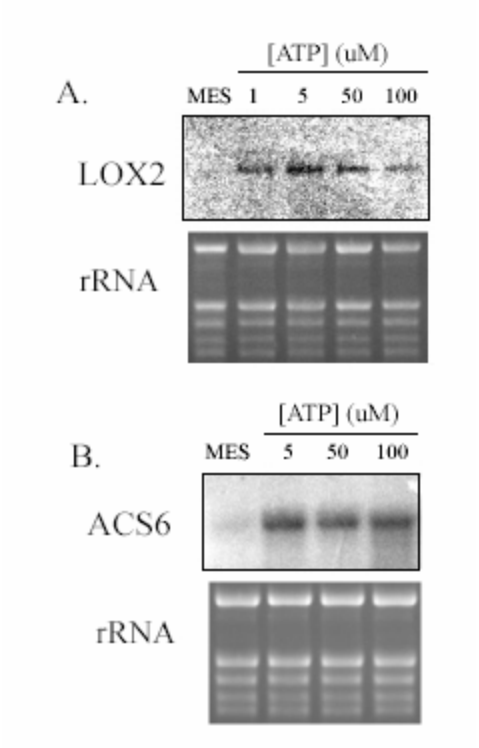

Figure 2.6 xATP induces gene expression changes for LOX2 and ACS6. Seedlings were treated with 1, 5, 50, or 100 µM ATP or MES buffer pH 5.7 as the negative control for blot A, and MES, 5, 50, 100 µM ATP or MES buffer as the negative control for blot B. Northern analyses were done to measure transcript levels of LOX2 and ACS6. rRNA is the loading control for the blots. A. LOX2 transcript levels at 90 minutes are induced at as low as 1 µM ATP and peaked at 5 µM ATP. The expression level decreases as the concentration of ATP increases above 5 µM ATP. B. ACS6 at 30 minutes was induced at all concentrations of xATP above the negative control. These experiments were done in duplicate with similar results.

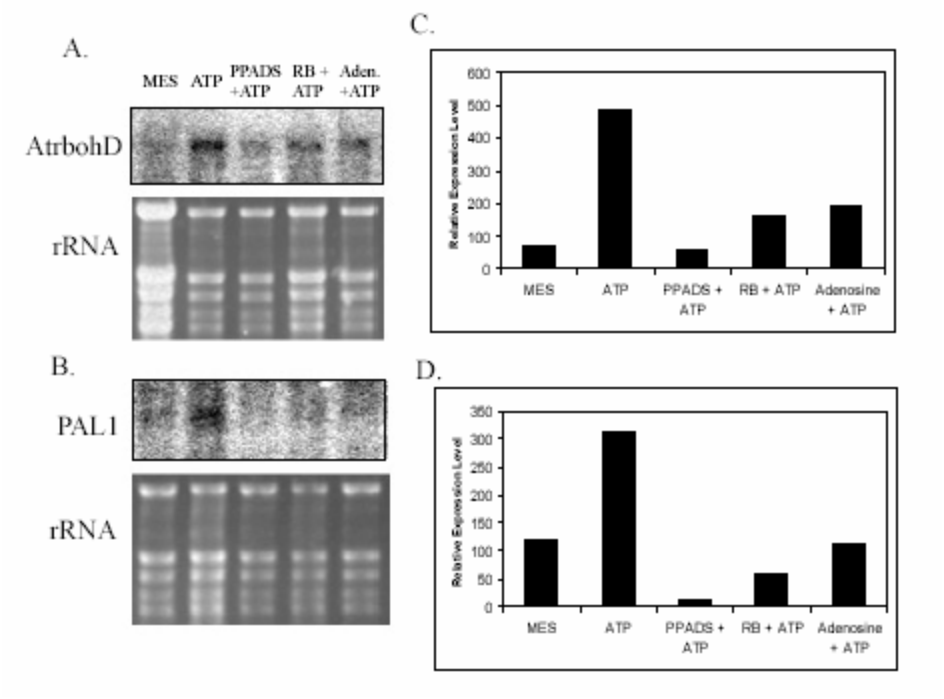

Figure 2.7 xATP induced gene expression of AtrbohD and PAL1 are reduced by P2 inhibitors.

Seedlings were pretreated for 10 minutes with MES buffer or inhibitor (PPADS, RB, or adenosine) and then treated with either MES buffer as a negative control or 50 µM ATP. Northern analyses were done to determine the transcript level of AtrbohD and PAL1. A. AtrbohD at 60 minutes is induced by xATP above the MES control. Treatments with PPADS, RB, or adenosine reduced the expression of AtrbohD. B. PAL at 90 minutes was induced by 50 µM ATP compared to the MES control. PPADS, RB, and adenosine reduced the xATP-induced transcript level. C. The relative expression level of AtrbohD when the bands were adjusted using the loading controls. The area of the bands was determined using ImageJ. D. The relative expression level of PAL1 when the bands were adjusted using the loading controls. These experiments were done in duplicate with similar results.

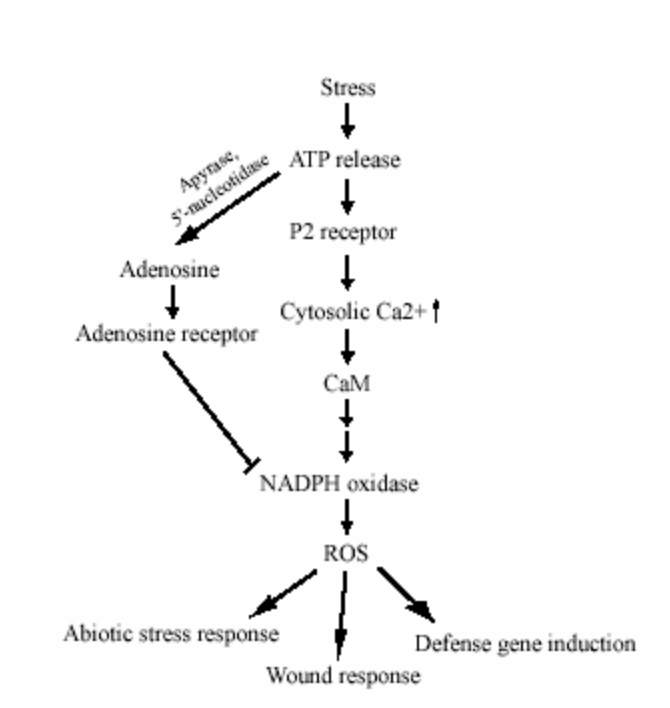

Figure 2.8 Model for xATP signaling in *Arabidopsis.*

CHAPTER 3

Possible Role for Apyrases in Wound and Defense Signaling

Introduction

Physical injury to plants can occur as a result of herbivory or environmental stresses, such as wind, rain, or hail (Leon et al. 2001). Wounding enhances the expression of a multitude of genes that function in the repair of damaged tissues and defense mechanisms to prevent further damage (Reymond et al. 2000). Characteristics of the wound response are the increased level of oligosaccharides, such as oligogalacturonic acid (OGA), from the cell wall by physical damage (Bishop et al. 1981) or from the activity of pectin-degrading enzymes (Bergey et al. 1999), production of reactive oxygen species (ROS) (Orozco-Cardenas and Ryan 1999, Orozco-Cardenas et al. 2001), increases in cytosolic Ca^{2+} levels (Knight et al. 1993) and activation of calmodulin (CaM) (Leon et al. 1998), induction of MAP kinase signaling (Rakwal and Agrawal 2003), and increased production of phytohormones, jasmonic acid (Creelman et al. 1992, Reymond and Farmer 1998) and ethylene (Nishiuchi et al. 2002, Cabrera and Saltveit 2003). In solanaceous plants, systemin, a 18-amino acid polypeptide wound signal, leads to the induction of proteinase inhibitor (pin) genes, but an equivalent pathway has not been found in *Arabidopsis* (Leon et al. 2001). Wounding also causes the breakage of intact cells and the release of cytoplasmic

contents, including ATP, into the extracellular matrix (Dubyak and El-Moatassim 1993).

Extracellular ATP (xATP) has been described as playing a regulatory role in physiological processes in plants (Thomas et al. 2000, Tang et al. 2003) and animals (Di Virgilio et al. 2001, Dubyak and El-Moatassim 1993). Recent papers have shown evidence of possible xATP signaling in plants. Lew and Dearnaley (2000) found that µM concentrations of exogenously applied ATP and ADP induced membrane depolarizations in *Arabidopsis* root hairs. Dose-dependent increases in cytosolic Ca^{2+} are induced by the addition of ATP in the range of 300 nM ATP up to 1 mM ATP in excised roots from *Arabidopsis* (Demidchik et al. 2003). Inhibition of pollen germination and root gravitropism were observed at mM concentrations of exogenously applied ATP in *Arabidopsis* (Steinebrunner et al. 2003, Tang et al. 2003).

Ectophosphatases, especially ectoapyrases, control the turnover of xATP (Zimmerman 1996). Apyrases (also known as ATP diphosphohydrolases and NTPDases), first described by Meyerhoff (1945), are enzymes that hydrolyze a variety of nucleoside di- and triphosphates. They are present in all investigated prokaryotes and eukaryotes (Komoszynski and Wojtczak 1996). They exhibit E-type ATPase activity, which is dependent on divalent cations, Ca^{2+} or Mg^{2+}, and are insensitive to specific inhibitors of P-type, F-type, and V-type ATPases (Plesner 1995). Apyrases usually have four domains of conserved amino acid

sequences termed apyrase conserved regions (ACRs) (Handa and Guidotti 1996). They can be divided into two categories depending on the location of the catalytic site. Ectoapyrases have the catalytic site outside of the cell, and endoapyrases have their catalytic site inside of the cell (Komoszynski and Wojtczak 1996). Ectoapyrases have been described to function in the quenching of a P2 receptor signal in neurotransmission (Todorov et al. 1997), blood platelet aggregation and thrombus formation (Marcus et al. 2002), inflammation (Di Virgilio et al. 1998), the respiratory burst (Zalavary et al. 1996), salvaging of extracellular nucleotides in animals (Che et al. 1992), and regulating the activity of 5'-nucleotidase (Komoszynski and Wojtczak 1996). Endoapyrases have been described to function in glycosylation in yeast (Gao et al. 1999).

Apyrases have been described in a wide variety of plants species including *Dolichos biflorus* (Etzler et al. 1999), *Pisum sativum* (Chen and Roux 1986), *Glycine soja* (Day et al. 2000), *Solanum tuberosum* (Handa and Guidotti 1996), *Cicer arietinum* (Vara and Serrano 1981), *Mimosa pudica* (Ghosh et al. 1998), *Medicago truncatula* (Cohn et al. 2001) and *Arabidopsis thaliana* (Steinebrunner et al. 2000). Plant apyrases have been proposed to function in phosphate transport (Thomas et al. 1999), toxin resistance (Thomas et al. 2000, Windsor et al. 2003), Nod signaling (Etzler et al. 1999, Day et al. 2000), wounding (Navarro-Gochicoa et al. 2003) or touch stimulation (Ghosh et al. 1998). Regarding apyrase regulation, light stimulates the activity of apyrases in *Mimosa pudica* (Ghosh et al.

1998) and pea (Chen and Roux 1986). A pea apyrase can be regulated by casein kinase II (Hsieh et al. 2000), and both a pea (Hsieh et al. 2000) and an *Arabidopsis* apyrase (Steinebrunner et al. 2000) are regulated by CaM. A pea apyrase, AtAPY1, activity is regulated by signaling molecules from pathogen, *Mycosphaerella pinodes* (Kawahara et al. 2003). A glycoprotein elicitor from the pathogen increases apyrase activity, and a mucin-type glycopeptide suppressor decreases apyrase activity (Kawahara et al. 2003).

During physical injury, intact cells can be lysed open resulting in ATP release (Dubyak and El-Moatassim 1993). Apyrases are importantly involved in quenching an xATP signal or in nucleotide salvaging in animals (Zimmerman 1996). Two *Arabidopsis* apyrases have been cloned and characterized, called Atapy1 and Atapy2 (Steinebrunner et al. 2000). In order to study whether these apyrases are up-regulated by a physiological condition that we would expect to elevate xATP concentrations in plants, we looked at their expression levels in response to wounding and OGA. We also tested to see if over-expression of apyrase could reduce xATP-mediated signaling.

Materials and Methods

Plant Material

Wild-type *Arabidopsis thaliana* ecotype Wassilewskija or Atapy2 over expressing (OE) plants were grown on MetroMix 350 soil (Hummert, EarthCity, MO) under continuous light for 4-5 weeks. Atapy2 OE lines were obtained from I. Steinebrunner. Atapy2 OE lines (2-2, 3-7, and 4-4) were confirmed by Northern analysis and semi-quantitative RT-PCR. For ATP and H_2O_2 treatments, wild-type *Arabidopsis* ecotype Wassilewskija seeds were sown on 1.5 % agar plates with ½ Murishige and Skoog basal salts (Sigma), 1 X B5 vitamin mixture (1 mg/l nicotinic acid, 10 mg/l thiamine·HCl, 1 mg/l pyrodoxine·HCl, 100 mg/l m-inositol), and 1 % sucrose. Seedlings were grown for 10 days under continuous light.

ATP Release Assay

We assayed wound sites of leaves to see if xATP was increased there compared to the surface of an intact leaf. For the control, a drop of either 5 µl or 10 µl water was added to the surface of an intact leaf and drawn off with a pipette, then immediately frozen in liquid nitrogen. The same leaf was crushed with a hemostat set to the first notch to maintain the same amount of pressure from leaf to leaf. Either 5 µl or 10 µl of H_2O was added to the wound site, drawn off, and immediately frozen. Ten samples each were collected of the intact leaf and the

wounded leaf. An ATP bioluminescent assay kit (Sigma) was used to measure ATP concentrations in the samples according to the manufacturer's instructions. The luminescence was detected using a luminometer (MLX Microtiter Plate Luminometer, Dynex Technologies).

Wound Time Course

Arabidopsis leaves were crushed with forceps repeatedly until about 40 % of the leaf area was crushed. The aerial portion of the plant was severed from the root using a razor blade after 0, 5, 15, 30, 60, 120, 240, 480, or 720 minutes. The collected plant material was immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. For the control, the plant was not wounded. The aerial portion of the plant was cut from the root and immediately frozen.

OGA Time Course

Arabidopsis rosette leaves were pressure infiltrated using a 1 ml syringe with no needle on one side of the leaf with 10 µg/ml oligogalaturonic acid (OGA) dissolved in dH_2O. Leaves were cut off with a razor blade after 0, 5, 15, 30, 60, or 120 minutes and frozen immediately in liquid nitrogen. For the negative control, leaves were infiltrated with dH_2O alone and collected after 0, 5, 30, or 120 minutes. Additionally, leaves that were not treated were collected and frozen immediately. All plant material was stored at -80 ºC.

ATP Treatments

Seedlings grown in Petri dishes were gently submerged in 10 ml of 5, 50, or 100 µM ATP dissolved in MES. For negative control, seedlings were submerged in MES buffer only. After 30 minutes, solutions of ATP and buffer were gently poured out. Seedlings were collected at 90 minutes and immediately frozen in liquid nitrogen. Plant material was stored at -80 °C.

RNA Isolation and Northern Analysis

For wounded plant material, tissue was ground using a mortar and pestle in liquid nitrogen until it was a fine powder. Total RNA was extracted according to Pawlowski et al. (1994). Frozen tissue was suspended in extraction buffer (100 mM LiCl, 1 % SDS, 100 mM Tris-HCl pH 9.0, 10 mM EDTA), 2 ml buffer for 1 g tissue. The tissue was then vortexed for 5 min and centrifuged at 4000 rpm for 30 min. A chloroform extraction was done twice, and the resulting solution was centrifuged at 12000 ***g***. The supernatant was collected into a fresh centrifuge tube. 8 M LiCl was added to a final concentration of 2 M LiCl. The RNA was precipitated overnight at 4 °C. The solution was centrifuged at 12000 ***g,*** and the pellet was resuspended in 300 µl sodium acetate pH 5.2 and transferred to an Eppendorf tube. A chloroform extraction was done on the solution, and the RNA was precipitated with 2.5 volumes of cold 96 % ethanol overnight at 4 °C. The RNA was pelleted by centrifugation for 20 min at 15000 rpm in a tabletop

centrifuge, dried under vacuum, and suspended in diethyl pyrocarbonate (DEPC; Sigma)-treated dH_2O. All other RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions.

Northern analysis was done using a Northern Max- Gly kit (Ambion) according to the manufacturer's instructions. 15 µg RNA was denatured by incubating in glyoxyl loading dye (Ambion) for 30 m at 50 °C, then electrophoretically separated in a 1.2 % agarose gel and transferred by downward capillary action onto a Bright star nylon membrane (Ambion). The RNA was cross-linked to the membrane with short UV light for 2 min. Specific cDNA probes for Atapy1, Atapy2, and LOX2 were hybridized to the membrane. The probes were randomly labeled according to the manufacturer's instructions with the DECAprime II kit (Ambion) using dCTP-a ^{32}P (NEN-Perkin Elmer). Radioactivity was detected using a Phosphorimager (model 445SI; Molecular Dynamics). The primers used for Atapy1 (AF093604) were 5'-TAGAAGCAGTATCCTCACC- 3' (forward) and 5'-ACAGAGGTTACGTATGCGG- 3' (reverse). The primers used for Atapy2 (AF156183) were 5'- CATAGTTGGGAGTTACCCATCTCCC- 3' (forward) and 5'- TACCAGACTCCAGGAGCTCAGTGG- 3' (reverse). The primers for LOX2 (L23968) were 5'-TATTGTAGAGAGTCCTTGTCG-3' (forward) and 5'-GACCAAGTTATGCCCTCCAG-3' (reverse).

Superoxide Detection

Superoxide was detected according to Jabs et al. (1996). Fully expanded leaves from wild-type plants or from Atapy2 OE plants were pressure infiltrated on one side with 50 µM ATP or phosphate buffer, pH 7.5 (0.16 mM KH_2PO_4, 1.1 mM K_2HPO_4) using a 1 ml syringe without a needle and incubated for 1 hr. Leaves were detached with a razor blade and immersed in 10 mM potassium buffer, pH 7.8 (9.15 mM K_2HPO_4, 0.085 mM KH_2PO_4) and 10 mM NaN_3. To visualize superoxide, an equal volume of 0.2 % NBT (Sigma) dissolved in 10 mM potassium buffer, pH 7.8 was added. The leaves were vacuum-infiltrated with the solution until all leaves were infiltrated (about 2 min), and the vacuum was broken quickly. All the leaves were incubated simultaneously until staining could be seen (about 5-10 min). The buffer was poured off, and 70 % ethanol was added to stop the staining. Leaves were boiled in 96 % ethanol to clear the leaves (Sang et al. 2001) and stored in 70 % ethanol until further analysis.

Computer-based Analyses

Arabidopsis leaves were analyzed with Image J for superoxide accumulation. The area of the leaf that was stained and the total leaf area were measured. The leaf area stained was divided by the total leaf area and multiplied by 100 to get the percentage of the leaf area stained.

The putative CaM-binding domain was determined using MacVector (Version 6.5, Oxford Molecular).

Statistical analyses were done using the student's T-test in Microsoft Excel.

Results

ATP release at wound sites

The amount of ATP was significantly higher in the fluid released at wound sites compared to that collected from the surface of intact leaves (Fig. 3.1). Wounded leaves that had 5 µl H_2O drawn off had a 16- fold increase over the control, which was statistically significant using the student's T-test ($P = 2.9 \times 10^{-6}$). Wounded leaves with 10 µl H_2O drawn off the wound site had a 9- fold increase over the control, which was also statistically significant ($P = 0.0006$). Samples of 10 µl drawn off wound sites had about half the luminescence of 5 µl samples.

Wounding induces expression changes for apyrases

The transcripts for both apyrases, Atapy1 and Atapy2, had increased accumulation in response to the wound stimulus (Fig. 3.2) by 15 min after a wound. Atapy1 message continued to accumulate and maximally accumulated at 1 ½ h to 2 h (Fig. 3.2 A). The transcript levels of Atapy1 gradually decreased at 8 h, and at 12 h. There was a noticeable reduction in transcript back down at the level of the control. Atapy2 transcript levels increased, but unlike Atapy1, the transcript levels stayed elevated up to 16 h (Fig. 3.2 B). Bell and Mullet (1993) observed that the LOX2 gene is induced by a wound and that the maximal accumulation of its transcript occurred within 6 h. We observed the maximal

accumulation of LOX2 message after wounding occurred between an hour and 4 h (Fig. 3.2 C).

OGA treatment increases Atapy2 transcript, but not Atapy1

The host-derived elicitor, OGA, could also induce the accumulation of at least one of the apyrase transcripts (Fig. 3.3). Atapy1 had no change in transcript levels compared to the H_2O control (Fig. 3.3 A), but Atapy2 did have increased transcript accumulation with OGA treatments (Fig. 3.3 B) compared to the H_2O controls, with this increase peaking at 15 min.

xATP-induced expression changes for apyrases

Transcripts of both apyrases have increased accumulation in the presence of xATP compared to the buffer treatment (Fig. 3.4). Atapy1 and Atapy2 have increased transcript accumulation at 5 µM and 50 µM ATP, and the transcript levels decrease at 100 µM (Fig. 3.4 A, B).

Plants overexpressing Atapy2 have reduced ATP-induced superoxide accumulation

To test whether the expression of an apyrase altered the sensitivity of plants to xATP, we used transformed *Arabidopsis* plants generated by Iris Steinebrunner (Steinebrunner and Roux, unpublished) that were expressing a

construct containing a CaMV promoter and the open reading frame (ORF) of Atapy2. Three independent lines were tested for increased expression levels of Atapy2. We found that all three lines; 2-2, 3-7, and 4-4; have increased Atapy2 transcript levels, much higher than wild-type plants (Fig. 2.6 A). We also tested the plants for Atapy1 transcript level and found that transcript levels of Atapy1 in Atapy2 over-expressing plants were similar to the wild-type (Data not shown). We tested whether increased Atapy2 expression may reduce xATP-induced superoxide accumulation. We have shown previously that xATP induces superoxide accumulation in *Arabidopsis* (Chapter 2; Fig. 2.2 A). The Atapy2 OE lines did reduce the amount of superoxide accumulation (Fig. 3.5 B). The Atapy2 OE lines did not completely block the superoxide accumulation, but the accumulation is significantly less than that induced by the xATP treatment of wild-type plants ($P = 0.0003$ for 2-2; $P = 0.0002$ for 3-7; $P = 0.002$ for 4-4). All three Atapy2 OE lines were not significant compared to each other, but were significant compared to buffer alone ($P = 0.0009$ for 2-2; $P = 0.0007$ for 3-7; $P = 0.0001$ for 4-4). Over-expression of apyrase reduces xATP-induced superoxide accumulation.

Discussion

ATP is a ubiquitous component inside of cells, where it typically reaches millimolar concentrations, so any cell could potentially be a source of xATP (Dubyak and El-Moatassim 1993). An obvious natural occurrence that would release the high cytoplasmic [ATP] into the extracellular space, where the [ATP] is typically nM, would be the breakage of intact cells (Di Virgilio et al. 2001, Dubyak and El-Moatassim 1993). Our results demonstrate that wounding does increase extracellular ATP concentrations (Fig. 3.1). When the wound exudates were directly measured, a range of 5 µM to 45 µM ATP was typically found at the wound site (Stout and Roux, unpublished). This is a physiologically significant amount of xATP because this range of xATP increases $[Ca^{2+}]_{cyt}$ (Demidchik et al. 2003) and superoxide production (Chapter 2, Fig. 2.2 B). The increases in ATP that occur at wound sites extracellularly may be important for the detection of a wound, as well as contributing to the downstream signaling for the wound response by the plant. In further support of this hypothesis, we detected gene expression changes in response to xATP of genes functioning in the biosynthetic pathway for jasmonic acid, LOX2, and ethylene, ACS6 (Chapter 2; Fig. 2.6). These phytohormones are critical in the response of plants to wounding (Reymond and Farmer 1998, Nishiuchi et al. 2002, Cabrera and Saltveit 2003).

In the event of xATP release, ectophosphatases, importantly apyrases, hydrolyze the ATP to AMP (Zimmerman 1996). Apyrases may function in the

wound response in *Medicago truncatula* (Navarro-Gochicoa et al. 2003) and in defense signaling in pea (Kawahara et al. 2003). Atapy1 and Atapy2 both have increased transcript accumulation when the plants are wounded, but they may have different functions in the wound response, because Atapy1 transcript decreased after 4 hours, while Atapy2 stayed increased for 16 hours. Atapy1 and Atapy2 may be playing a crucial role during the wound response by regulating the amount of substrate, ATP, for P2 receptors in plants. Another possibility is that apyrases are important in purine salvaging because ATP would be escaping from the wounded cells, and the plant may try to salvage the released purines (Che et al. 1992).

OGA fragments are released from plant cell walls by wounding events (Bishop et al. 1981) and are important in inducing defense-related signaling (Hahlbrock et al. 1995). We found that the transcript level of Atapy1 did not have any apparent change in plants treated with OGA (Fig. 3.3 A), but Atapy2 does appear to have increased transcript accumulation with OGA treatments (Fig. 3.3 B). To the extent that apyrase transcript levels predict ultimate activity levels (a relationship not yet tested), Atapy1 and Atapy2 may be playing different roles in the plant, with Atapy2 being more important in defense signaling than Atapy1.

There is evidence that xATP is a signaling molecule in plants (Lew and Dearnaley 2000, Demidchik et al. 2003, Steinebrunner et al. 2003, Tang et al. 2003). Apyrases would be important in hydrolyzing the ATP and quenching the

ATP signal (Komoszynski and Wojtczak 1996). Transcripts encoding Atapy1 and Atapy2 increase in abundance after xATP treatments (Fig. 3.4), which is consistent with a role for apyrases as quenchers of an xATP signal. It could be important for plants to carefully control their apyrase activity depending on how much ATP signal they needed. Alternatively, when plants detect xATP, they may salvage the purines, increasing apyrase expression to accomplish this task (Che et al. 1992).

In order to see whether xATP-induced accumulation of superoxide can be reduced by apyrases, we treated three independent lines of *Arabidopsis* confirmed to be overexpressing Atapy2 mRNA with xATP and measured the superoxide accumulation in their leaves. The increase in apyrase protein level and increased overall activity in these three lines have yet to be tested. We found that in all three lines, the amount of superoxide accumulation induced by ATP was significantly reduced compared to the wild-type plants, but the reduction was not completely down to the level of the buffer control (Fig. 3.5 B). It is likely that in the overexpressing lines, the reduced xATP level was sufficient to bind to receptors, (as yet hypothetical) and induce some superoxide accumulation. The other possibility is that Atapy2 is hydrolyzing xATP to xAMP, and the increased xAMP is serving as a substrate for increased production of adenosine, which is suppressing the ATP responses through adenosine receptors (also as yet hypothetical) by negative feedback, a well described phoneomenon in animal

systems (Cronstein et al. 1985, Bengtsson et al. 1996, Zalavary et al. 1996). An analysis of the levels of 5'- nucleotidase activity and adenosine in the ECM of apyrase-overexpressing lines would provide valuable data to resolve whether the adenosine feedback model helps explain why apyrase overexpressing plants have reduced capability to induce superoxide production after xATP treatment.

Atapy1 was identified by its homology to pea apyrase (NTPase), and Atapy2 was identified by its similarity to Atapy1 (Steinebrunner et al. 2000). The pea apyrase, psNTP9, was found to localize to the plasma membrane (Thomas et al. 1999), and hydropathy analysis of Atapy1 and Atapy2 predicts that they might be ectoapyrases with catalytic sites facing the extracellular matrix (Steinebrunner et al. 2000), like most animal apyrases. These previous data suggest a possible function of *Arabidopsis* apyrases as ectoapyrases, and our data are consistent with the hypothesis that these apyrases are ectoapyrases. However, direct localization studies, now in progress, are required to resolve definitively whether these apyrases are localized to the plasma membrane.

The level of H_2O_2 in leaves increases in response to wounding (Orozco-Cardenas and Ryan 1999, Orozco-Cardenas et al. 2001), pathogen attack (Lamb and Dixon 1997), and abiotic stresses (Xiong et al. 2002). H_2O_2 is known to induce stress-related gene expression changes (Desikan et al. 1998, Desikan et al. 2001). To see whether Atapy1 and Atapy2 have any gene expression changes in response to H_2O_2, we treated plants with H_2O_2 and measured transcript levels. We

found that the level of mRNAs encoding either apyrase does not appear to undergo any changes in response to H_2O_2 (Appendix, Fig. 2).

In human endothelial cells, Robson and colleagues (1997) found that the activity of an apyrase was reduced with treatment of superoxide. When antioxidants were used, apyrase inhibition was abrogated (Robson et al. 1997). They also noted that they did not have any change in protein level of their apyrase (Robson et al. 1997). Certainly, the possibility exists that although there are no changes in message level of *Arabidopsis* apyrases, but the apyrases may be regulated at the protein level by a reduction of activity with increased superoxide, like in human endothelial cells. If apyrase activity is reduced by superoxide, then more ATP would accumulate and may induce more superoxide accumulation. In the case of a defense response, the plant requires the massive burst of ROS which may directly kill the pathogen and induce defense signaling (Lamb and Dixon 1997). In this case, it would be advantageous for the plant to increase the signal, xATP, which can contribute to the oxidative burst.

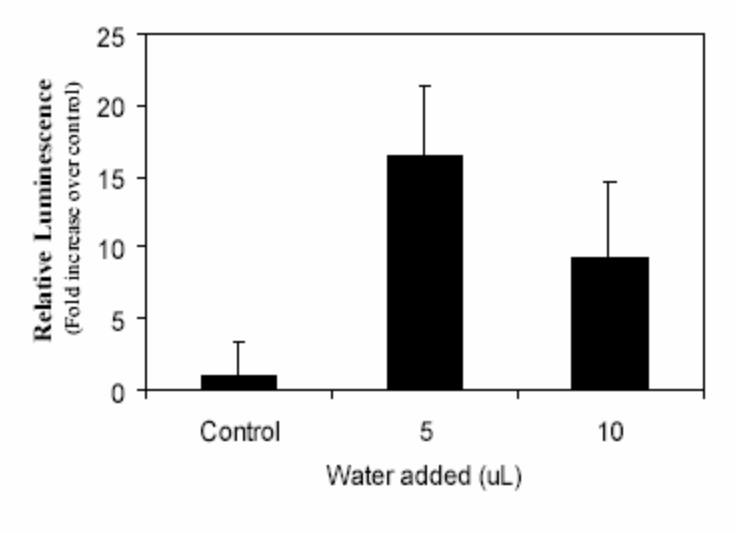

Figure 3.1 ATP released from a wound site.
5 µl or 10 µl H_2O was added to a wound site or an intact leaf. The exudates were tested for ATP concentration using luminometry. The amount of ATP corresponds with the relative luminescence. The relative concentration of ATP is represented as fold increases over the control. n = 10 for wounded leaves and n = 20 for the intact leaves. The [ATP] measured after both 5 µl and 10 µl H_2O dilutions at the wound site was statistically different than the same dilutions on the surface of intact leaves, P = 0.0006. The error bars represent the standard deviation. Results were obtained in triplicate with similar results for each experiment.

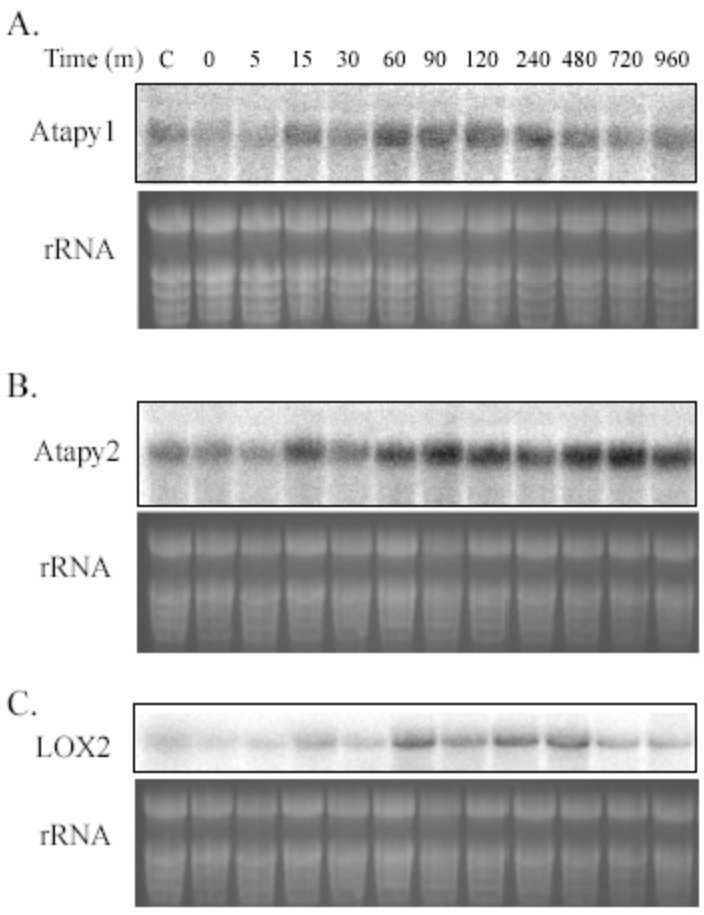

Figure 3.2 Atapy1 and Atapy2 expression upon wounding.
A. Atapy1 transcript accumulation at various times after wounding. Lane C is the negative control for no wound. B. Atapy2 transcript accumulation at various times after wounding. C. LOX2 transcript accumulation after wounding. rRNA is for the loading control. This experiment was done in triplicate with similar results.

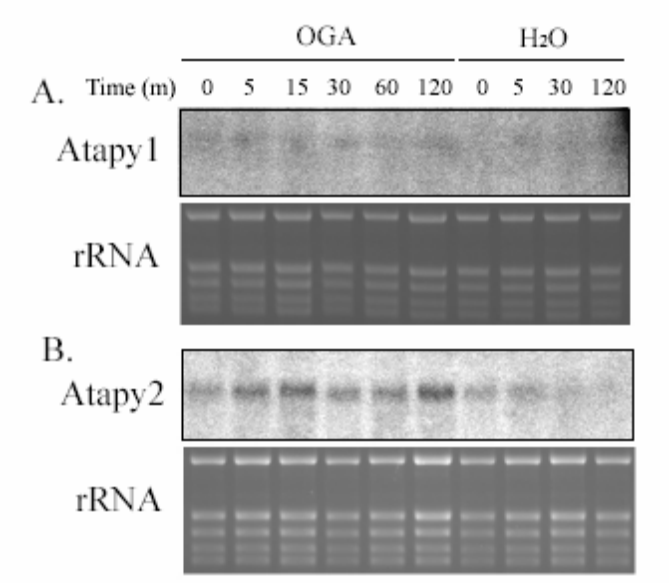

Figure 3.3 Atapy1 and Atapy2 expression after OGA treatments.
Arabidopsis leaves were infiltrated with either 10 µg/ ml OGA or H_2O on one side of the leaf, and tissue was collected at various times. Northern analyses were done to measure transcript level. A. Atapy1 did not have any apparent change in transcript level with OGA treatment. B. Atapy2 has increased transcript accumulation at 5 min and 15 min. rRNA shows the equal loading control. This experiment was done in duplicate with similar results.

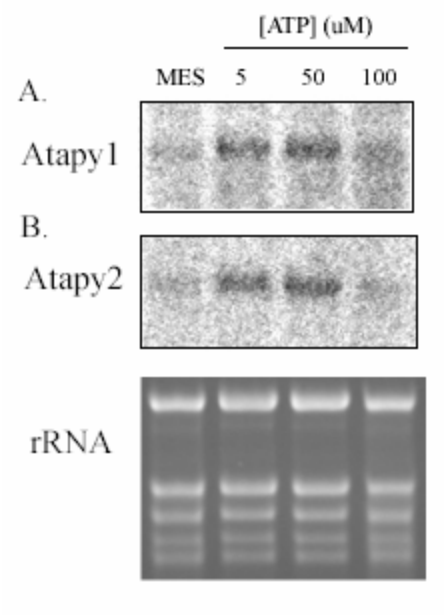

Figure 3.4 xATP induces transcript accumulation of Atapy1 and Atapy2. Seedlings were treated with either MES or various concentrations of ATP. Northern analyses were done to determine message level. A. Atapy1 at 90 min had increased transcript accumulation at 5 µM and 50 µM ATP. B. Atapy2 at 90 min had increased transcript accumulation at 5 µM and 50 µM ATP. rRNA shows equal loading. This experiment was done in duplicate with similar results.

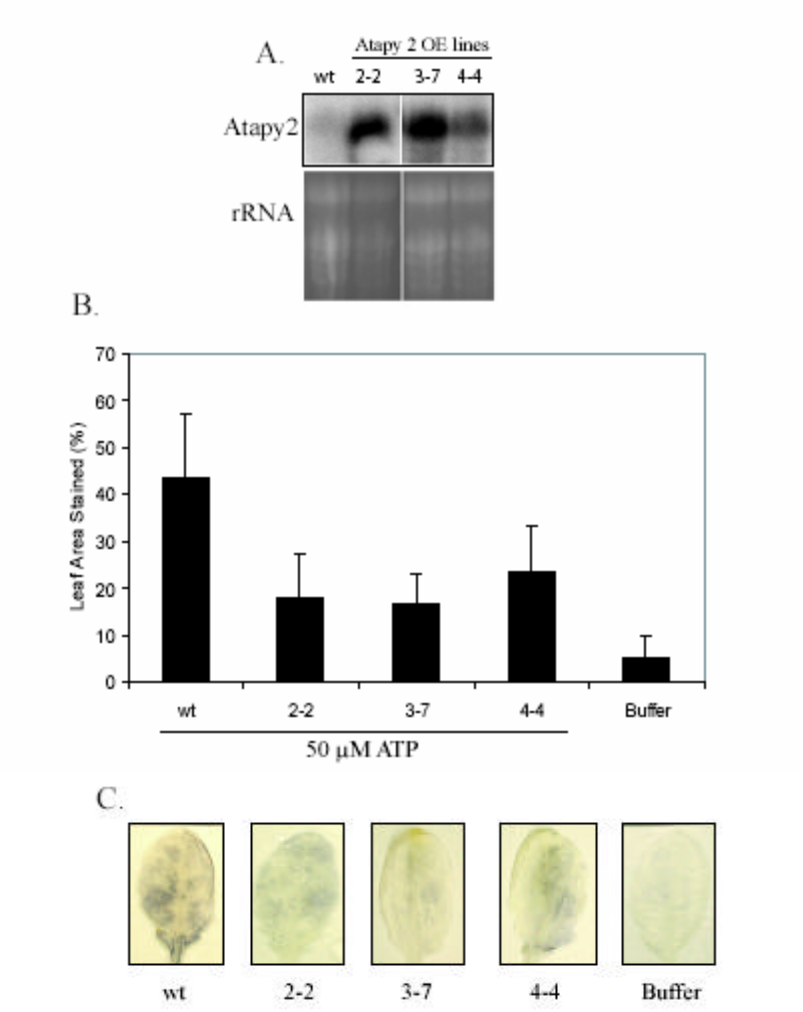

Figure 3.5 Transgenic lines overexpressing Atapy2 have reduced xATP-induced superoxide accumulation.
A. Northern analysis shows the expression level of Atapy2 in three independent lines of transgenic plants overexpressing (OE) Atapy2 and in wild-type plants. B. Three independent lines of Atapy2 OE plants have reduced superoxide accumulation when induced with 50 µM ATP. Superoxide was detected with NBT reduction. Dark spots on the leaf are areas with superoxide. The leaf area stained representing the presence of superoxide was measured with Image J. For all values, n = 8. The error bars represent standard deviation. C. Representative images of leaves with superoxide accumulation

CHAPTER 4

Conclusion

Plants are constantly assaulted with a variety of abiotic and biotic stresses. They have adopted diverse ways to sense these stresses, and typically respond using multiple, intricate signal transduction pathways with cross-talk between different pathways (Klessig et al. 2000). Plants and animals share some common mechanisms to cope with stress (Bergey et al. 1996, Staskawicz et al. 2001), including, prominently, the production of ROS (Lamb and Dixon 1997, Vignais 2002). The respiratory burst in animals and the oxidative burst in plants are both described as a massive burst of ROS.

NADPH oxidase in phagocytes is responsible for the production of ROS in animals (Vignais 2002), and homologous NADPH oxidases have been found in plants that function in plant stress responses, such as wounding and pathogen infection, and are required for the accumulation of ROS (Torres et al. 2002, Kwak et al. 2003). xATP has been shown to induce a respiratory burst in different mammalian phagocytes (Kuroki and Minakami 1989, Murphy et al. 1993, Dichmann et al. 2000), while adenosine is known to inhibit this respiratory burst (Cronstein et al. 1985, Bengtsson et al. 1996, Zalavary et al. 1996).

We have found that xATP induces superoxide accumulation in a biphasic and dose-dependent manner in *Arabidopsis* similar to its effects in phagocytes, and adenosine inhibits this accumulation of superoxide. These data suggest xATP

is acting as a signal inducing the production of ROS. The relatively low concentration of xATP, between 1 µM and 50 µM, that induced the accumulation of superoxide and the dose-dependence of the response suggest that xATP is acting through a receptor. Plant P2 receptors may also exist and may mediate similar responses in plants, although to date none have been found. In support of this hypothesis, we found that inhibitors of animal P2 receptors were able to block the superoxide accumulation induced by xATP and xATP-induced gene expression of two genes, PAL1 and AtrbohD.

Because xATP induces superoxide by activating NADPH oxidases in animals, we considered plant NADPH oxidases good candidates for mediating the xATP-induced superoxide accumulation. We found that *Arabidopsis* NADPH oxidases are responsible for the production of superoxide in response to xATP. In support of this hypothesis, we found that mutant plants with disruption of NADPH oxidase subunits, AtrbohD and AtrbohF, were reduced in their capability to accumulate superoxide with xATP treatment compared to wild-type plants, and that the NADPH oxidase inhibitor, DPI, abolished the xATP-induced accumulation of ROS. Additionally, AtrbohD, an NADPH oxidase subunit, had dose-dependent transcript accumulation in response to xATP. Previous studies found that *atrbohD* and *atrbohF* mutants were not able to fully respond to pathogen infection (Torres et al. 2002) or close stomata in response to ABA (Kwak et al. 2003). Our results suggest that xATP may be upstream of NADPH

oxidase subunits, AtrbohD and AtrbohF, in response to pathogen or abiotic stresses.

We also found that transcripts of PAL1, an important defense- and wound-induced gene, accumulated in response to xATP. This accumulation paralleled the superoxide accumulation and lent further support to the hypothesis that xATP is an upstream signal in the defense response.

Ca^{2+} and CaM are involved in numerous signal transduction pathways in plants (Yang and Poovaiah 2003). In animals, P2 receptors mediate their diverse responses through increases in cytosolic Ca^{2+} (Dubyak and El-Moatassim 1993), so we knew that if P2 receptor signaling in plants is similar to that in animals it would most likely be mediated through Ca^{2+} increases. Relevant to this point, Demidchik and colleagues (2003) have already demonstrated that low concentrations of xATP can induce significant increases in cytosolic Ca^{2+}. In this context, it is significant that we found that Ca^{2+} and CaM are intermediate signals between xATP and superoxide accumulation. Taken together, these findings are consistent with the hypothesis that an ATP-signaling pathway may exist in plants.

Other possible downstream components of the ATP signal transduction pathway are the phytohormones, jasmonic acid and ethylene. xATP induced LOX2 transcript accumulation, a gene in the biosynthetic pathway of jasmonic acid, in a dose-dependent manner and induced ACS6, a gene in the biosynthetic pathway for ethylene. Both jasmonic acid and ethylene are important in plants'

response to abiotic and biotic stresses, and both also play critical roles in developmental processes (Turner et al. 2002, Wang et al. 2002). Our results indicate that xATP may be functioning upstream of these hormones in response to a wound or infection. Recent papers indicate that xATP may also be important in developmental processes. Steinebrunner et al. (2003) found that xATP inhibited pollen germination, and Tang et al. (2003) found that xATP influenced auxin transport, root growth, and root gravitropism.

An important enzyme that regulates the level of extracellular nucleotides in animals is apyrase (Zimmerman 1996). Two *Arabidopsis* apyrases, Atapy1 and Atapy2, have been cloned and characterized (Steinebrunner et al. 2000). The disruption of these genes resulted in the inhibition of pollen germination (Steinebrunner et al. 2003), but the exact function of the enzymes is still unknown. We propose that one or both of these enzymes may be hydrolyzing extracellular nucleotides. Consistent with this hypothesis, lines overexpressing Atapy2 had reduced responsiveness to xATP in the induction of superoxide accumulation. Full apyrase activity would hydrolyze xATP to AMP, quenching an xATP signal. The increase in [AMP] could then stimulate the activity of 5'- nucleotidase, which hydrolyzes AMP to adenosine. In animals, adenosine can bind to adenosine receptors, resulting in negative feedback and inhibiting further accumulation of superoxide. Evidence that this feedback system may exist in plants is that adenosine suppresses the effects of xATP on superoxide production. Thus, an

adenosine negative feedback system could be a second explanation for why apyrase overexpression reduces the ability of plants to show the superoxide response to xATP. However, no *Arabidopsis* homologs to the mammalian ecto-5'-nucleotidase were found in the database, and no adenosine receptors or adenosine signaling has been documented in plants.

The Atapy1 and Atapy2 messages have expression level differences in response to wounding and OGA, suggesting differential regulation of the apyrases, but also suggesting a role for apyrases in wound and defense signaling.

Different mechanisms exist in animals to release ATP to the extracellular matrix (Gordon 1986, Dubyak and El-Moatassim 1993). During physical injury, intact cells may be lysed to release their contents, including mM concentrations of ATP (Di Virgilio et al. 2001). Other sources of xATP are from exocytosis and plasma membrane transporters (Abraham et al. 1993, Dubyak and El-Moatassim 1993, Roman et al. 1997). In plants, two mechanisms for xATP increases outside of the cell have been demonstrated. Thomas and colleagues (2000) were able to overexpress an ABC transporter protein and found that these plants had increased ATP on the surface of the leaves compared to wild-type plants. We have shown that in the case of wounding, there is a significant increase in ATP at a wound site compared to the surface of intact leaves. We found that the range of ATP released is high enough to induce gene expression changes and induce superoxide accumulation. Apyrases have increased transcript accumulation in response to a

wound and to xATP treatment, which is consistent with the interpretation that the ATP signal can induce a response that terminates itself, a common phenomenon in stimulus-response pathways.

Based the data in this dissertation, we propose a model for the tight regulation of superoxide production by NADPH oxidases in *Arabidopsis* through xATP signaling. Plants have very intricate signal transduction pathways with complex interactions though crosstalk between pathways and regulatory mechanisms to tightly control the concentration of specific signals. We suggest that xATP is a signal and that its accumulation and duration in the ECM is tightly regulated by ectoenzymes, especially apyrases and nucleotidases. We propose that the ATP released during wounding or pathogen-induced cell breakage induces the production of superoxide by binding to P2-like receptors, , and that this response is terminated when xATP is hydrolyzed to AMP by apyrases and to adenosine by 5'- nucleotidases. We propose that adenosine can serve as a negative feedback regulator by turning on a receptor-mediated pathway that inhibits superoxide production.

Regarding the role of apyrases in the control of [xATP], we propose that plants differentially regulate apyrases to further control the activity of these enzymes and downstream effectors. Atapy1 and Atapy2 genes have different expression patterns in response to wounding and OGA. The activity could also be affected by ROS itself. In animals, the apyrase activity of endothelial cells was

inhibited by superoxide, but antioxidants were able to restore the function of the enzymes. xATP signaling may be important in diverse responses of the plants, including response to wounding, infection, abiotic stress, and developmental processes.

Appendix

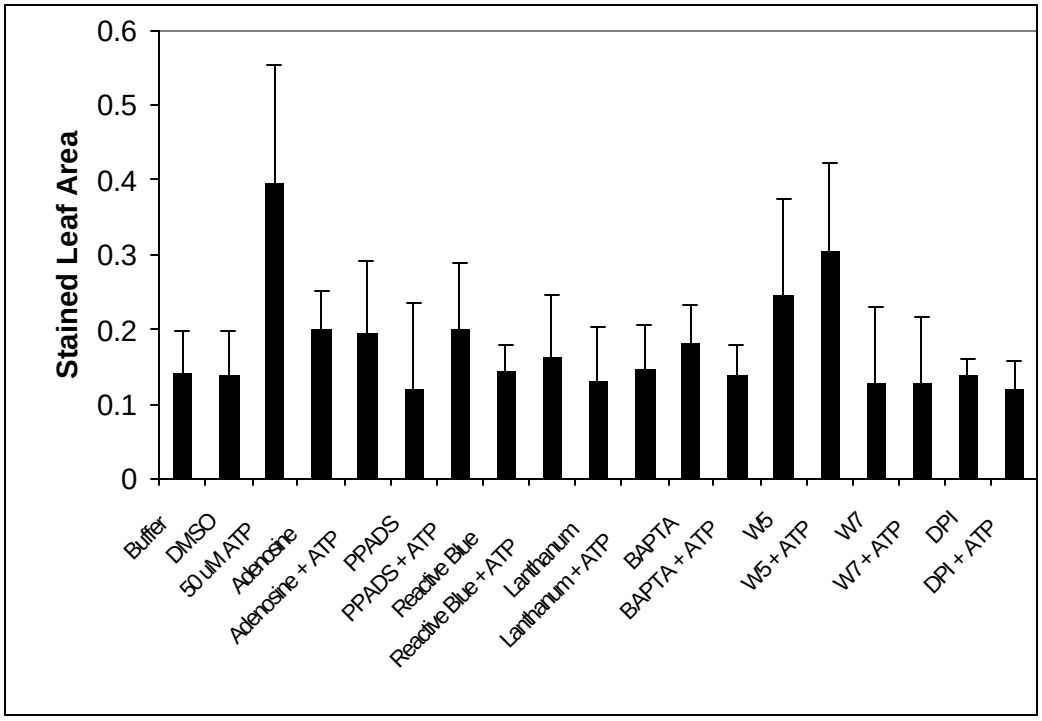

Figure 1 Superoxide detection after co-treatment of ATP with various inhibitors. *Arabidopsis* rosette leaves were co-treated with ATP and inhibitor, inhibitor alone, buffer alone, or ATP alone. Superoxide was detected with NBT reduction. The stained leaf area was measure and divided by the total leaf area. "Stained leaf area" represents the ratio of stained area to total area. For all values, $n > 5$.

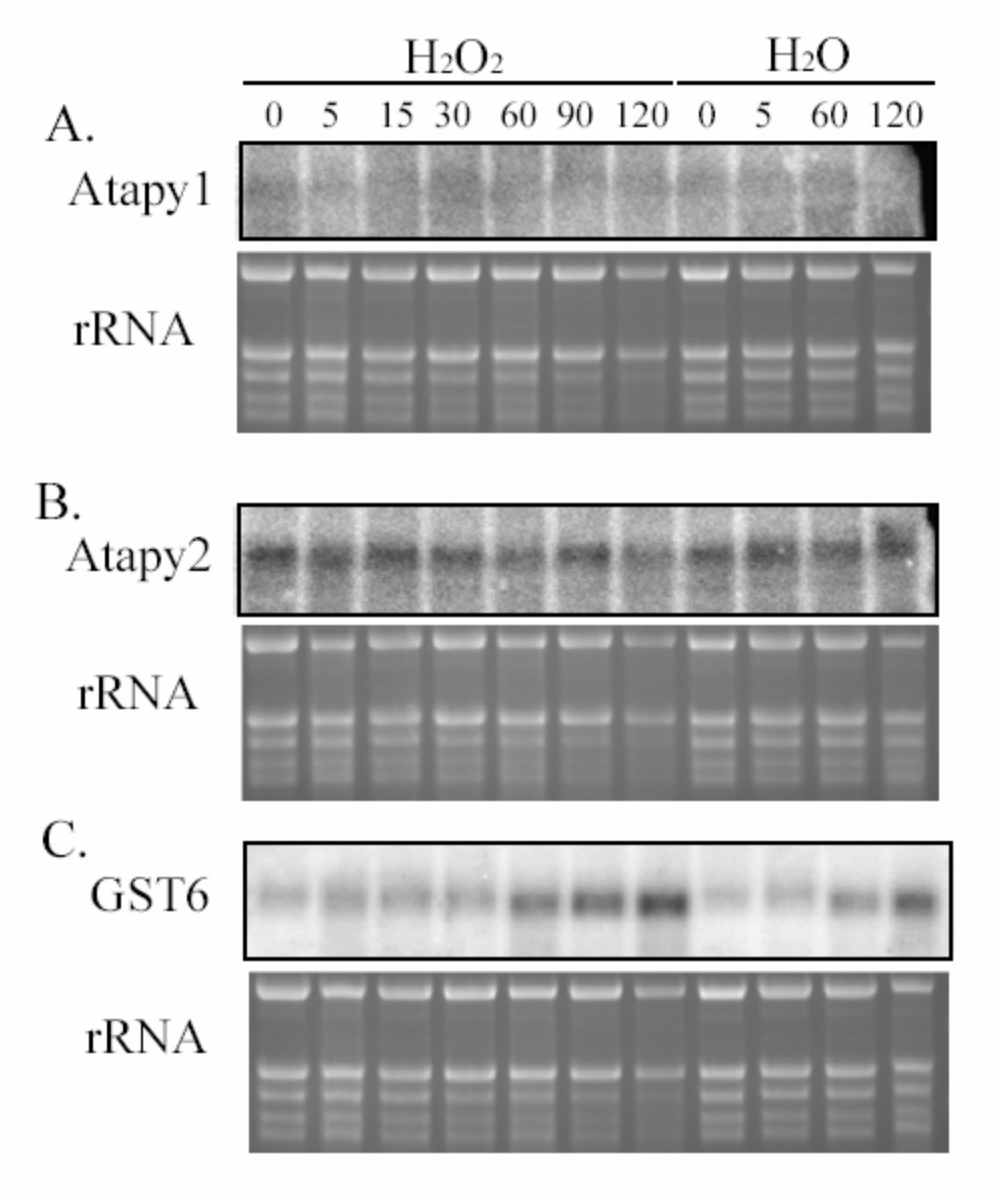

Figure 2 H_2O_2 treatments do not induce changes in Atapy1 or Atapy2 transcript accumulation. Seedlings were treated with either 20 mM H_2O_2 or H_2O and collected at various times. Northern analyses were done to measure transcript accumulation. A. Atapy1 transcript levels do not change with H_2O_2 treatments. B. Atapy2 transcript levels do not change with H_2O_2 treatments. C. GST6, the positive control, has increased transcript accumulation at 90 min and 120 min.

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