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Title: Extracellular nucleotide signaling in plants

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Current Project Personnel: Gary Stacey, Ph.D., Principal Investigator
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Abstract: Over the life of this funded project, our research group identified and characterized two key receptor proteins in plants; one mediating the innate immunity response to chitin and the other elucidating the key receptor for extracellular ATP. In the case of chitin recognition, we recently described the quaternary structure of this receptor, shedding light on how the receptor functions. Perhaps more importantly, we demonstrated that all plants have the ability to recognize both chitin oligomers and lipochitooligosacchardes, fundamentally changing how the community views the evolution of these systems and strategies that might be used, for example, to extend symbiotic nitrogen fixation to non-legumes.

Our discovery of DORN1 opens a new chapter in plant physiology documenting conclusively that eATP is an important extracellular signal in plants, as it is in animals. At this point, we cannot predict just how far reaching this discovery may prove to be but we are convinced that eATP signaling is fundamental to plant growth and development and, hence, we believe that the future will be very exciting for the study of DORN1 and its overall function in plants.

Past postdoctoral associates or graduate students trained during the current grant period:

Dr. Jinrong Wan, Ph.D., Nanjing University, 2002-2011, Currently employed as a senior research scientist, Division of Plant Science, University of Missouri, Columbia, MO

Dr. Oswaldo Valdés López, University of Mexico, 2009-2012, Currently employed as an Assistant Professor, UNAM, Mexico City.

Dr. Jeremy Dahmen, Washington State University, 2011-2013, currently employed at a company in Seattle, WA

Dr. Kiwamu Tanaka, Kagoshimia Univ., Japan, 2006-2014, currently an Assistant Professor in the Department of Plant Pathology at Washington State University, Pullman, WA, USA.

Geon-Hui Son, Ph.D., Division of Plant Sciences, 2012, Identification of an interaction network of Arabidopsis transcription factors and MAP kinases involved in chitin signaling and pathogen defense. Currently at Indiana University, Bloomington, IN

Ha Mi Le, Ph.D., Division of Plant Sciences, 2012. Downstream chitin signaling mediated by CERK1 in Arabidopsis. Currently living in Seattle, WA.

Jeongmin Choi, Ph.D. Division of Plant Sciences, 2008-2013, Identification of an Extracellular ATP receptor in *Arabidopsis thaliana*. Dissertation chosen to receive the 2013 University of Missouri, Outstanding Dissertation Award through a University-wide competition. Jeongmin received a prestigious EMBO postdoctoral fellowship and is currently working at the Sainsbury Lab at the Univ. of Cambridge, England

Dr. Yan Liang, University of Vermont, 2011-2015, currently Professor, Institute of Biotechnology, Zhejiang University, 866 Yuhangtang Road, Hangzhou, 310058, China

Dr. Yangrong Cao, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. 2012-2015. Currently Professor, State Key Lab of Agricultural Microbiology, College of Life Science Technology, Huazhong Agricultural University, Wuhan, Hubei, 430070

Expected unexpended funds: none

Project publications arising from the current funding period (i.e., since June 1`, 2011; peer reviewed publications listed first; reprints available upon request)

1. Tanaka, Kiwamu, Tran H.N. Nguyen, G. Stacey (2011) Enzymatic role for soybean ecto-apyrase in nodulation. Plant Signaling and Behavior 6(7): 1034-1036
2. Oswaldo Valdés-López, Sandra Thibivilliers, Jing Qiu, Wayne Wenzhong Xu, Tran H.N. Nguyen, Marc Libault, Brandon H. Le, Robert Goldberg, Curt Hill, Glen Hartman, Brian Diers, and Gary Stacey (2011) Identification of quantitative trait loci controlling gene expression during the innate immunity response of soybean Plant Physiol. 157: 1975-1986
3. Xue-Cheng Zhang, Zheng Wang, Mi Ha Le, Xinyan Zhang, Jianguo Sun, Dong Xu, Jianlin Cheng, and Gary Stacey (2012) Evolutionary dynamics of protein domain architecture in plants. BMC Evolutionary Biology 12:6.
4. Son, Geon Hui, Jinrong Wan, Hye Jin Kim, Xuan Canh Nguyen, Woo Sik Chung, Jong Chan Hong, and Gary Stacey. 2012. Ethylene-Responsive Element-Binding Factor 5, ERF5, is involved in chitin-induced innate immunity response. Mol. Plant-Microbe Int. 25(1): 48-60.

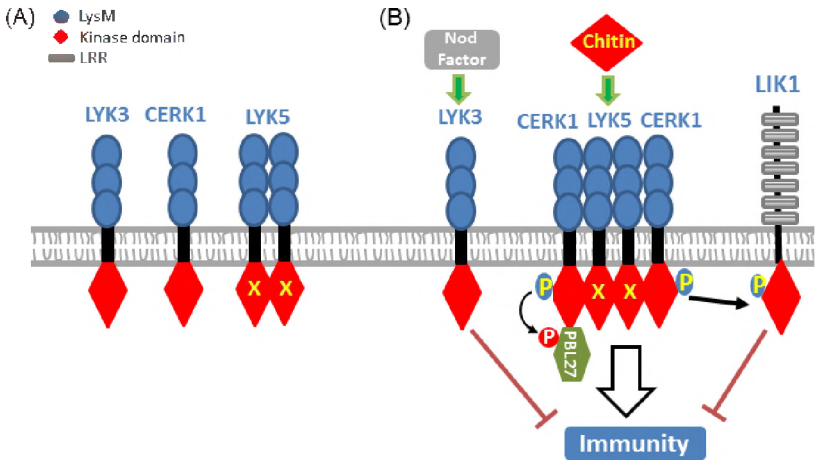

Figure 1. Chitin-triggered immunity in plants. (A) In the absence of chitin, lysin-motif receptor like protein kinase 5 (LYK5) forms a homodimer, mediated by disulfide bonds, while the other LYK proteins, LYK3 and CERK1 do not interact with LYK5. (B) Upon addition of chitin (d.p.>6), LYK5 binds chitin and associates with CERK1, likely forming a heterotetramer, activating CERK1 kinase activity, which ultimately induces MTI. LIK1, an LRR-RLK is phosphorylated by CERK1 kinase and acts as a negative feedback regulator to suppress innate immunity. PBL27, a BIK1 homolog protein, which was shown to be phosphorylated by CERK1, directly associates with CERK1 to stimulate an immune response. Another LYK protein, LYK3 was shown to mediate Nod factor or short-chain chitin (dp: 3-5) response in non-leguminous plants resulting in suppression of MTI.

5. Jinrong Wan, Xue-Cheng Zhang, Kiwamu Tanaka, Geon-hui Son, Laurent Brechenmacher, Tran Hong Nha Nguyen, Gary Stacey. (2012) The LysM receptor-like kinase AtLysM RLK4 is important for chitin signaling and plant innate immunity in Arabidopsis. Plant Physiol. 160: 396-406.
6. Dahmen, J.L., G. Stacey, H.K. Hunt (2013) Current and emerging analytical technologies for analyzing chitin-protein binding interactions. Reviews in Analytical Chemistry 32(1): 35-63.
7. Tanaka, K., C.T. Nguyen, Y. Liang, Y. Cao, G. Stacey (2013) Role of LysM domain receptors in chitin-triggered plant innate immunity. Plant Signal. Behavior 8:1, e22598
8. Yan Liang, Yangrong Cao, Sandra Thibivilliers, Jinrong Wan, Kiwamu Tanaka, Jeongmin Choi, Changho Kang, Gary Stacey (2013) Non-legumes respond to Rhizobial Nod Factors by suppressing MAMP-triggered innate immunity. Science 341: 1384-1387.
9. Oswaldo Valdes-Lopez, Saad M Khan, Robert J. Schmitz, Shiqi Cui, Jing Qiu, Trupti Joshi, Dong Xu, Brian Diers, Joseph R. Ecker, and Gary Stacey (2014) Genotypic variation of gene expression during the soybean innate immunity response. Plant Genetic Resources. 12: S27-S30.

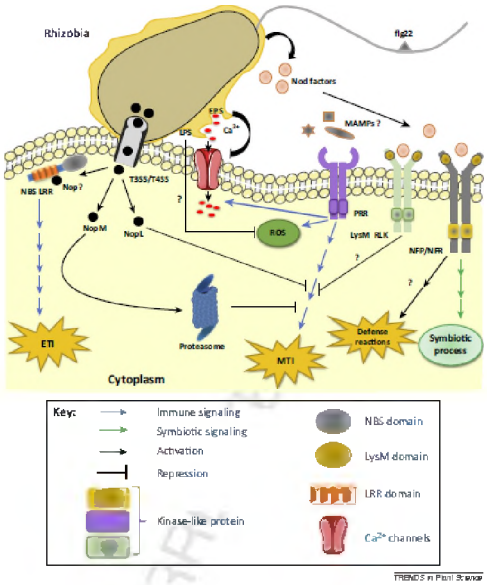

Figure 2. Defense suppression during early symbiotic interactions. Plants can perceive bacterial MAMPs using pattern recognition receptors (PRRs ; e.g., CERK1/LYK5) that trigger MTI. Rhizobia can escape MTI by producing modified MAMPs not recognized by PRRs (e.g., flagellin), through Nod factor-(LCO) mediated suppression of MTI (52), by other means (e.g., calcium chelation using bacterial exopolysaccharides). LCO perception by symbiotic LysM RLK receptors (NFR/NFP) induces the symbiotic process. Particular rhizobia can inject protein effectors into the host cytoplasm to short circuit immune signaling. Plants can recognize Nop effectors via NBS-LRR R genes that will turn on ETI . It is important to note that the figure presents an ensemble of responses induced by rhizobia in different plant species and different plant cells. Abbreviations: ETI, effector-triggered immunity; LysM RLK, lysin motif receptor-like kinase; MAMP, microbe-associated molecular pattern; MTI, MAMP-triggered immunity; NBS-LRR, nucleotide-binding site/leucine-rich repeat protein; NF, Nod factor; Nop, nodulation outer protein; PRR, pattern recognition receptor; R, resistance gene; ROS, reactive oxygen species; T3SS/T4SS, type III/type IV secretion system. Figure taken from [3])

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Hunt. (2014) Interfacing whispering gallery mode optical microresonator biosensors with the plant defense elicitor chitin. Colloids and Surfaces B: Biointerfaces 122: 241-249.
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17. Kiwamu Tanaka, Jeongmin Choi, Yangrong Cao, and Gary Stacey. 2014. Extracellular ATP as a damage associated molecular pattern (DAMP) signal in plants. Frontiers in Plant Science 5: 446.
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Progress relevant to the aims of the proposal: The original, specific aims of the funded project were:
1. Investigate the mechanism of CO recognition and translation into a cellular signal.
2. Investigate the mechanism by which chitotetraose and LCO suppress plant innate immunity.

Short summary of published work relevant to specific aims: Aim 1. Figure 1 presents the current model of chitin signaling, coupled to innate immunity, in Arabidopsis. We contributed significantly to this model by demonstrating that CERK1/LYK1 is a key component of the chitin receptor complex [4]. CERK1 provides the active, intracellular kinase domain to the receptor but functions as a heterodimer with a second LysM receptor-like kinase. For example, we showed that LYK4 interacts with CERK1 and is important for chitin signaling [5]. However, most recently, we showed that LYK5 is the major chitin binding protein in this complex and interacts with CERK1 to form an active heterotetrameric receptor [6]. The interaction of CERK1 with LYK5 is induced by chitin addition. This leads to activation of CERK1 kinase activity and downstream signaling (e.g., through the MAPK cascade; [7]. In addition, we demonstrated that LIK1 is a negative regulator of chitin signaling through direct interaction with CERK1 [8].

Aim 2. The model presented in Figure 1 likely has direct relevance to the recognition of lipochitooligosaccharide (LCO) signals that are critical to legume nodule development. LCO recognition is also mediated by a heterodimer (perhaps tetramer) composed of two LysM RLKs, NFR1 and NFR5, of which only NFR1 has an active intracellular kinase domain [9]. The dogma in the field, until recently, has been that LCO recognition is essential for nodulation and the lack of recognition of LCO by non-legumes likely explains their inability to nodulate [10, 11]. However, recently, we demonstrated that most, if not all, non-legumes do indeed recognize LCO signals, likely mediated through a LysM RLK [12]. Indeed, at a very recent meeting in Budapest, Giles Oldryod admitted this and they have now redirected their efforts to engineer cereals to fix nitrogen, abandoning the focus on initial LCO recognition, and instead focusing on later steps in symbiotic development as our [12] paper suggested. However, LCO recognition by non-legumes is not mediated by the normal Nod factor (LCO receptors); that is, NFR1/NFR5 and orthologs. Instead, LCO recognition in non-legumes is coupled to suppression of the innate immunity response. We proposed that this function of LCO may have predated its role as a signal in symbiotic plant-microbe interactions [13]. That is, the rhizobial-legume symbiosis may have begun as a pathogenic interaction with LCO acting to suppress innate immunity to allow infection. Through evolution and adaptation this symbiosis became more benign with LCO taking on an additional role of inducer of symbiotic developmental programs essential for nodule formation. We believe that the role of innate immunity in the rhizobial-legume symbiosis plays a much more important role than currently appreciated and is deserving of greater research interest (Figure 2, [3])

Unpublished results relevant to aims 1 and 2:

A. Proteosome mediate turnover of the LYK5 receptor (under review at New Phytol.):

Turnover of plasma membrane receptors is one means by which the cell can mediate its response to extracellular signal. Therefore, we undertook a study to investigate the means by which this may occur to AtLYK5, the major chitin-binding receptor (see above). We found that basal LYK5 protein levels are controlled by the ubiquitin/26S proteasome pathway, since treatment with MG132, an inhibitor of the 26S proteasome, resulted in the significant increase in LYK5 protein levels (Figure 3A). The ubiquitin/26S proteasome pathway is a common post-

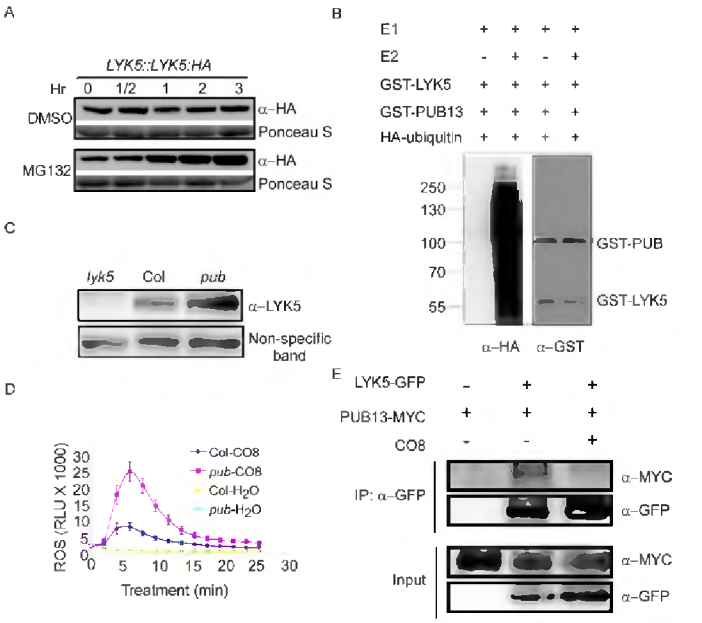


Figure 3: (A) MG132 stabilizes basal LYK5 protein levels; (B) PUB13 ubiquinates LYK5 *in vitro*; (C) *pub12/13* mutants contain higher levels of LYK5 proteins compared to wild type. (D) *pub12/13* mutants showed hypersensitivity to chitin-induced ROS production. (E) Chitin induces the dissociation of PUB13 and LYK5.

translational modification that involves an enzymatic cascade of Ub-activating (E1), Ub-conjugating (E2) and Ub-ligase (E3) enzyme which mediates the covalent binding of the small ubiquitin (Ub) to target proteins destined for degradation by the 26S proteasome [14]. In Arabidopsis, 1415 E3 ubiquitin ligases have been predicted, which are classified into two groups depending on the presence of a HECT (Homologous to E6-associated protein C-Terminus), or a RING (Really Interesting New Gene)/U-Box domain [15]. In this study, plant U-Box E3 ubiquitin ligases PUB12/PUB13 are identified that could ubiquitin LYK5 (Figure 3B) and regulates the steady state levels of AtLYK5 protein (Figure 3C). *pub12/13* double mutants showed hypersensitivity to chitin-induced early innate immunity, such as the production of reactive oxygen species (ROS) (Figure 3D) and MAP kinase phosphorylation, but exhibited normal responses to chitin-induced late responses, such as gene expression and callose deposition. Consistent with the mutant phenotype, chitin induced the dissociation of PUB13 and LYK5 (Figure 3E). The data suggest that AtPUB12/13 control the steadystate turnover of AtLYK5, which then is terminated upon chitin addition to allow for the stable production of the chitin receptor hetertetrameric complex (AtCERK1, AtLYK5). We are currently studying the chitin-induced turnover of this complex, which appears to involve a distinct AtPUB-mediate pathway.

B. Connection between chitin and salt stress signaling (under review at New Phytol.): Salinity stress and plant disease caused by pathogenic fungi are detrimental for agriculture and pose a current and growing threat to food security. The ability of plants to respond to multiple environmental stresses requires cross-talk and fine-tuning of stress signaling pathways. Here, we report a crosstalk between fungal-derived chitin and salt stress responses and demonstrate that members of the chitin receptor complex, *CERK1* and *LYK4* are necessary for salt stress responses in Arabidopsis. Gene responses to chitin and salt were highly co-expressed (z=30.27) and 92% of the genes induced by chitin were also induced by salt. This co-expression was unique to salt and chitin, since other PAMP, flg22 and osmotic stress did not show significant co-expression. We hypothesized that salt and chitin might share similar calcium permeable channels, or calcium reserves, required for the initial $[Ca^{2+}]_{cyt}$ increases. Consistent with this idea, pre-treatment with NaCl reduced by 38% the subsequent chitin-induced $[Ca^{2+}]_{cyt}$ increases, while pre-treatment with chitin reduced by 16% the subsequent NaCl-induced $[Ca^{2+}]_{cyt}$ increases. *CERK1* and *LYK4* expression was increased in roots during salinity stress but not in response to other abiotic stresses. Mutant plants, either *cerk1*, *lyk4,* or the double mutants, were hypersensitive to NaCl but not to osmotic stress (D-sorbitol) or ABA. These results, together with gene expression data, indicate that *CERK1* and *LYK4* are required for the ionic stress component of salinity stress. We found that CERK1 and LYK4 associates at the plasma membrane with ANNEXIN 1, a calcium permeable channel and component of the NaCl-induced $[Ca^{2+}]_{cyt}$ signal [16]. Mutant *cerk1* plants also showed higher NaCl-induced $[Ca^{2+}]_{cyt}$ in the roots in comparison with the wild type, suggesting that CERK1 may be a negative regulator of the calcium response to salt. Taken together, these data suggest a model where salinity stress or fungal pathogen-derived chitin are recognized and cause a cross stress regulation, perhaps mediated

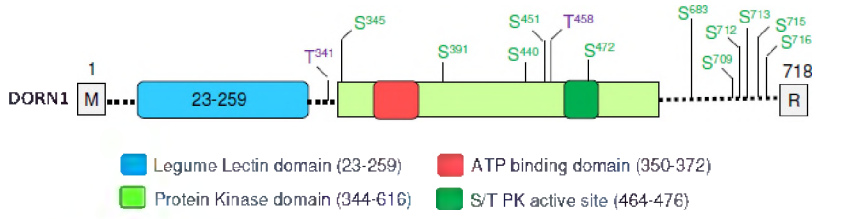


Figure 4, Cartoon depiction of the structure of DORN1 (P2K1) highlighting the autophosphorylation sites identified using mass-spectrometry. The various domains of DORN1 are color coded.

through phosphorylation of calcium channels. Furthermore, components of the chitin receptor, CERK1 and LYK4, are required for salinity stress responses, and in complex with ANN1 might control the salinity-induced $[Ca^{2+}]_{cyt}$ increases necessary to trigger an adequate salt stress response in the plant.

The last year of the DOE proposal represented a change in focus with work shifting to examine the role of extracellular ATP as a signal in plants. The sole objective of this final year, as described in the revised work plan, was as follows:

Objective 1. Identify other plant extracellular nucleotide receptors and define their role in plant growth, development and energy metabolism. *Hypothesis: By analogy to animal systems, DORN1 is part of a large lectin receptor-like kinase family and other members of this family likely also function in extracellular nucleotide signaling.*

ATP is an extracellular signal in both plants and animals: ATP is a ubiquitous compound in all living cells; it not only provides the energy to drive many biochemical reactions fundamental to survival, but also functions in signal transduction as a substrate for kinases, adenylate cyclases, etc. However, ATP is also an essential signaling agent outside of cells, where it is referred to as extracellular ATP (eATP). eATP is involved in numerous cellular processes, including neurotransmission, immune response, cell growth, and cell death [17]. In mammalian cells, the plasma membrane-purinoceptors P2X and P2Y bind eATP, as well as other nucleotides, at the cell surface to activate intracellular signaling cascades. Binding of eATP to P2X receptors gates ion influx; whereas activation of P2Y receptors recruits heterotrimeric G-proteins to trigger cytoplasmic signaling and gene expression. As a common phenomenon, the activated receptors induce elevation of cytoplasmic calcium $[Ca^{2+}]_{cyt}$, which in turn activates production of downstream messengers, such as nitric oxide (NO) and reactive oxygen species (ROS) [17, 18]. A multibillion dollar market exists for drugs that target the mammalian purinergic signaling pathway [e.g., plavix (clopidogrel) targeting P2Y12 in platelet aggregation].

A possible physiological role for eATP in plants was first suggested by the ability of exogenously applied ATP to stimulate closure of the Venus flytrap [19], to induce the formation of nucleases in excised Avena leaves [20], and to induce potassium ion uptake into maize leaf slices [21]. More recently, eATP was shown to induce various plant responses, including root-hair growth [22, 23], stress responses [24-26], gravitropism [27], cell death [28, 29], response to pathogens [30] and thigmotropism [31]. These studies clearly suggest that eATP exists in plants and plays an important role throughout plant growth and development. However, no P2 receptor homologs have been identified in plants, despite the fact that plants share a number of cellular responses to eATP with animal cells, including elevation of $[Ca^{2+}]_{cyt}$, NO, and ROS.

In 2014, our laboratory identified and characterized the first plant, eATP receptor, *do*esn't *r*espond to *n*ucleotides (DORN1; Figure 4). DORN1 defines a new receptor-kinase family (P2K) of purinoreceptors [32]. The identification of this receptor, as well as access to mutants that disrupt eATP signaling, now provide a unique opportunity to compare and contrast animal and plant purinergic signaling systems to define components and responses that are evolutionarily conserved. Ultimately, our comparative approach should broaden our knowledge of purinergic signaling in higher organisms, hopefully aiding the design of

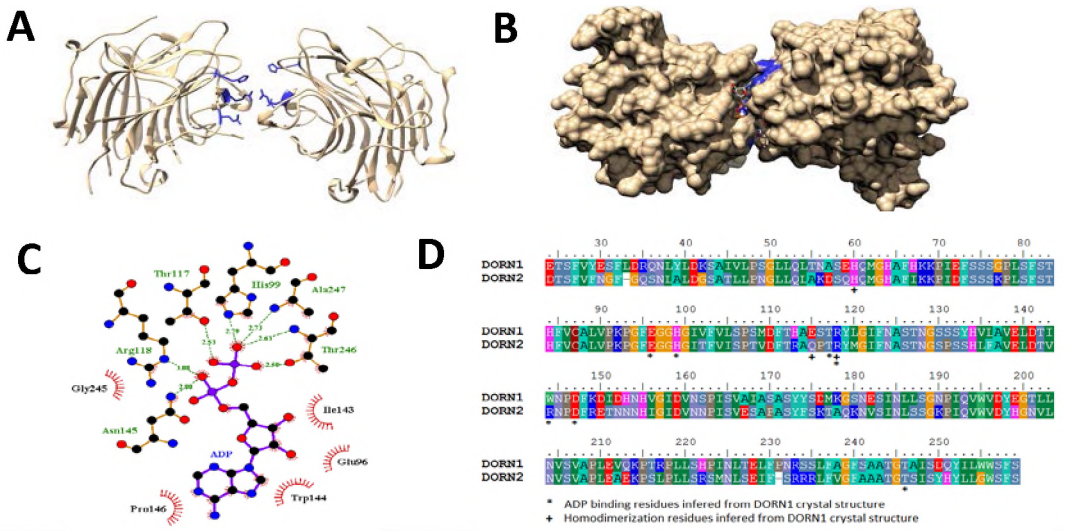


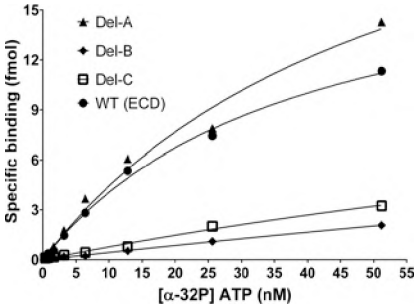
Figure 5. A. Crystal structure of DORN1 homodimer (lectin extracellular domain; blue residues are those involved in dimerization; see also panel D); B. Space-filling model of DORN1 crystal structure showing binding of two ADP molecules along the dimer interface; C. Map of ADP-amino acid interactions based on the DORN1 crystal structure. All of these residues have been mutagenized and the resulting mutant proteins tested for 32P-ATP binding (either α-32P or γ-32P labeled) and ability to complement the *dorn1* mutant phenotype (e.g., effects on cytoplasmic calcium levels; see Fig. 3); D. Alignment of the DORN1 and DORN2 amino acid sequence. Amino acids involved in ADP binding (see panel C), as well as homodimerization (see panel A) are identified and are largely conserved among the two proteins.

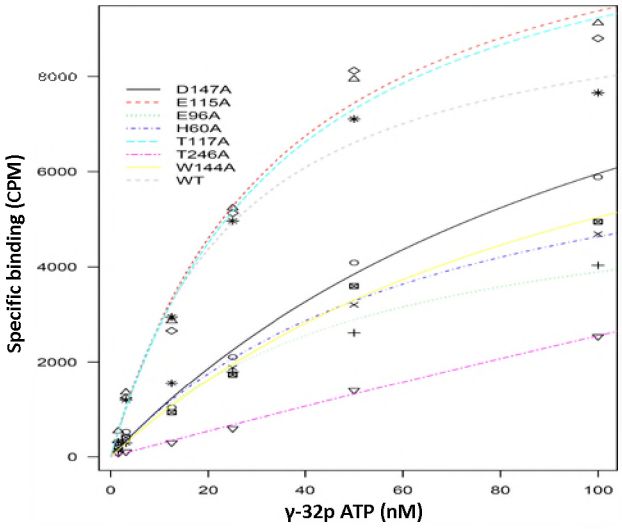
therapeutic treatments that can alleviate a wide variety of disease states associated with the response to eATP (e.g., inflammation and cardiovascular disease).

Purinergic signaling: an ancient and widely distributed trait. Multicellular organisms have assembled complex signaling networks that mediate specific and dynamic responses following various environmental stimuli. Among these are mechanisms that recognize a potentially life-threatening event as a danger signal. Danger signals include endogenous molecules and fragments from damaged cells and tissues, referred to as damage-associated molecular patterns (DAMPs). Eukaryotes use DAMPs to evoke immune inflammatory responses and damage healing.

Extracellular ATP (eATP) is a DAMP signal in both animals and plants [33, 34]. ATP is the primary source of high-energy phosphate bonds to support cellular metabolism. However, once ATP is released from cells following cellular damage, it acts as a DAMP signal. ATP is a good choice for such a role since cells contain a high concentration of ATP (1-10 mM), which is highly reactive and involved in more chemical reactions than any other compound except H_2O. In animals, eATP has been studied for over 60 years. The released ATP is recognized by plasma membrane-localized purinergic receptors (P2X and P2Y) that are involved in a wide range of animal physiology [35]. The animal P2X purinergic receptors have an ancient origin of at least 1 billion years [36]. Genomic sequence-based surveys for canonical P2X and P2Y receptors identified a P2X-like receptor with 28% identity in the photosynthetic algae, *Ostreococcus tauri* [36]. While ATP addition modulated ion channel activity of this protein when expressed in human embryonic kidney cells, this protein functions intracellularly based on its endomembrane localization and lack of ion flux upon ATP treatment in algal cell cultures [36]. The lack of identification of any homologous P2 receptors in insects, roundworm and land plants opens the possibility that other proteins may be involved in eATP signaling [37].

Extracellular ATP initiates inflammatory-like responses in both plants and animals. There are many cases of where plant and animal physiology converge and where studies in one system have been informative in the other. Examples include studies of small RNA [38] but also studies of organismal responses to stress; pathogen-host interactions are a particularly good example [39]. Thus, it is not





	Kd (nM)	Bmax (fmol)	R^2
WT (ECD)	38.3	19.6	0.99
Del-A	54.7	28.7	0.98
Del-B	533.7	23.7	0.99
Del-C	189.9	15.4	0.99

Wildtype or mutant protein	Kd	Kd Std. Error	Bmax	Bmax Std. Error
WT	25.87	5.02	10021.87	727.48
H60A	69.71	17.81	7863.86	1068.04
E96A (Loop-A)	53.34	18.58	5967.28	1007.58
E115A (Loop-B)	35.06	6.73	12659.11	1013.92
T117A (Loop-B)	35.54	9.96	12504.02	1469.53
W144A (Loop-C)	111.51	32.77	10659.04	1937.66
D147A	121.58	30.12	13201.33	2027.28
T246A (Loop-D)	1350	163.6	37013	422.23

Figure 6. Representative data showing the binding of [α-32P]ATP or [γ-32P]ATP to the purified, extracellular lectin domain of DORN1. Refer to Fig. 2 for correspondence to whole protein. Left panel: deletion (del) A removes residues 94-97, del B, 116-118, and del C, 140-145; right panel shows the effects of specific alanine substitution mutations. Note, H60A mutation does not affect ATP binding but appears to affect homodimerization (see study 3). Data are consistent with a critical role for R118, I143, W144, N145, D147, and T246 in nucleotide binding (see Fig. 2C).

surprising that animals and plants both have purinergic signaling pathways. In animals, eATP is considered a DAMP that is released by physical injury [40] or severe microbial infection [41, 42]. Some nucleotide receptors mediate anti- and pro-inflammatory responses in various tissues depending on the concentration of ATP released [33, 43-45]. These responses, found in both animals and plants, include the production of ROS, NO, and induction of secondary messengers (e.g., elevated cellular calcium). eATP is also a plant DAMP, released after wounding [24, 46] or in response to various stress agents. These include pathogen associated elicitors [e.g., chitin [22]] and the stress-associated plant hormone, abscisic acid (ABA) [46, 47]. Furthermore, abiotic stimuli [e.g., mechanical pressure [31], cold [26] and hypertonic solution treatment [46, 48-50]] cause ATP release. Eukaryotic cells have evolved elaborate systems involving a combination of exocytosis, transporters and ATP-hydrolyzing enzymes to maintain appropriate levels of eATP. Too little or too much eATP can affect pathogen virulence and even induce cell death (reviewed in [29, 51]) .

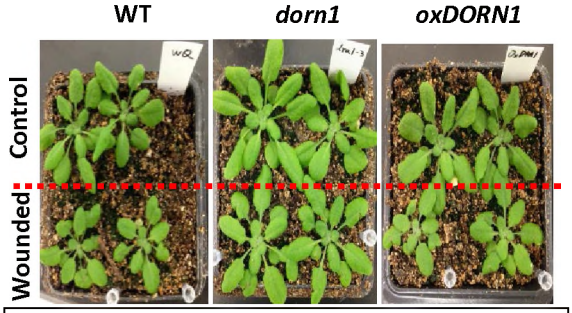


Figure 7. Effect of successive wounding on wild-type and *dorn1* mutant plants. WT: control (top) and wounded (bottom) wild-type plants showing that successive (daily) wounding results in dwarfing, which is slightly enhanced in plants ectopically expressing DORN1 (oxDORN1). However, *dorn1* mutant plants do not show dwarfing upon successive wounding.

DORN1 (P2K1) defines a novel purinoreceptor. The first plant receptor for eATP (DORN1; doesn't respond to nucleotides) was identified from a genetic screen for mutants of *Arabidopsis thaliana* insensitive to ATP, when monitored for cytoplasmic calcium levels using aequorin [32]. DORN1 has an extracellular legume (L)-type lectin domain, a transmembrane domain, and an intracellular serine/threonine kinase domain (Figure 4). Recently, in conjunction with Dr. Jijie Chai, Tsinghau University, we elucidated the X-ray crystal structure of the DORN1 extracellular domain in the presence ADP (Figure 5). Usable crystals in the presence of ATP were not obtained. DORN1 binds ATP and ADP with roughly equal affinity [32]. The elucidated structure is a homodimer with each monomer binding a single nucleotide. The nucleotide binding sites lie along the face of the interacting monomers. Extensive site-directed mutagenesis has defined the specific nucleotide binding residues that are critical for activity, as well as key residues necessary for homodimerization (Figures 4 & 5). The crystal structures resembles closely the computationally derived structure of DORN1 that we recently published [52].

The L-type lectins were originally found in legume seeds and generally defined by their ability to bind carbohydrates [53]. However, we and others [54] found no apparent interaction between DORN1 and a variety of sugars (unpubl.), likely due to a substitution at the ion binding site (Asp79 to His) that is critical for the formation of hydrogen bonds to stabilize the monosaccharide binding site [54, 55]. Instead, the extracellular lectin domain of DORN1 binds ATP (Kd = 45.7 nM) and ADP (Ki against ATP= 38.3 nM) with high affinity [32]. This affinity is within the physiological range since nanomolar levels of eATP are released from Arabidopsis roots when mechanically stimulated [31], while micromolar levels (~40 μM) are found at sites of wound damage [24]. Although we sometimes use μM concentrations of ATP in some experiments, some of our other studies have clearly shown that plants respond to ATP in the nanomolar range, consistent with the *in vitro* measured affinity of the receptor [31, 56]. We should also note that we find no evidence of ATP hydrolysis by DORN1 and, as shown in Figure 6, DORN1 binds strongly to both [α-32P]ATP or [γ-32P]ATP.

DORN1 was previously identified as lectin receptor kinase I.9, a positive regulator of plant defense against the oomycete pathogens, *Phytophthora infestans* and *Phytophthora brassicae* [2, 54, 57]. In this case, lectin receptor kinase I.9 (DORN1) was identified through its ability to directly interact with an oomycete pathogen secreted protein, *in plant induced-O* (IPI-O). The interaction between IPI-O and DORN1 reduced the plant immunity response, promoting infection [2]. This role is not limited to Arabidopsis since ectopic expression of DORN1 in tobacco and potato plants conferred greater resistance to *Phytophthora infestans*, the famous 'potato famine' pathogen causing bacterial leaf blight [57]. These data underline the central role of DORN1 and, presumably, purinergic signaling in the plant response to a variety of biotic and abiotic stresses.

DORN1 defines the P2K family of receptor kinase purinoreceptors. The *dorn1* (*p2k1*) gene was identified by positional cloning of mutations that disrupted the normal cellular calcium response to eATP [32]. These same mutations blocked various downstream signaling events, including MAP kinase activation and gene expression. For example, transcriptome analysis comparing wild-type and *dorn1-1* mutant seedlings identified 332 and 242 genes significantly up- and down- regulated, respectively, in response to eATP. None of these genes were regulated in response to eATP in the *dorn1-1* mutant

A total of ten mutant alleles of *dorn1* were identified throughout the coding sequence; all of which blocked the response to ATP addition (e.g., Ca^{2+}_{cyt} response) [51]. If *DORN1* encodes the essential receptor for eATP, then *dorn1* mutants should not show defects in other signaling processes, other than the response to ATP. Indeed, the Ca^{2+}_{cyt} response of *dorn1* mutants to various biotic (flg22, chitin, elf26 and pep1) and abiotic (NaCl, mannitol, D-glucose and cold water) calcium elicitors was similar to wild-type plants [32].

Some animal P2Y receptors recognize other nucleotides, e.g., ADP and UTP, in addition to ATP [58]. The *dorn1* mutant seedlings showed strong defects in the calcium response to ADP and slowly hydrolysable forms, ATPγS and ADPβS, and also to other nucleotides. In contrast, the response to a pyrimidine nucleotide, CTP, was comparable to the level of wild-type plants, suggesting a unique plant receptor for CTP. Strong ectopic expression of DORN1 (*oxDORN1* transgenic plants) led to a stronger

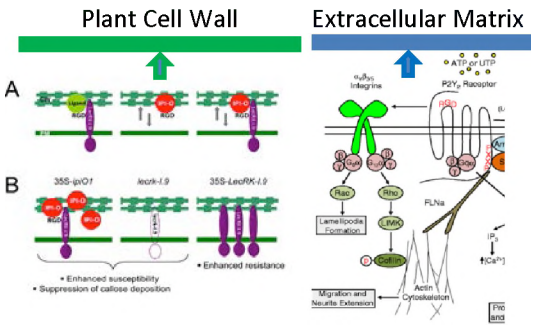


Figure 8. Plant and animal purinergic receptors also function to facilitate a tight connection between the plasma membrane and extracellular matrix (i.e., cell wall in the case of plants). In both cases, these connections are mediating through interaction with RGD domain proteins. Figures taken from [1, 2].

(e.g., ~20-fold higher) cellular response to ATP. DORN1 is localized to the plasma membrane [32]. The ligand binding specificity *in vitro* reflects the order of the calcium response in wild-type Arabidopsis seedlings [56].

To monitor ATP binding activity *in planta*, we conducted an ATP-binding assay using Arabidopsis protoplasts. The *DORN1* protein linked with biotinylated 8-azido-ATP was pulled-down by streptavidin beads. Biotinylated 8-azido-ATP crosslinking to DORN1 was abolished by competition with unlabeled ATP, whereas CTP had no effect [32]. Taken together, our data suggest that *DORN1* directly binds eATP on the cell surface.

Phenotypes resulting from mutation of purinergic receptors. *DORN1* mutants do not show a strong growth or developmental phenotype, which is similar to knock-out (KO) mice of mammalian P2Y and P2X receptors. For example, KO mice lacking the P2Y1 [59], P2Y2 [60], P2Y12 [61], P2X3 [62] or P2X7 [63] receptors show no apparent growth/development phenotype with the same true of KO mice lacking P2X1 [64], although these mice exhibit male infertility. However, all of these mutant mice exhibit conditional phenotypes, especially upon nucleotide challenge, at the biochemical and/or cellular level. Again, this is similar to our *dorn1* (and *dorn2*) mutant plants [32]. For example, a dwarfing phenotype is apparent when wild-type plants are wounded daily (Figure 7). This dwarfing is not seen in the *dorn1* mutant plants but is slightly more pronounced in plants ectopically over-expressing the DORN1 receptor. Hence, loss of purinergic signaling in mammals or plants does not grossly affect growth and development. However, the importance of these receptors is amply demonstrated under the appropriate biochemical/ cellular conditions.

Do purinergic receptors function to mediate plasma membrane-extracellular matrix connections? The P2K (DORN1) family of plant purinergic receptors is biochemically and evolutionarily distinct from the P2Y and P2X families of receptors found in mammals. What form of selection resulted in the convergence of purinergic signaling systems in both plants and animals via such different receptors?

Integrins are a class of animal proteins that possess a RGD (arginine-glycine-aspartate) peptide binding domain, which is important for mediating cell membrane attachment to the extracellular matrix (ECM). This function of integrin is important to surface adhesion, aggregation and motility [65]. A variety of ECM proteins have a RGD peptide. In mammalian cells, the eATP receptor, P2Y2, has a RGD peptide, which binds to integrin [66]. Although not essential for eATP signaling, mutants of P2Y2 lacking the RGD domain do show reduced ATP binding activity [43]. Plants lack canonical animal integrins but possess integrin-like proteins, which are also thought to mediate cell membrane attachment to the plant cell wall [67]. DORN1 is predicted to have two RGD binding domains, ASYY (residues 151-154) and PHPR (residues 257-260), both in the extracellular lectin domain

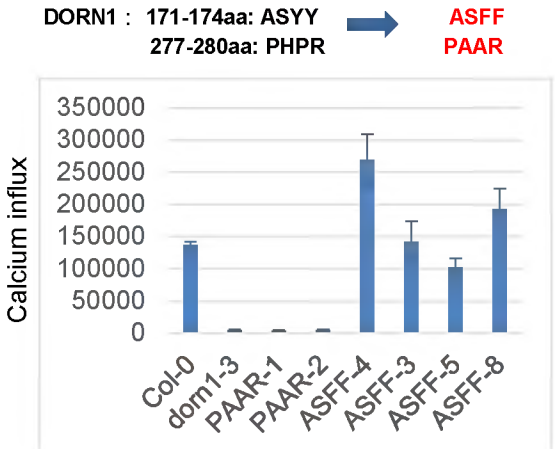


Figure 9. Complementation of the Ca^{2+}_{cyt} response of *dorn1-3* mutant plants upon addition of ATP by expression of DORN1 proteins modified at either of the two RGD binding domains (top). In each case, no significant response was found in the absence of ATP. Note that only mutations that affect the PHPR (->PAAR) domain compromise the ability to complement the *dorn1* mutant phenotype. Col-O = wildtype.

[54]. Photoaffinity labeling and peptide inhibitor studies demonstrated that these DORN1 (LecRK I.9) domains bind directly to RGD-containing peptides [54] [68]. These same domains mediate DORN1 (LecRK I.9) interaction with the oomycete *Phytophthora* effector protein IPI-O [54, 68]. Of the two DORN1 RGD-binding domains, the ASYY domain appeared to bind to RGD peptides (e.g., AGRGDSP) and IPI-O with greater affinity but the authors suggested this may be due to the PHPR domain lying close to the predicted DORN1 transmembrane domain. The addition of IPI-O or synthetic RGD peptide was shown to disrupt plant cell membrane-cell wall adhesion zones, as visualized after plasmolysis of the plant cells [2]. The authors suggested that this 'loosening' of cell wall integrity contributes to pathogen invasion. Alternatively or, perhaps, in addition, blocking of DORN1-RGD interaction may also disrupt eATP signaling, which might also reduce plant resistance. Indeed, *it is interesting to speculate that the selection of a lectin domain purinoreceptor in plants was driven by the need to interact with a polysaccharide-rich ECM, as opposed to the protein-glycoprotein rich ECM of animal cells* (Figure 8).

In order to study the importance of the DORN1 RGD binding domains, the two RGD binding domains were independently mutated ASYY → ASFF and/or PHPR → PAAR, respectively. Stable transgenic plants expressing these mutant DORN1 proteins expressed from the native promoter were tested for their ability to complement the *dorn1* mutant phenotype (as measured by Ca^{2+}_{cyt}). Only DORN1 proteins disrupted in the PHPR motif failed to complement the *dorn1* mutant phenotype when challenged with eATP (Figure 9).

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