Arabidopsis Lectin Receptor Kinase P2K2 Is a Second Plant Receptor for Extracellular ATP and Contributes to Innate Immunity

An Quoc Pham, Sung-Hwan Cho, Cuong The Nguyen, and Gary Stacey

In animals, extracellular ATP is a well-studied signaling molecule that is recognized by plasma membrane-localized P2-type purinergic receptors. However, in contrast, much less is known about purinergic signaling in plants. P2 receptors play critical roles in a variety of animal biological processes, including immune system regulation. The first plant purinergic receptor, Arabidopsis (Arabidopsis thaliana) P2K1 (L-type lectin receptor kinase-I.9), was shown to contribute to plant defense against bacterial, oomycete, and fungal pathogens. Here, we demonstrate the isolation of a second purinergic receptor, P2K2, by complementation of an Arabidopsis p2k1 mutant. P2K2 (LecRK-I.5) has 74% amino acid similarity to P2K1. The P2K2 extracellular lectin domain binds to ATP with higher affinity than P2K1 (dissociation constant \( K_d \) = 44.47 ± 15.73 nM). Interestingly, p2k2 and p2k1 p2k2 mutant plants showed increased susceptibility to the pathogen Pseudomonas syringae, with the double mutant showing a stronger phenotype. In vitro and in planta studies demonstrate that P2K2 and P2K1 interact and cross-phosphorylate upon extracellular ATP treatment. Thus, similar to animals, plants possess multiple purinergic receptors.

ATP is well known as the source of cellular energy in all organisms. Under normal conditions, cells maintain millimolar levels of intracellular ATP, but also nanomolar levels of extracellular ATP (eATP). In response to the appropriate stimulus, ATP is released from the cytosol to the extracellular matrix and becomes an essential signaling molecule for growth, development, and stress responses (Khakh and Burnstock, 2009; Tanaka et al., 2010a; Cho et al., 2017). In mammals, P2Xs (ion channel receptors) and P2Ys (G-protein-coupled receptors) are plasma membrane purinoreceptors that can bind eATP and trigger various downstream signaling cascades. Humans possess seven P2X receptors and eight P2Y receptors (Burnstock, 2018). The action of these multiple P2X and P2Y receptors explains the critical roles of eATP in various biological processes in animals, such as inflammation, neurotransmission, immune response, cell proliferation, cell differentiation, and cell death (Burnstock and Verkhratsky, 2010; Cekic and Linden, 2016; Diezmos et al., 2016; Ferrari et al., 2016).

Plants lack canonical P2X and P2Y receptors but still respond to eATP by, for example, triggering an increase in cytosolic calcium (\( Ca^{2+} \)), as well as in nitric oxide and reactive oxygen species (Reichler et al., 2009; Tanaka et al., 2010a). Indeed, eATP signaling has been implicated in a variety of plant processes, including root growth (Tang et al., 2003; Weerasinghe et al., 2009; Clark et al., 2010), stress responses (Thomas et al., 2000; Song et al., 2006), and pollen germination (Reichler et al., 2009; Rieder and Neuhaus, 2011).

The question of how plants recognize eATP was answered by the cloning of the first plant eATP receptor, DORN1 (DOES NOT RESPOND TO NUCLEOTIDES1), defining a new kinase class of purinoreceptor (Choi et al., 2014a). In keeping with the animal P2 receptor nomenclature, we now prefer the designation “P2K1” for this first plant purinergic receptor. The P2K1 protein was previously referred to as L-type lectin receptor kinase I.9 (LecRK-I.9) based on sequence comparisons of various Arabidopsis (Arabidopsis thaliana) LecRK proteins. Subsequent studies from our lab showed that upon eATP addition, P2K1 regulates the stomatal aperture by direct phosphorylation of NADPH oxidase.
P2K1 also phosphorylates protein acyltransferases (i.e. PAT5 and PAT9) to regulate their activity (Chen et al., 2019).

The LecRK protein family members contain a lectin-like ectodomain, a single transmembrane domain, and an intracellular Ser/Thr kinase domain. In Arabidopsis, the LecRK family contains 45 members. Thirty-eight are divided into nine subclades, while seven singleton members do not belong to any clade (Bouwmeester and Govers, 2009). The diversity of the LecRK family in plants was proposed to be the result of tandem- and whole-genome duplication (Hofberger et al., 2015). As a result of duplication events, proteins can have diverse functions or may share similar functions (Moore and Purugganan, 2003). For example, in Arabidopsis, LecRK-I.9 is an ATP receptor, while LecRK-I.8 was shown to be a NAD (NAD\(^+\)) sensor (Choi et al., 2014a; Wang et al., 2017). In clade IX, LecRK-IX.1 and LecRK-IX.2 share analogous function, regulating Phytophthora resistance and plant cell death (Wang et al., 2015).

Under normal growth conditions, only P2K1 (LecRK-I.9), LecRK-IV.1, and LecRK-VIII.1 are expressed at a high level in most plant tissues, whereas the other LecRK genes show lower expression and only in specific tissues. However, the expression of individual LecRK genes was found to be responsive to specific hormone treatments, abiotic stresses, elicitor treatments, or pathogen infections, suggesting a functional role for these receptors under specific conditions (Bouwmeester and Govers, 2009). While the specific functions, as well as ligands, of most LecRK family members remain unknown, there appears to be a general association of these receptors with the plant response to both abiotic and biotic stress. LecRK-I.3 was reported to be an active kinase that is regulated by ethylene in response to salt stress (He et al., 2004). LecRK-IV.3, which is strongly induced by abscisic acid, methyl jasmonate, salicylic acid, or stress treatments, plays critical roles in both abiotic and biotic stress responses (Huang et al., 2013). Besides P2K1 (LecRK-I.9), LecRK-V.2 and LecRK-VII.1 were also reported to play essential roles in regulating stomatal closure (Yekondi et al., 2018). Recently, it was reported that P2K1 is involved in modulation of jasmonic acid-mediated gene expression for plant defense responses (Tripathi et al., 2018).

 Animals possess multiple P2X and P2Y receptors, consistent with the wide and varying functions that purinergic signaling plays in physiology (Puchalowicz et al., 2014). Given that eATP was shown to mediate a variety of plant processes (Tanaka et al., 2010a), it seemed reasonable to suggest that plants also encode multiple purinergic receptors. In this study, we identify P2K2 (LecRK-I.5) as a second Arabidopsis eATP receptor by screening various AtLecRK clade I RLKs for their ability to complement an Arabidopsis p2k1 mutant. The P2K2 lectin ectodomain binds ATP with higher affinity than P2K1 and is an active kinase with the ability to autophosphorylate and transphosphorylate other proteins. A variety of assays showed that P2K1 and P2K2 can interact. Similar to p2k1 mutant plants, plants defective in P2K1 or P2K1/P2K2 function are significantly more susceptible to pathogen infection.

RESULTS

Identification of the Second eATP Receptor, P2K2, by Screening All Members of LecRK Clade I

Among nine subclades of the LecRK family of Arabidopsis, the first plant eATP receptor, P2K1 (LecRK-I.9), belongs to clade I, which has 11 members (Supplemental Fig. S1; Bouwmeester and Govers, 2009). Compared to P2K1, all of the other LecRK clade I members are expressed at relatively low levels in Arabidopsis under normal conditions but can be induced by various stimuli (Bouwmeester and Govers, 2009). Among clade I members, LecRK-I.5 (At3g45430) and LecRK-I.10 (At5g60310) are the most closely related to P2K1, based on sequence comparisons.

In order to test whether LecRK clade I members other than P2K1 could function in eATP perception, we ectopically expressed each of the clade I LecRK genes in the Arabidopsis p2k1-3 mutant, which expresses the calcium reporter protein aequorin, and then monitored the Ca\(^{2+}\) response upon eATP addition. Among the LecRKs tested, ectopic expression of LecRK-I.8 showed a lethal phenotype, while LecRK-I.10 showed no expression (Supplemental Fig. S2). Only LecRK-I.5 (P2K2) could partially restore the Ca\(^{2+}\) response of the p2k1-3 mutant plants (Fig. 1). These results led us to test the Ca\(^{2+}\) response of p2k2 (lecrk-I.5) mutant plants to eATP (Supplemental Fig. S3). The results indicated that the loss of P2K2 function reduced the ability of the plants to elevate Ca\(^{2+}\) levels in response to ATP, although this defect was not as severe as found with p2k1 mutant plants. The calcium response of the p35S:P2K2 overexpression plants was higher than that of the wild type (Fig. 1; Supplemental Fig. S3). Given these results, we renamed LecRK-I.5 as P2K2, reflecting a functional annotation for this protein beyond the strictly sequence-based assignment of LecRK-I.5.

ATP Binds to the P2K2 Extracellular Domain

If P2K2 is a bona fide purinergic receptor, then it should bind ATP with a physiologically relevant affinity. In order to test this experimentally, we purified the P2K2 ectodomain (Supplemental Fig. S4) and conducted in vitro binding studies using radiolabeled \(\gamma\)\(^{32}\)P-ATP. The P2K2 protein showed a typical saturation curve for ATP binding with high affinity (dissociation constant \([K_d] = 44.47 \pm 15.73\) nM and maximum binding capacity \([B_{max}] = 625.8 \pm 86.48\) pmol mg\(^{-1}\)) (Fig. 2).

To determine whether P2K2 could bind other nucleotides or ligands, in vitro competitive binding assays were performed. We tested the ability of unlabeled
ligands to compete against the binding of radiolabeled \( ^{32}\text{P}-\text{ATP} \). Unlabeled ATP and ADP were found to be strong competitors with low equilibrium dissociation constant (\( K_i \); ATP \( K_i = 31.7 \) nM; ADP \( K_i = 97.3 \) nM; Fig. 2). Unlabeled GTP was a weak competitor, while the other ligands, AMP, adenosine, adenine ITP, CTP, TTP, and UTP, showed no competition (Fig. 2). These results differ from our previous studies with P2K1 and suggest that P2K2 may have higher specificity for ATP and ADP than P2K1 has (Choi et al., 2014a).

The P2K2 Intracellular Domain Has Strong Kinase Activity, Which Is Essential for Downstream Signaling

The P2K2 intracellular domain is predicted to have kinase activity. In order to confirm this prediction, we cloned the P2K2 intracellular domain (Supplemental Fig. S4), including the putative kinase domain (P2K2-KD). Subsequent purification of the P2K2-KD demonstrated strong autophosphorylation activity in vitro, as well as the ability to transphosphorylate myelin basic protein (MBP; Fig. 3). Comparison of the sequence of P2K1-KD and P2K2-KD allowed us to predict critical amino acid residues for kinase activity. Specific mutations were generated within the P2K2-KD sequence using site-directed mutagenesis, specifically \( ^{\text{D}}\text{A}^{\text{G}}\text{e} \) (kinase activation motif mutation) and \( ^{\text{D}}\text{S}^{\text{S}}\text{N} \) (similar to the \( ^{\text{K}}\text{l}^{\text{E}}\text{I} \) mutant version; Choi et al., 2014a). Subsequent in vitro assays demonstrated a lack of kinase activity for the \( ^{\text{D}}\text{A}^{\text{G}}\text{e} \) protein and very low kinase activity for the \( ^{\text{D}}\text{S}^{\text{S}}\text{N} \) protein (Fig. 3). Electrophoretic separation of the wild-type P2K2 and kinase domain mutant P2K2 proteins demonstrated differences in their mobility, which we attributed to their relative ability to autophosphorylate (Fig. 3). Consistent with this notion, treatment of the wild-type and kinase mutant forms of P2K2 with \( \lambda \)-protein phosphatase (PPase) to release the phosphate group resulted in all three proteins showing similar electrophoretic mobility (Supplemental Fig. S5).

In order to ascertain whether P2K2 kinase activity was critical for function, we expressed the wild-type P2K2 protein (as control) and the full-length \( ^{\text{D}}\text{A}^{\text{G}}\text{e} \) and \( ^{\text{D}}\text{S}^{\text{S}}\text{N} \) mutant versions in the \( ^{\text{K}}\text{l}^{\text{E}}\text{I} \) mutant background and assayed the ability of the resulting transgenic plants to elevate \( \text{Ca}^{2+} \) upon ATP addition. Ectopic expression of the full-length \( ^{\text{D}}\text{A}^{\text{G}}\text{e} \) or \( ^{\text{D}}\text{S}^{\text{S}}\text{N} \) mutant proteins in \( ^{\text{K}}\text{l}^{\text{E}}\text{I} \) mutant plants failed to complement the \( ^{\text{K}}\text{l}^{\text{E}}\text{I} \) phenotype (Fig. 3). We conclude therefore that P2K2 kinase function is required for P2K2 receptor function, which is similar to the results obtained for P2K1 (Choi et al., 2014a).

P2K2 Self-Associates and Also Interacts with P2K1 on the Plasma Membrane

Like P2K1, P2K2 has a putative transmembrane domain (Supplemental Fig. S4). To determine whether P2K2 localizes to the plasma membrane, we fused full-length P2K2 with yellow fluorescent protein (YFP) and expressed P2K2-YFP or free YFP in Arabidopsis protoplasts. We used FM4-64 for plasma membrane staining. The resulting data indicate that, again like P2K1, P2K2-YFP localizes to the plasma membrane (Fig. 4; Supplemental Fig. S6). Plasma membrane receptors often form homodimer or heterodimer complexes. For example, P2K1 was previously shown to self-associate on the plasma membrane (Chen et al., 2017). Therefore, we performed bimolecular fluorescence complementation (BiFC) assays in Arabidopsis protoplasts expressing P2K2-YFP\(^\text{e} \) and P2K2-YFP\( ^\text{c} \), as well as P2K2-YFP\( ^\text{e} \) and P2K1-YFP\( ^\text{c} \). The data indicate that P2K2 colocalized with the FM4-64 plasma membrane marker (Fig. 4). Full-length P2K2 and P2K1 were cloned into pCambia-Nluc or pCambia-Cluc and the resulting luciferase fusion constructs were

![Figure 1](image-url)
coexpressed with P2K2-Cluc in *Nicotiana benthamiana* leaves. Consistent with the BiFC results, P2K2-Nluc and P2K1-Nluc showed a strong interaction signal in the split-luciferase assay when coexpressed with P2K2-Cluc, but not with empty vector controls (Fig. 4). Furthermore, the strength of the P2K2-P2K2 and P2K2-P2K1 interactions was increased by the exogenous addition of ATP (Fig. 4). Our in vitro pull-down experiments also showed that P2K2-KD-His directly interacted with GST-P2K2-KD and GST-P2K1-KD, but not with GST-LYK5-KD, which served as our negative control (Fig. 4). Coupled with our previous results regarding P2K1, the data indicate that both P2K1 and P2K2 can self-associate but also have the ability to form heterocomplexes within the plasma membrane. In our experiments, these associations were enhanced upon addition of ATP (Fig. 4).

P2K1 Can Phosphorylate P2K2, But Not Vice Versa

The data above indicate that both P2K1 and P2K2 can autophosphorylate, as well as transphosphorylate other proteins. Hence, there is the possibility that these two proteins could interact and transphosphorylate each other. In order to test this, we incubated kinase-dead versions of GST-P2K1D525N, D572N-KD, and GST-P2K2D467N-KD proteins with kinase-active versions of GST-P2K1-KD and GST-P2K2-KD and assayed for phosphorylation using radiolabeled 32P-ATP. GST-P2K1-KD and GST-P2K2-KD phosphorylated GST-p2k2D467N-KD in vitro (Fig. 5). However, P2K1 and P2K2 failed to transphosphorylate the two kinase-dead versions of P2K1, GST-p2k1D525N-KD and GST-p2k2D572N-KD (Fig. 5). The data indicate that P2K1 is incapable of transphosphorylating itself but also cannot be phosphorylated by P2K2, and they also suggest that if P2K1 and P2K2 form an active heterocomplex, then activation of P2K1 is likely the initial step leading to transphosphorylation of P2K2, as well as other downstream proteins.

P2K2 Plays a Partially Redundant Role with P2K1 during Plant Immunity

Previous reports indicated that P2K1 positively regulates plant defense against *Pseudomonas syringae* (Balagué et al., 2017; Chen et al., 2017), *Phytophthora infestans*, *Phytophthora brassicae* (Gouget et al., 2006; Bouwmeester et al., 2011, 2014), and *Botrytis cinerea* (Tripathi et al., 2018). Given that P2K1 and P2K2 interact, and that P2K2 can partially complement the P2K1 phenotype, it is likely that these two proteins may function redundantly in ATP signaling, either in separate complexes or in association with one another. Therefore, we hypothesized that P2K2 might also be involved in pathogen resistance. To test the role of P2K2 directly, we examined plant susceptibility to *P. syringae* upon flood inoculation. The level of bacterial infection was monitored by bioluminescence and confirmed by direct counting to quantify the level of pathogen colonization (Fig. 6). The *p2k1-3* and *p2k2* single-mutant and *p2k1p2k2* double-mutant plants showed significantly greater colonization than wild-type plants (Fig. 6). Consistent with the visual assays, direct bacterial counts showed that *p2k1-3* and *p2k2* single-mutant and *p2k1p2k2* double-mutant plants were significantly more...
susceptible to bacterial infection compared to the wild type, whereas the P2K2 complemented line showed no significant difference compared to the wild type (Fig. 6). Interestingly, those plants ectopically expressing P2K2 showed elevated resistance to bacterial infection relative to the wild type (Fig. 6). These results are consistent with P2K2 playing an important role in plant innate immunity. The fact that the p2k1 p2k2 double mutant showed the highest level of susceptibility suggests that, at least for this specific phenotype, the two receptors show some level of functional redundancy.

To further understand how P2K2 contributes to bacterial pathogen resistance, we measured the activity or expression of a few genes known to respond to bacterial infection. We used wild-type (ecotype Columbia of Arabidopsis [Col-0]) plants as the positive control and the p2k1-3 mutant line as a negative control while testing the pathogen response of p2k2 single-mutant and p2k1p2k2 double-mutant plants. A key aspect of the pathogen response pathway is the activation of mitogen-activated protein kinase (MAPK) signaling (Bi and Zhou, 2017). Therefore, after ATP treatment, we measured activation of MPK3 and MPK6 in p2k2 and p2k1 p2k2 mutant plants relative to controls. As shown in Figure 7, wild-type plants showed strong phosphorylation of MPK3 and MPK6 upon ATP treatment, whereas the p2k1-3, p2k2, or p2k1 p2k2 mutant plants exhibited lower phosphorylation. Interestingly, the phenotype of the p2k1 p2k2 double mutant was stronger than that of either of the single mutants, suggesting that the two receptors are at least partially redundant in function. In addition, we measured the expression of MYC2 and ZAT10, previously reported to be regulated by P2K1 and known to respond to pathogen infection (Balagué et al., 2017; Tripathi et al., 2018; Jewell et al., 2019). The expression of both genes was significantly reduced in the p2k2 and p2k1 p2k2 mutant plants after ATP treatment (Fig. 7). Taken together, the results suggest that, like P2K1, P2K2 is a critical component of ATP signaling through the pathogen response pathway.

**DISCUSSION**

The first evidence that eATP plays a signaling role in plants was found in 1973 when exogenous addition of ATP stimulated faster closure of the specialized leaves of the Venus fly trap (Dionaea muscipula; Jaffe, 1973). About four decades after this report, the first plant eATP receptor, P2K1 (LecRK-I.9), was identified (Choi et al., 2014a). The p2k1 mutant plants are unable to recognize exogenous ATP and activate various downstream responses. In both animals and plants, eATP
Figure 4. P2K2 is localized to the plasma membrane, self-associates, and interacts with P2K1. A, Fluorescence microscopy images of Arabidopsis protoplasts transiently expressing the indicated constructs. Bright-field images show protoplasts without fluorescence. The protoplast plasma membrane was labeled with FM4-64 dye. Chlorophyll was detected by autofluorescence. Free YFP was used as a control for P2K2 localization. EFR-YFP was used as a control for the BiFC assay. Scale bars = 20 μm. B, Split-luciferase assay image of N. benthamiana leaves coinfiltrated with the agrobacterial strains containing P2K2-NLuc/CLuc, P2K1-NLuc, and EFR-NLuc. Circles indicate leaf patches that were infiltrated with Agrobacterium containing each construct. ATP, Leaves infiltrated with 20 μM ATP; Mock, leaves infiltrated with 2 mM MES (pH 5.7). Asterisks indicate values significantly different from P2K2/EFR (top, n = 4) or mock treatment (bottom, n = 7; *P < 0.05, Student’s t test). C, P2K2 directly interacts with P2K1 and itself in vitro. Purified GST-P2K1-KD, GST-P2K2-KD, and GST-LYK5-KD (negative control) recombinant proteins were incubated with or without His-P2K2-KD for 1 h. The results of GST-bead pull-down were detected by anti-His and anti-GST immunoblot (top and middle). The His-P2K2-KD loading control was detected by anti-His immunoblot (bottom). All experiments were repeated with similar results.
appears to play a variety of important roles. Hence, it seemed reasonable to consider the possibility that both animals and plants possess multiple eATP receptors. The identification of P2K2 (LecRK-I.5) confirms this possibility. As expected, P2K2 can bind ATP (Fig. 2) with high affinity, roughly equivalent to that measured previously for P2K1 (Choi et al., 2014a) and consistent with ATP levels required to induce measurable physiological responses (Tanaka et al., 2010b). Interestingly, in vitro competitive binding data indicate that P2K2 has a higher specificity than P2K1 for ATP and ADP (Fig. 2; Choi et al., 2014a). The physiological relevance of this is unknown but could be important, especially since P2K1 and P2K2 may function in planta as a heteromeric complex (Fig. 4).

P2K1 and P2K2 are likely the result of a tandem genome duplication. Therefore, they were suggested to be ohnologous genes (paralogous genes that diverged at the same time through whole-genome duplication; Hofberger et al., 2015). Ohnologous genes can share similar functions, but more often they diverge and develop different functions (Moreira and López-García, 2011; Hofberger et al., 2015). Our current data argue that both P2K1 and P2K2 have a similar function in that:

**Figure 5.** P2K1 can phosphorylate P2K2. A, P2K1 and P2K2 can phosphorylate a kinase-dead version of P2K2. Purified GST-p2k2D467N-KD (kinase dead) or GST-LYK5-KD (negative control) was incubated with GST-P2K1-KD, GST-P2K2-KD, or GST (negative control) in an in vitro kinase assay. B, P2K1 and P2K2 cannot phosphorylate kinase-dead versions of P2K1. Purified GST-p2k1D525N-KD, GST-p2k1D572N-KD, or GST-p2k2D467N-KD (positive control) was incubated with GST-P2K1-KD, GST-P2K2-KD, or GST in an in vitro kinase assay. Autophosphorylation and transphosphorylation were detected by incorporation of 32P labeled-ATP (top). The protein loading was measured by Coomassie brilliant blue staining (bottom). LD, Ladder. These experiments were repeated three times with similar results.

**Figure 6.** P2K2 plays a critical role in plant resistance to P. syringae DC3000. The wild type (Col-0) and the p2k3-1 mutant were used as controls to compare to p2k2, p2k1 p2k2, pP2K2::P2K2 (the p2k2 complemented line using the P2K2 native promoter), and p35S::P2K2 (the P2K2 overexpression line) plants. Fourteen-day-old seedlings were flood inoculated with a P. syringae DC3000 lx suspension (OD600 = 0.002) containing 0.025% (v/v) Silwet L-77. At 1 d after inoculation, bright-field photographs were taken by a normal camera while bacteria invasion was detected by a CCD camera (Bio-luminescence images; A) and bacterial colonization was determined by plate counting (B). Values represent the mean ± SE, n = 6 biological replicates. ANOVA with multiple comparisons analysis was calculated by GraphPad Prism 7. Lowercase letters over bars indicate significantly different means (P < 0.05). The experiment was repeated with similar results.
they bind and respond to eATP and can, at least partially, complement one another (Figs. 1 and 2; Supplemental Fig. S7; Choi et al., 2014a).

Our data suggest that P2K1 and P2K2 may function in planta as part of a heteromeric complex. However, it should be noted that under normal growth conditions, P2K1 is strongly expressed in most tissues, which is not the case for P2K2 (Bouwmeester and Govers, 2009). Our initial mutant screen for plants unable to increase intracellular calcium levels upon ATP addition yielded largely mutants in P2K1 (Choi et al., 2014a). Therefore, P2K1 appears to be the primary eATP receptor in Arabidopsis. The expression of P2K2, although quite low under normal growth conditions, is induced under stress conditions, including upon addition of ATP or pathogen treatment (Supplemental Fig. S8; Bouwmeester and Govers, 2009; Balague et al., 2017). Therefore, the possibility remains that a heteromeric complex of P2K1 and P2K2 could function during such stress conditions, perhaps as a means to increase the specificity and/or intensity of the response. Such an occurrence would be similar to the situation in animals, in which heteromeric purinergic receptor complexes are well documented (Nicke et al., 1998; Virginio et al., 1998; Kawate et al., 2009). Given that P2K1 can transphosphorylate P2K2, but not vice versa, if such a heteromeric complex is formed, it seems likely that activation of P2K1 is the primary event leading to transphosphorylation of P2K2 and other downstream signaling components.

Plants recognize conserved molecular patterns derived from pathogens (i.e., pathogen-associated molecular patterns [PAMPs]), resulting in the activation of an innate immune response called PAMP-triggered immunity (PTI; Jones and Dangl, 2006). These PAMPs are recognized by specific pattern recognition receptors. In a similar manner, specific pattern recognition receptors can also recognize molecules released from plants due to cellular damage, or damage-associated molecular patterns (DAMPs; Gust et al., 2017). The responses of plants to DAMPs are similar to PTI responses (Yamaguchi and Huffaker, 2011). Consequently, plants trigger a variety of secondary, intracellular events, such as cytosolic calcium increase, reactive oxygen species generation, and protein phosphorylation (such as MAPKs and calcium-dependent protein kinases). As a result, various transcription factors (such as MYBs, MYCs, and ZATs) are activated that contribute to pathogen resistance (Bigeard et al., 2015). In both animals and plants, eATP is defined as a DAMP (Choi et al., 2014b; Tanaka et al., 2014; Tripathi and Tanaka, 2018). Recognition of eATP by its receptors triggers very similar responses, as seen in the plant response to well-characterized PAMPs (Choi et al., 2014a; Chen et al., 2017). Indeed, given that PAMPs appear to induce the release of ATP, we previously proposed that at least a portion of the PTI response is actually due to purinergic signaling (Choi et al., 2014a; Chen et al., 2017). It is now clear that both P2K1 and P2K2 contribute to the ability of ATP to induce plant immune responses. An important role of P2K2 in innate immunity is consistent with an increase in P2K2 expression upon either ATP or pathogen treatment (Supplemental Fig. S8). The data are consistent with a model in which P2K2, normally expressed at a low basal level, is induced upon pathogen challenge (perhaps specifically due to eATP), resulting in an elevation of purinergic signaling and the formation of a stress-specific, heteromeric P2K1-P2K2 complex.

Humans possess seven P2X receptors and eight P2Y receptors, and therefore, it is possible for plants to function with only two purinergic receptors, P2K1 and P2K2. We are cognizant of the fact that the identification of these two plant receptors relied on the ability to...
detect an increase in intracellular calcium upon ATP addition. Hence, if other receptors exist that are not coupled to this calcium response, our assays would not have detected them. The fact that P2K2 is not expressed except under stress conditions likely explains why this particular receptor was not identified in our initial mutant screens, suggesting that other receptors may only be detectable under very specific growth conditions. There are 45 LecRK proteins encoded within the Arabidopsis genome, 60 t-type LecRKS in soybean (Glycine max; Liu et al., 2018), 50 t-type LecRKS in Populus (Yang et al., 2016), 84 t-type LecRKS in wheat (Triticum aestivum; Shumayla et al., 2016), 72 t-type LecRKS in rice (Oryza sativa; Vaid et al., 2012), and 53 t-type LecRKS in foxtail millet (Setaria italica; Zhao et al., 2016). If only a fraction of these LecRK proteins function in purinergic signaling, then the field is indeed very rich in future discoveries as the community continues to explore all of the various functions of eATP in plant growth and development.

MATERIALS AND METHODS

Phylogenetic Tree Analysis

11 LecRK clade I protein sequences were collected from The Arabidopsis Information Resource web site (https://www.arabidopsis.org/). The sequences alignment was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenetic tree was generated in MEGA7.0 (www.megasoftware.net) by using the Maximum Likelihood method based on the JTT matrix-based model.

Plant Materials

All Arabidopsis (Arabidopsis thaliana) ecotype Col-0 plants used in this study are aequorin-expressing lines in the Col-0 background. Stable transgenic lines of wild type and p2k1-3 mutants (Sailk_M22209) with Aequorin (AEQ) were described in our previous studies (Choi et al., 2014a; Chen et al., 2017). The lecK1.5 transfer DNA (T-DNA) mutant, p2k2 (GK-77718680), was obtained from the Arabidopsis Biological Resource Center (Ohio State University) and crossed with wild-type/AEQ and p2k1-3/AEQ to generate p2k2/AEQ and p2k1p2k2/AEQ. Homozygosity for the T-DNA insertion and the aequorin transgene in F2 progeny was confirmed by PCR-based genotyping and reverse transcription quantitative PCR (RT-qPCR) using the specific primers listed in Supplemental Table S1. Arabidopsis seeds were sterilized and submerged in autoclaved water and incubated at 4°C for 4 to 7 d before sowing onto one-half strength Murashige and Skoog medium plates. Each of the wells contained 50 mL of CITZ buffer, including 10 μM coelenterazine (Nanolight technology), 2 mM MES, and 10 mM CaCl2 (pH 5.7). The plates were incubated at room temperature overnight in the dark, followed by the addition of 30 mL of 2× treatment solution directly applied into the wells using a multichannel pipette. This process can be done ~10 to 20 s before measurements are initiated. The production of luminescence was immediately measured using an image-intensifying CCD camera (Photek 216). After these measurements, the remaining unchelated aequorin was estimated by applying discharging buffer, including 2 μM CaCl2 and 20% (v/v) ethanol, and luminescence was measured by the CCD camera. The photon-counting data were normalized and converted into calcium concentration using the procedures of Mithöfer and Mazars, 2002.

Plasmid Construction and Protein Purification

The full-length genomic DNA of P32K in the pDONR-Zeo vector was used as the template for further cloning. The extracellular domain and kinase domain of P32K were amplified by PCR using gene-specific primers (Supplemental Table S1). The PCR products were digested by restriction enzymes BamH1 and XhoI for extracellular domain cloning after gel extraction and purification. The DNA products were cloned into the pGEX-2T vector (in the case of extracellular domain cloning) and pET-41a or pET-28a vectors (in the case of kinase domain cloning) using T4 DNA ligase enzyme. To generate mutant versions, the wild-type P2K2 extracellular domain in pGEX-5X-1 of the kinase domain in pET-41a was used as a template.

The constructs used for protein purification were transformed into Escherichia coli BL21-AI (Invitrogen). The bacteria were cultured in Luria-Bertani medium to reach OD600 = 0.6 to 0.9 at 37°C. The bacteria were shaken at 25°C for 30 min before the target proteins were incubated by addition of 0.1 mM isopropylthio-β-D-galactoside (IPTG) at 25°C for 6 h. The culture was then centrifuged at 4,500 rpm for 10 min and 4°C. After decanting the Luria-Bertani medium, the bacterial pellet for GST-fused protein purification was resuspended in Tris-buffered saline (TBS) containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Triton X-100, 1× protease inhibitor (Pierce), and 2 mM phenylmethylsulfonyl fluoride (PMSF); while cells for HIS-fused protein purification were resuspended in HIS-lysis buffer containing 50 mM sodium phosphate (pH 7.5), 300 mM NaCl, 8 mM imidazole, 0.05% (v/v) NP-40, 1× protease inhibitor (Pierce), and 2 mM PMSF. The cells were broken by sonication followed by centrifugation at 12,000 rpm for 4°C at 10 min. The supernatant was then applied to glutathione sepharose 4B R10 resin (GE Healthcare) for GST-fused proteins, while TALON Metal Affinity Resin (Clontech) was used for HIS-fused proteins. The supernatant was incubated with gentle rotation with the resin for 2 h at 4°C; the resin was collected by centrifugation at 5,000 rpm for 5 min at 4°C. The resin was washed three times with 1× TBS (for GST-fused proteins) or HIS washing buffer containing 50 mM sodium phosphate (pH 7.5), 300 mM NaCl, and 8 mM imidazole. After removing the washing buffer, elution buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 25 mM reduced glutathione (for GST-fused proteins) or 150 mM imidazole (for His-fused proteins), and 10% (v/v) glycerol was added. Purified proteins were stored at ~80°C until use.

In Vitro ATP Binding Assay

The purified P2K2 extracellular domain protein was mixed in a 90 μL reaction including 10 mM HEPES (pH 7.5), 5 mM MgCl2, in the presence or absence of 100-fold unlabeled ATP (for the specific binding assay) or unlabeled other nucleotides (for the competitive binding assay), and γ32P-ATP (800 Ci mmol−1. PerkinElmer). The reactions were incubated at 4°C for 30 min. After that, the reactions were loaded onto Sephadex G-25 gel filtration columns (GE Healthcare).
The free nucleotides were trapped in the column, while the bound radioligand went through the column and was collected by scintillation vials. After mixing with scintillation cocktail (MP Biomedicals), the signal of bound radioligand was measured using liquid scintillation counting (Tri-Carb 2810TR, PerkinElmer). The data were analyzed using GraphPad Prism 7.

In Vitro Kinase Assay

The kinase assays were performed as described by Choi et al. (2014a), with minor modifications. For the kinase assay, 5 μg of GST or GST-fusion proteins were mixed, or not, with 2 μg MBP in kinase assay buffer (50 mM Tris-Cl [pH 7.5], 50 mM KCl, 10 mM MgCl₂, 4 μM ATP, 0.2 μL γ-32P-ATP [PerkinElmer; specific activity, 6,000 Ci mmol⁻¹] and incubated for 30 min at 30°C. After 10% SDS-PAGE, the gels were exposed for 3 h for autoradiography. After the kinase assay, samples were treated with 0.5 μL of Lambda PPase (NEB). Results were detected by 10% SDS-PAGE and Coomassie brilliant blue staining.

BIFC

Full-length genomic DNA of P2K2 from the pDONR-Zeo, described above, was cloned into pAM-PAT-35s:YFP-GW (for identification of P2K2 subcellular localization) and pam-PAT-35s:YPF (for BIFC assay) designation vectors. For the free YFP control, a 66-bp fragment of YFP was subcloned into the pDONR-Zeo vector and then cloned into pAM-PAT-35s:YPFGW, pam-PAT-35s:YPFmGW, or pam-PAT-35s:YPFe:GW. The full-length complementary DNA of P2K1 in pAM-PAT-35s:YPFGW was described in our previous study Chen et al. (2017). These constructs were transformed or cotransformed into Arabidopsis protoplasts. The protoplast isolation and transformation were performed as previously described (Cao et al., 2016). YFP fluorescence was observed under a Leica DM 5500B compound microscope using a Leica DFC290 color digital camera. FM4-64 dye (TS166, Invitrogen) was directly added into the W5 solution containing protoplasts for plasma membrane staining before visualization under the fluorescence microscope.

Split-Luciferase Complementation Assay

The vectors pCambia-NLuc and pCambia-Cluc were kindly provided by Dr. Jian-Min Zhou (Chen et al., 2008). EFR, P2K1 and P2K2 in pDONR-Zeo constructs were used for LR reactions to generate EFR, P2K1 and P2K2 in pCambia-NLuc and P2K2 in pCambia-Cluc. These constructs were electroporated into the A. tumefaciens GV3101. These constructs were co-infiltrated into 3-week-old Nicotiana benthamiana plants (Tri-Carb 2810TR, PerkinElmer) and incubated for 30 min at 30°C. After 10% SDS-PAGE, the gels were exposed for 3 h for autoradiography. After the kinase assay, samples were treated with 0.5 μL of Lambda PPase (NEB). Results were detected by 10% SDS-PAGE and Coomassie brilliant blue staining.

GST-HIS Pull-Down Assay

The LYK5 in pGEX-5X-1 was kindly provided by Dr. Dongqin Chen (Chen et al., 2017). The p2K1-1 kinase-dead and wild-type P2K1 kinase domain in pGEX-5X-1 was kindly provided by Dr. Jeongmin Choi (Choi et al., 2014a). The in vitro pull-down assay was performed as described by Chen et al. (2017), with minor modifications. In brief, 2 μg of HIS-fused and GST-fused proteins were mixed in 1 mL pull-down buffer containing 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 0.5% (v/v) Triton X-100, followed by incubation for 1 h at 4°C. After that, 25 μL glutathione resin was added and incubated for 1 h at 4°C. The resin was washed at least 10 times with pull-down buffer. The proteins were eluted using GST elution buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 25 μM reduced glutathione. The results were detected by SDS-PAGE, followed by immunoblotting using anti-HIS (SABI30538, Sigma-Aldrich) and anti-GST (A01380-40, GeneScript) antibody.

Bacterial Inoculation Assay

The assay was modified from the Arabidopsis seedling flood inoculation assay (Ishiga et al., 2011). In detail, 3-week-old Arabidopsis seedlings grown in square petri dishes were used for this assay. Forty milliliters of Pseudomonas syringae pv. tomato DC3000 Lux (OD600 = 0.002; Fan et al., 2008) bacterial suspension in sterile water with 0.025% (v/v) Silwet t-77 was dispensed into the dishes for 2 to 3 mins. After removing the bacterial suspension, the plants were incubated in a growth chamber. At 1 d postinoculation, the seedlings, without roots, were collected and the weight was determined, followed by washing with sterile water for 5 min. Bacterial growth was visualized and analyzed under a CCD camera (Photoet 216). The seedling tissue was ground in 10 mM MgCl₂, diluted serially, and dropped onto King B agar plates containing rifampicin and kanamycin. The number of colonies was counted and analyzed after incubation at room temperature for 2 d.

MAPK Assay

Ten-day-old Arabidopsis seedlings were incubated in sterile water at room temperature overnight. After treatment with 200 μM ATP, total protein was extracted from whole seedlings by homogenization in Radioimmunoprecipitation assay buffer (RIPA buffer) containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.1% (v/v) NP-40, 1 mM dithiothreitol, 0.5% (w/v) sodium deoxycholate, 2 mM PMSE, and 1× protein inhibitor (Pierce). The clear lysate was mixed with 5× Laemmli loading buffer (10% [w/v] SDS, 50% [v/v] glycerol, 0.01% [w/v] bromophenol blue, 10% [v/v] β-mercaptoethanol, 0.3 M Tris-HCl [pH 6.8]), and heated in boiling water for 10 min. The total extracted proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The blots were blocked by 5% (w/v) skim milk in TBS plus Tween 20 (TBST) buffer and then incubated with rabbit antiphospho-p44/p42 MAPK antibody (Cell Signaling Technology). After washing three times with TBST, the immunoblots were incubated with horseradish peroxidase-conjugated goat antirabbit IgG antibody (Jackson ImmunoResearch Laboratories). After washing three times with TBST, the final signal on immunoblots was visualized using a Fuji LAS3000 luminescence imaging system (Fujifilm).

RT-qPCR Assay

Five-day-old Arabidopsis seedlings were incubated in sterile water at room temperature overnight. After treatment with 200 μM ATP, samples were collected for total RNA extraction using Trizol reagent (Invitrogen). One microgram of total RNA was treated with Turbo DNA-free DNase (Ambion). The RNA was then used for complementary DNA synthesis using the M-MLV kit (Promega). SYBR Green and the 7500 Realtime PCR system (Applied Biosystems) was used to perform the qPCR with specific primer sets defined in Supplemental Table S1. RNA levels were normalized against the expression of the reference gene, SADI (At2g28390; Choi et al., 2014a).

Statistical Analyses

The average and SE of all results were calculated and one-way ANOVA and Student’s t tests were performed using IBM SPSS statistics version 25. The Kᵦ, Kᵦ, Kᵦ, and model goodness of fit (R²) values in vitro ATP binding assay were calculated using a one site – fit Kᵦ, nonlinear regression model, GraphPad Prism 6.

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource (https://www.arabidopsis.org/) under accession numbers At3g45330.1 (LeRK11); At5g34410.1 (LeRK9); At3g43090.1 (LeRK12); At5g54510.1 (LeRK5); At5g34260.1 (LeRK14); At5g43500.1 (LeRK5); At5g45440.1 (LeRK16); At3g50270.1 (LeRK7); At5g60280.1 (LeRK18); At5g60300.3 (LeRK19); and At5g20480.1 (EFR); At2g33580.1 (LYK5); and At2g28390.1 (SADI).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Molecular phylogenetic analysis of AtLeRK clade 1 members.

Supplemental Figure S2. Ectopic expression of other clade-1 lekKs in p2K1-3 mutant plants cannot restore the ability to response to eATP.

Supplemental Figure S3. P2K2 could play a partially redundant role with P2K1 during ATP-induced responses.
Supplemental Figure S4. Domain structure of P2K2.
Supplemental Figure S5. P2K2 kinase domains, wild-type, and mutant versions were treated with PPase.
Supplemental Figure S6. P2K2 localized to the plasma membrane in the root.
Supplemental Figure S7. Expression of P2K1 and P2K2 in mutant lines.
Supplemental Figure S8. ATP treatment triggered elevated expression of P2K2.
Supplemental Table S1. List of primers.

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