

Expression and phosphorylation analysis of soluble proteins and membrane-localised receptor-like kinases from *Arabidopsis thaliana* in *Escherichia coli*

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Abstract Molecular and functional characterization of proteins and their levels is of great interest in understanding the mechanism of diverse cellular processes. In this study, we report on the convenient *Escherichia coli*-based protein expression system that allows recombinant of soluble proteins expression and cytosolic domain of membrane-localised kinases, followed by the detection of autophosphorylation activity in protein kinases. This approach is applied to regulatory proteins of *Arabidopsis thaliana*, including 14-3-3, calmodulin, calcium-dependent protein kinase, TERMINAL FLOWER 1 (TFL1), FLOWERING LOCUS T (FT), receptor-like cytoplasmic kinase and cytoplasmic domain of leucine-rich repeat-receptor like kinase proteins. Our Western blot analysis which uses phospho-specific antibodies showed that five putative LRR-RLKs and two putative RLCKs have autophosphorylation activity *in vitro* on threonine and/or tyrosine residue(s), suggesting their potential role in signal transduction pathways. Our findings were also discussed in the broader context of recombinant expression and biochemical analysis of soluble and membrane-localised receptor kinases in microbial systems.

Keywords Calcium-dependent protein kinase, Leucine-rich repeat receptor-like kinase (LRR-RLKs), Receptor-like cytoplasmic kinases (RLCKs), Post-translational modification, *Arabidopsis thaliana*, *Escherichia coli*

Introduction

Plants, as sessile organisms, are adapted to cope with adverse

environmental conditions by employing complex signalling and control networks. Following stimuli perception by cells, signals are transmitted to downstream components by a variety of mechanisms, including activation of second messenger systems and post-translational modification, to elicit appropriate responses. Extensive research efforts over the past decades on functional analysis of various proteins have enriched our understanding of how plants respond to abiotic and biotic stresses, but many of their underlying mechanisms is still remain to be elucidated.

Protein phosphorylation is one of the most widespread post-translational modifications in both prokaryotes and eukaryotes. It is used as a regulatory signal by altering the conformational and dynamic state of the protein (Johnson and Barford, 1993). Phosphoproteome analysis of *Arabidopsis thaliana* revealed about 4000 phosphopeptides from all sub-cellular compartments, suggesting the involvement of protein phosphorylation in diverse cellular processes (Sugiyama et al. 2008; Reiland et al. 2009). In general, kinases and phosphatases regulate protein phosphorylation by reversibly catalysing the transfer of a phosphate group of ATP to hydroxyl groups of amino acid residues, predominantly on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues.

In eukaryotes, protein kinases are divided into three families: Tyr kinase, Ser/Thr kinase and Ser/Thr/Tyr kinase (also termed dual-specificity kinase) (Lindberg et al. 1992). Unlike animals, plants have no genes encoding classical Tyr kinase homologs, and only about 4% of plant phosphopeptides are Tyr-phosphorylated (Sugiyama et al. 2008; Rudrabhatla et al. 2006). Nevertheless, there is accumulating evidence that Tyr phosphorylation in plants contributes to the regulation of various pathways, including gibberellin response, brassinosteroid signalling, innate immunity and root nodule symbiosis (Macho et al. 2014; Lin et al. 2014; Oh et al. 2009; Nemoto et al. 2017; Saha et al. 2016). To date, several members of kinase, including

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calcium-dependent protein kinase (CPK), leucine-rich repeat (LRR)-receptor-like kinase (RLK), receptor-like cytoplasmic kinase (RLCK) and mitogen-activated protein kinase (MAPK), have been reported to be responsible for Tyr phosphorylation signalling in plants by acting as the dual-specificity kinase, and kinase themselves, 14-3-3 proteins and ubiquitin ligases have been found as target substrates (Rameneni et al. 2015; Nemoto et al. 2017; Saha et al. 2016; Matsuoka et al. 2018; Swatek et al. 2014; Oh et al. 2012).

In *Arabidopsis*, RLKs belong to a monophyletic gene family, containing over 600 members, which represent about 60% of all kinase proteins, and generally consist of an extracellular domain, a transmembrane domain and a cytoplasmic domain (CD) (Shiu and Bleeker, 2003). The group of LRR-RLKs is the largest RLK subfamily with more than 200 members and only found in plants (Shiu and Bleeker 2003; Hwang et al. 2011). LRR motif on the ectodomain mediates protein-protein interaction via its unique repeat sequences, allowing the perception of various external ligands, including hormone, pathogen molecules and small peptides (Kobe and Deisenhofer, 1994; Shiu et al. 2004). The CD is composed of a juxtamembrane domain, kinase domain and carboxyl-terminal domain. In BRI1, the LRR-RLK involved in brassinosteroid signalling, phosphorylation at the juxtamembrane domain and carboxy-terminal domain residues is required for oligomerization of RLKs and/or signal transduction to downstream components, indicating the regulation of RLKs as a multimeric complex (Wang et al. 2008; Wang et al. 2005). On the other hand, about 25% of RLKs, called RLCKs, carry no extracellular and transmembrane domains (Jurca et al. 2008). Many RLCKs, including BSK1, PBS1 and BIK1, are still closely associated with membrane-localised RLKs and play a role in intracellular signal transduction of plant development and immunity (Tang et al. 2008; Lu et al. 2010; Shao et al. 2003; Zhang et al. 2010).

Given the important role of phosphorylation in regulating cellular processes, sensitive and specific approaches for detecting protein phosphorylation are critical for understanding signal transduction pathways. Herein, we employed an *Escherichia coli* protein expression system to investigate signalling networks between substrates and receptor kinases in *Arabidopsis*. We successfully produced a soluble form of various regulatory proteins, including 14-3-3, calmodulin (CaM), CPK, TERMINAL FLOWER 1 (TFL1), FLOWERING LOCUS T (FT), the CD of LRR-RLK and RLCK proteins, some of which were further analysed by immunoblots with phospho-specific antibodies to determine autophosphorylation activities *in vitro*. We identified five putative LRR-RLKs and two putative RLCKs that might play a role in signal transduction through phosphorylation at

threonine and/or tyrosine residue(s). This approach is useful for not only the identification of specific post-translational modification but also for the characterization of the regulatory role at a given modification.

Materials and methods

Recombinant protein expression and immunopurification

To produce recombinant proteins, cDNA sequences of *Arabidopsis* 14-3-3s, CaMs, AtCPK4, FT, and TFL1 were cloned into the pET15b vector (His-tag) for soluble protein expression, and those of the CD of the LRR-RLKs and RLCKs were cloned into the Gateway-modified pFlag-Mac vector (Flag-tag). The resulting constructs were introduced into *E. coli* BL21 (DE3) cells (Novagen, Rockland, MA, USA), and the transformants were grown in LB broth with ampicillin at 50 µg/ml. When the cell culture reached the optical density (OD₆₀₀) 0.6, protein expression was induced with 300 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h at room temperature. Expression and purification of recombinant His- and Flag-tagged proteins was performed according to previously described methods (Chae et al. 2016). After elution of the recombinant proteins from beads, protein solutions were dialysed against a 1000× volume of buffer containing 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS) (pH 7.5) and 1 mM DTT, as previously described (Oh et al. 2000). After dialysis of purified recombinant proteins, protein concentration was measured by the Bradford assay.

Electrophoresis and Western blot analysis

All immunopurified recombinant proteins were prepared according to previously described methods (Oh et al. 2009; Oh et al. 2018). Polyvinylidene fluoride (PVDF) membranes were probed with anti-Flag, pThr polyclonal antibodies and pTyr (pY) monoclonal antibodies that recognise phosphorylated threonine and tyrosine residues. Alternatively, PVDF membranes were stained with ProQ Diamond reagent (Invitrogen, Grand Island, NY, USA). After incubation with specific primary antibodies for 6 h at room temperature, membranes were washed three times with PBST for 5 min each time and incubated with an Alexa Fluor 680-conjugated secondary antibody (Thermo Fisher Scientific, Rockford, IL, USA) diluted at 1:10,000 in PBST. Western blot hybridisation images were obtained using a LI-COR C-DIGIT scanner (LI-COR Biosciences, Lincoln, NE, USA).

Results and Discussion

In biochemical analysis of protein of interest, production of recombinant proteins should be proceeded to fully determining its kinetics and post-translational modifications. Since obtaining enough amounts of proteins in a functionally active form is critical, diverse expression platforms have been developed and studied. Among them, *E. coli* is one of the most popular expression platforms by offering several advantages: its culture reaches high cell density in relatively simple growth condition, and a number of vectors that allow for controlling the rate of protein expression have been well established (Rosano and Ceccarelli, 2014). In this study, using *E. coli* BL21 (DE3) strain and two different protein expression vectors, pET15b and pFlag-Mac, *A. thaliana* regulatory proteins, including cytosolic proteins (14-3-3s, CaMs, AtCPK4, TFL1 and FT) and cytoplasmic domain of membrane proteins (LRR-RLKs and RLCKs), were purified and analysed by Western blot to determine protein expression levels and post-translational modifications.

The 14-3-3 proteins are a family of highly conserved, soluble, acidic proteins found in all eukaryotes (Fu et al. 2000). They specifically bind to phosphoserine and/or phosphothreonine residues of target proteins and mediate diverse signalling pathways, including cell metabolism, hormone signalling, protein trafficking, abiotic stress tolerance and defence response (Yaffe and Smerdon, 2001). Among multiple isoforms of 14-3-3 proteins in Arabidopsis (Chae et al. 2016), we cloned cDNA sequence of kappa and lambda 14-3-3 isoforms into pET15b, which carries an N-terminal His-tag sequence, and introduced the expression vector into *E. coli* BL21(DE3) strain. His₆-tagged 14-3-3 proteins were immunopurified using Ni-NTA resin and detected by Coomassie Brilliant Blue staining, following separation by SDS-PAGE (Fig. 1A).

In plants, transient fluctuation of Ca²⁺ concentration inside cells is widely observed upon exposure to stress conditions, suggesting that Ca²⁺ plays an important role in cell signalling networks (Virdi et al. 2015). There are several known families of Ca²⁺ sensory proteins, including CaM and CPK. Ca²⁺ binds to CaM via EF-hand motifs and triggers conformational changes, leading to interaction with the target proteins (Babu et al. 1988; DeFalco et al. 2010). On the other hand, since CPK carries both EF-hand motif and kinase domain, it can directly respond to Ca²⁺ influx without interaction with CaM (Cheng et al. 2002). We selected two isoforms of CaM (CaM2 and CaM7) and one isoform of CPK (AtCPK4) in Arabidopsis for the production of recombinant proteins. Their cDNA sequences were cloned into the pET15b, expressed in *E. coli* BL21 (DE3) strain and purified using Ni-NTA resin. All the three His₆-tagged proteins were isolated in the soluble

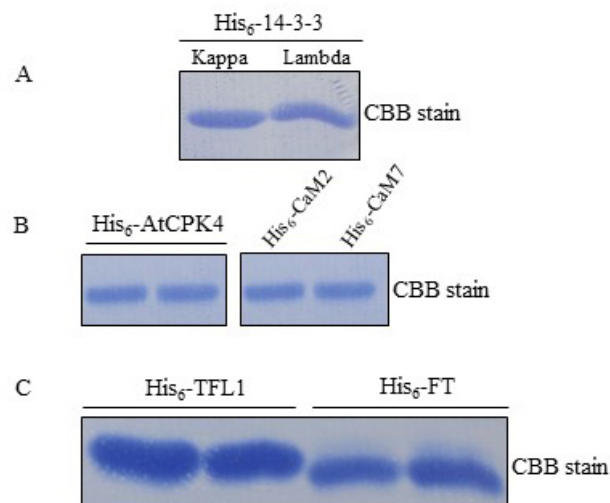


Fig. 1 Recombinant protein production using the *Escherichia coli* expression system. Coomassie Brilliant Blue (CBB) R-250 staining clearly showing the successful recombinant expression of (A) 14-3-3s, (B) AtCPK4, CaM2, CaM7, (C) TFL1 and FT. Protein expression induced by adding IPTG to the cell culture for 16 hours at room temperature. Total crude extracts were affinity purified using Ni-NTA resin, separated using 12% SDS-PAGE and stained with CBB

fraction and confirmed by SDS-PAGE (Fig. 1B).

FT and TFL1 proteins of Arabidopsis were also obtained successfully in a similar way using *E. coli* expression platform (Fig. 1C). Both of them are members of phosphatidylethanolamine-binding protein (PEBP) family that regulates the activity of diverse enzymes by functioning as a scaffold for the interaction between proteins (Pnueli et al. 2001; Schoentgen et al. 1987). Although FT and TFL1 share a similar amino acid sequence identity with about 60%, FT and TFL1 act antagonistically in the regulation of flowering-time genes (Hanzawa et al. 2005; Wickland and Hanzawa, 2015). However, much remains to be revealed about molecular mechanisms of not only FT/TFL1, but also 14-3-3, CaM and CPK. Isolating soluble forms of these proteins could serve as the first step to further elucidate the structural and functional properties.

In addition to cytosolic proteins, the CD expression of plasma membrane-localised LRR-RLKs was also tested in the *E. coli* system (Park et al. 2015). Plant LRR-RLKs are involved in the tight control of growth, morphogenesis, pathogen resistance and abiotic stress responses (Becraft, 2002; De Lorenzo et al. 2009; Tör et al. 2009; Torii et al. 2004). Among three domains of the CD, kinase domain is mainly responsible for phosphoryl transfer from ATP to amino acid residues or other protein substrates of downstream components. However, the carboxyl-terminus region of receptor kinases, carrying juxtamembrane domain and carboxyl-terminal domain, often plays an important regulatory role, which might be modulate

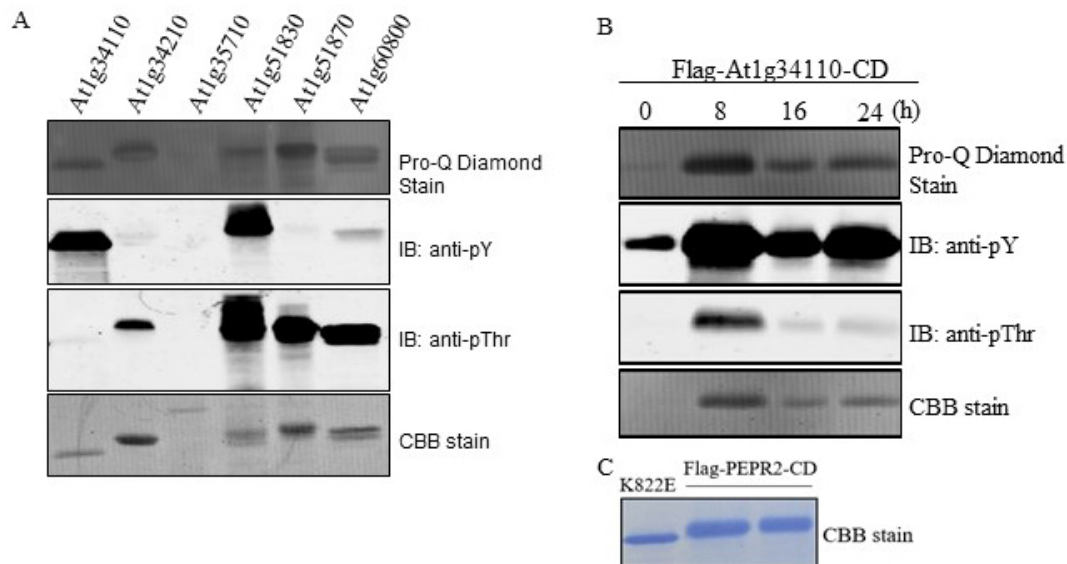


Fig. 2 Production and phospho-analysis of cytoplasmic domain (CD) of LRR-RLKs in *Escherichia coli* expression platform. (A) Analysis of CD of six different Flag-tagged LRR-RLKs (Atlg34110, Atlg34210, Atlg35710, Atlg51830, Atlg51870, Atlg60800) at single times following addition of IPTG (B) Analysis of Flag-Atlg34110-CD phosphoprotein production at different induction times after addition of IPTG. For immunoblot analysis, PVDF membranes were probed with phosphothreonine and phosphotyrosine-specific antibodies

by the phosphorylation of serine, threonine, and tyrosine residues (Oh et al. 2018; Bentem and Hirt, 2009).

In the present study, we cloned cDNA sequences of LRR-RLK CDs into the pFlag-Mac expression vector, introduced them into *E. coli* BL21 (DE3) strain and performed comparative biochemical assays. The phosphorylation levels of the expressed recombinant proteins were monitored at different time points after induction using anti-phosphothreonine (pThr) and anti-phosphotyrosine (pTyr) antibodies along with ProQ Diamond stain (Fig. 2A, B). Among six different LRR-RLKs tested, four LRR-RLKs (Atlg34210, Atlg51830, Atlg51870 and Atlg60800) were autophosphorylated on threonine residue(s) and three LRR-RLKs (Atlg34110, Atlg51830 and Atlg60800) were autophosphorylated on tyrosine residue(s). No phosphorylation on threonine and tyrosine residue(s) was observed in Atlg35710. Interestingly, Atlg51830 showed strong autophosphorylation on both threonine and tyrosine residue(s), suggesting the presence of dual-specificity kinase activity (Fig. 2A). Similar observations of autophosphorylation activity in *E. coli* have been also reported for CDs of Arabidopsis BRI1 and BAK1 (Oh et al. 2009; Oh et al. 2018). We also found that the level of Atlg34110 autophosphorylation on threonine and tyrosine residue(s) was changed at different induction times (Fig. 2B). In particular, phosphorylation at threonine residue(s) reached its peak intensity at 8 h after induction, but was barely detected at other time points. These autophosphorylation and transphosphorylation activities on tyrosine residues have been reported in many plant receptor

kinases, including PRK1, AtSERK1 and light-repressible kinases, suggesting that Atlg34110 receptor kinase might play an important role in cell signaling pathway as upstream component in *Arabidopsis thaliana*.

RLCK proteins, belonging to RLK superfamily, lack extracellular and transmembrane regions. Many studies have revealed that plant RLCKs play a central role in defense response by activating signal transduction between PRR complexes and MAPK (Couto and Zipfel 2016). In addition to kinase domain, RLCKs contain N- and C-terminal extensions possibly implicated in association with membrane-bound RLKs or for regulatory purposes via post-translational modification (Antolín-Llovera et al. 2012). Although few RLCKs, such as BSK1, PBS1 and BIK1, have been well-characterized, most RLCKs remain to be explored at the molecular level.

Here, we tested eight Arabidopsis RLCKs for autophosphorylation kinase activities *in vitro* using *E. coli* system (Fig. 3). We cloned full-length cDNA sequences of Arabidopsis RLCKs into the pFlag-Mac expression vector, introduced them into *E. coli* BL21 (DE3) strain and analyzed by immunoblots with phospho-specific antibodies. Among eight RLCKs tested, only two RLCKs (Atlg06700 and Atg13190) had distinct autophosphorylation activity in *E. coli*. Immunoblots of Atlg06700 showed autophosphorylation at tyrosine and threonine residue(s), indicating multi-specific kinase activity, while that of Atg13190 showed autophosphorylation only at threonine residue(s). Given that phosphorylation of RLKs generally initiates and mediates signal transduction pathways,

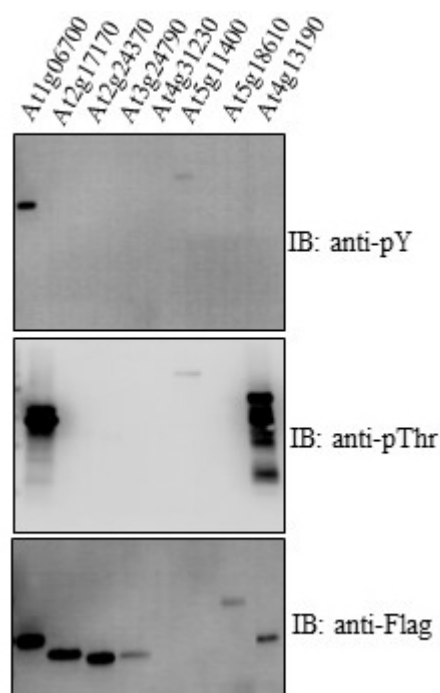


Fig. 3 Autophosphorylation of recombinant *Arabidopsis* RLCK proteins expressed in *Escherichia coli*. Anti-phosphothreonine and anti-phosphotyrosine immunoblots showing patterns of autophosphorylation. Not all RLCKs were capable of autophosphorylation following induction in *E. coli*. Autophosphorylation activity of recombinant proteins was monitored 16 hours after induction of receptor kinase expression. Each sample showed total crude proteins, including FLAG-tagged receptor kinase, extracted from *E. coli* and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to polyvinylidene difluoride (PVDF) membranes. Membranes were immunoblotted (IB) using the antibodies indicated

these two RLCKs might be also involved in sensing and responding to cellular needs in *Arabidopsis*.

Overall, our results demonstrated the successful expression and immunopurification of recombinant proteins combined with subsequent protein phosphorylation analysis using *E. coli in vitro* system. It is applicable for both soluble proteins and membrane-localised receptor kinases, and can be employed as a useful tool for studying molecular mechanisms and functions of protein of interest. Importantly, our assays for autophosphorylation of receptor kinases could be applied more broadly to a wide range of recombinant proteins, and provides a convenient and powerful system for elucidating kinase specificity *in vitro*.

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Conflicts of Interest

The authors declare no conflict of interest.

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