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Update

Protein kinase sensors: an overview of new designs for visualizing kinase dynamics in single plant cells

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Abstract

Protein kinase dynamics play key roles in regulation of cell differentiation, growth, development and in diverse cell signaling networks. Protein kinase sensors enable visualization of protein kinase activity in living cells and tissues in time and space. These sensors have therefore become important and powerful molecular tools for investigation of diverse kinase activities and can resolve long-standing and challenging biological questions. In the present Update, we review new advanced approaches for genetically encoded protein kinase biosensor designs developed in animal systems together with the basis of each biosensor's working principle and components. In addition, we review recent first examples of real time plant protein kinase activity biosensor development and application. We discuss how these sensors have helped to resolve how stomatal signal transduction in response to elevated CO_2 merges with abscisic acid signaling downstream of a resolved basal SnRK2 kinase activity in guard cells. Furthermore, recent advances, combined with the new strategies described in this Update, can help deepen the understanding of how signaling networks regulate unique functions and responses in distinct plant cell types and tissues and how different stimuli and signaling pathways can interact.

Background

Understanding intracellular signal transduction is a timely and important goal in diverse plant signaling pathways and networks (Taj et al., 2010; Moeder et al., 2019). In plants, protein kinases function in diverse processes including cell differentiation, cell growth, and development (Liu et al., 2017; Liang and Zhou, 2018; Wu et al., 2018). Furthermore, in abiotic and biotic stress responses, protein kinases play key roles in initiating plant responses through specific reversible protein phosphorylation of their substrate proteins. Relevant conformational transitions contribute to the functional state of active kinases, which are evolutionarily conserved and show structural plasticity (Johnson et al., 1996; Huse and Kuriyan, 2002; Nolen et al., 2004; Kornev et al., 2006; Endicott et al., 2012; Tong and Seeliger, 2015). In the present Update, we review major advances in the design principles of real-time live-cell protein kinase activity sensors developed in animal systems. In addition, we review recent first examples of development of real-time kinase sensors in plants and discuss how these sensors mechanistically resolve where the early CO_2 and abscisic acid signal transduction pathways merge in guard cells. Currently, *in-gel* kinase assays using radioactive isotopes including ^{32}P-ATP are the popular method for detecting protein kinase activity; these rely on copolymerization of a substrate protein or peptide within a sodium dodecyl sulfate (SDS)–polyacrylamide gel matrix

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ADVANCES

- Genetically encoded protein kinase biosensors allow high temporal and spatial resolution and provide insights into signaling dynamics and contribute to understanding signaling networks in plants.
- The latest and powerful protein kinase activity sensor designs were developed for animal systems and we discuss their potential for plant systems.
- MAPK and SnRK2 kinase biosensors were recently developed for plants and enable time-resolved monitoring of protein kinase activity in living single plants cells and tissues (Zaman et al., 2019; Seitz and Krysan, 2020; Zhang et al., 2020).
- Protein kinase biosensors help to address challenging plant signaling network questions, enabling the clarification of stomatal CO_2 signal transduction mechanisms (Zhang et al., 2020).

(Wooten, 2002; Wang and Zhu, 2016). This method has revealed important functions of diverse subfamilies of protein kinases in plant signal transduction research including mitogen-activated protein kinases (MAPKs; Zhang and Klessig, 1997; Asai et al., 2002; López-Bucio et al., 2018), sucrose nonfermenting-1-related protein kinases (SnRK2s; Mori and Muto, 1997; Li et al., 2000; Mustilli et al., 2002; Yoshida et al., 2002; Boudsocq et al., 2004; Fujii et al., 2007), and calcium-dependent protein kinases (Romeis et al., 2000; Boudsocq et al., 2010; Brandt et al., 2015). However, since *in-gel* kinase assays detect protein kinase activity after proteins are separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), this method may not be applicable to determining activity changes for all protein kinases because this method relies on SDS-tolerating modifications of protein kinases of interest, such as phosphorylation. Furthermore, cell extraction or protein kinase immunoprecipitation procedures disrupt cellular integrity, including subcellular localizations of proteins and protein complex formations, which could result in loss of *in vivo* information. In addition, for cell type-specific analyses, large numbers of purified cells are required for *in-gel* kinase assays. Guard cell protoplasts have been used for biochemical analyses of protein phosphorylation. However, biochemical experiments for stomatal signaling research require many highly purified protoplasts from many fresh healthy plants. For example, a single *in-gel* kinase assay lane requires isolation of 100,000 purified guard cells from more than 100 rosette leaves in Arabidopsis (*Arabidopsis thaliana*; Mustilli et al., 2002; Takahashi et al., 2017). Application of biosensor technology may help overcome these drawbacks by reporting time-resolved changes in protein kinase activation states in single living cells; biosensors may also help monitor kinases that are present in very small amounts in cells that nevertheless mediate important cellular functions. Protein kinase biosensors provide rapid, sensitive, quantifiable, and locally visible real-time responses *in vivo*.

A brief history of protein kinase activity reporters

For over 20 years, several types of protein kinase biosensors have been reported and designed; these have constantly broken down barriers in understanding biological signaling pathways and answered important biological questions in animal systems. At first, a fluorescence probe for cyclic AMP (cAMP) that monitors the dissociation of the catalytic and regulatory subunits of protein kinase A when the kinase is activated was developed (Adams et al., 1991). The fluorescence probe was made up of two separately purified subunits connected to the fluorophores, such as rhodamine and fluorescein, respectively. cAMP caused a change in energy transfer efficiency, providing firsthand evidence of where and when cAMP was produced inside living cells. After early development of genetically encoded reporters for cAMP (Adams et al., 1991; Zaccolo et al., 2000), the first genetically encoded biosensor that reports protein kinase A (PKA) activity, A-kinase activity reporter (AKAR), was designed (Zhang et al., 2001). This AKAR sensor contains the PKA substrate domain and a shortened 14-3-3 protein (14-3-3τ), inducing Förster resonance energy transfer (FRET) through conformational changes between enhanced cyan fluorescent protein (ECFP) and yellow fluorescent protein (YFP) (Figure 1A). In parallel, in the same year, the same laboratory of Roger Tsien designed and reported three additional biosensors for monitoring the activities of Sarcoma-related (Src), epidermal growth factor receptor (EGFR), and Abelson (Abl) protein kinases with the same conformational change principle (Ting et al., 2001).

Since then, many biosensors based on those first prototypes have been developed for animal protein kinases through modifications in the phosphorylation recognition domain and substrate domain. Furthermore, a wide range of biological questions in animal systems and advances in the field are reflected upon in several recent reviews on genetically encoded biosensors (e.g. Mehta and Zhang, 2011; Miyawaki, 2011; Newman et al., 2011; Zhou et al., 2020; Yoshinari et al., 2021). One of the main reasons for the apparent delay in developing such protein kinase activity sensors in plants has been the very limited knowledge of specific well-defined phosphorylation sites targeted uniquely by specific (clades of) plant protein kinases. In addition, the relatively unusual large expansion and number of protein kinase family members encoded in plant genomes can increase the difficulty of developing isoform specific plant protein kinase biosensors.

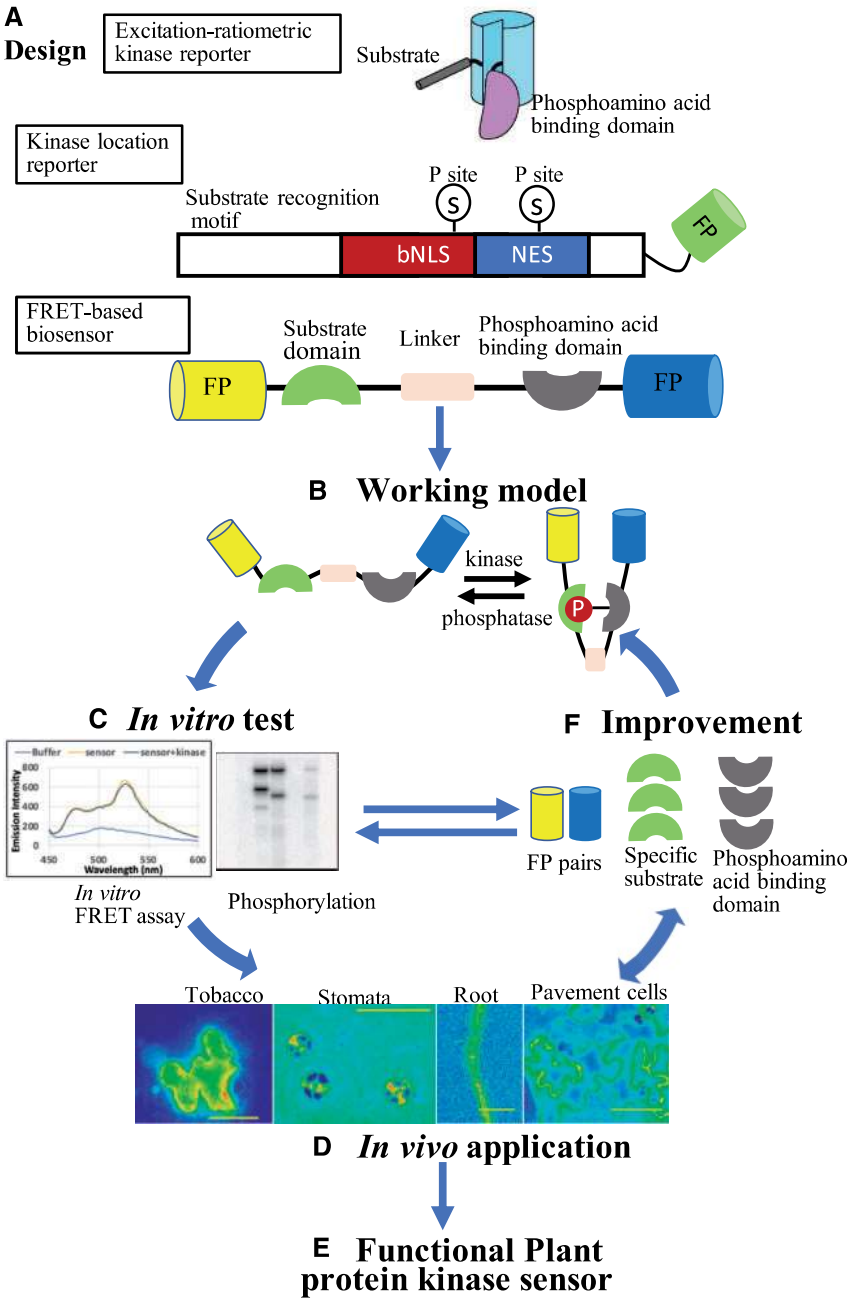

Figure 1 Illustration of the design of genetically encoded protein kinase biosensors (FRET-biosensor as example). A, The design of biosensors mentioned in this Update. (Top) excitation–ratiometric kinase reporter contains a cpGFP (blue), substrate domain (gray), and phosphoamino acid binding domain (purple). (Middle) KLR contains substrate recognition motif, bNLS, NES, and FP. "S" indicates phosphorylation sites. Lower part: FRET-based biosensor contains two FPs, substrate domain, phosphoamino acid binding domain and linker domain. B, Working model (FRET-based biosensor as example). Protein phosphorylation-dependent molecular switch induces FRET efficiency changes between the two FPs. C, Initial *in vitro* testing of the new biosensors, usually by *in vitro* emission intensity analyses in response to protein kinases, and/or in vitro phosphorylation assays investigating biosensor phosphorylation levels as a function of active protein kinase. D, Testing biosensors in plant cells. Tobacco or *N. benthamiana* epidermal cells, Arabidopsis cells (guard cells, roots, and pavement cells), scale bar = 50 μm. Note that pseudocolored images would have distinct scales each and have not been included for these examples. E, Functional Plant protein kinase biosensors can be used for single cell resolution and real-time investigations of kinase activity changes and analyses of spatiotemporal regulation, or crosstalk among signaling pathways. F, Improvement of biosensors may involve changing the FP pairs, linker domains, specified substrate domains, or changing phosphoamino acid binding domains that recognize the phosphorylation sites.

Presently developed and applied protein kinase sensor approaches

Briefly, here we discuss recent advances in the development of two major types of fluorescent biosensors: (1) FRET-based biosensors, where phosphorylation causes conformational changes between two fluorescent proteins (FPs) and (2) single FP biosensors. In animal cells, genetically encoded FRET-based biosensors are the most commonly used biosensors (Depry and Zhang, 2010; Mehta and Zhang, 2011). FRET-based biosensors for several types of protein kinases have been designed and applied to study several protein kinases in animal cells, including Src (Ting et al., 2001; Su et al., 2013; Mo et al., 2020), Akt (Ser/Thr kinase AKT, also named protein kinase B; Sasaki et al., 2003; Gao and Zhang, 2008; Belal et al., 2014; Zhou et al., 2015; Mo et al., 2020), Abl (Ting et al., 2001), AMP-activated protein kinase (AMPK; Depry et al., 2015; Konagaya et al., 2017; Pelosse et al., 2019), EGFR (Ting et al., 2001; Nakajima et al., 2008), c-Jun N-terminal kinase (JNK; Bakal et al., 2008; Shcherbakova et al., 2018), MAPK (Timm et al., 2011), and other kinases (Newman et al., 2011; Zhou et al., 2015). These protein kinase biosensors have been used to address cellular biological questions in specific signal transduction pathways.

A sensitive and specific biosensor can be developed by choosing unique sensing and appropriate reporting units. In the case of FRET-based biosensors, three main aspects of improvements were made by using more suitable reversible phosphoamino acid binding domains, by changing the fluorophore coupling and/or by switching the positions of FPs. For example, an ERK kinase activity reporter (KAR), EKAR4, was developed with swapped positions of FPs (YPet and ECFP) within the EKAR-EV (Komatsu et al., 2011; Keyes et al., 2020). The ERK biosensor EKAR4 has YPet at the C-terminal end and ECFP at the N-terminal end, which shows a substantially improved dynamic range in HEK-293 cells (Harvey et al., 2008; Komatsu et al., 2011; Keyes et al., 2020).

AKAR, as the first genetically encoded A-kinase activity reporter, demonstrated protein kinase A activity in live cells (Zhang et al., 2001), which provided a meaningful advance for protein kinase A research and protein kinase biosensor development (Zhang et al., 2001). However, the first AKAR version was irreversible and thus incompatible for continuous monitoring of protein kinase A activity reductions, probably because of the strong binding between the 14-3-3 protein domain and the phosphorylated substrate domain. To overcome this problem, a forkhead-associated (FHA) domain was used instead of the 14-3-3 protein domain in the next PKA reporter generation, named AKAR2. AKAR2 showed a reversible behavior (Zhang et al., 2005). Other domains have also been used as the phosphoamino acid binding domain in FRET-based biosensors (see below).

In parallel, researchers made efforts to improve FPs. Recently, a single-residue mutant D159V of the red FP TagRFP-T (Merzlyak et al., 2007) was developed, named super-TagRFP ("stagRFP"), which shows increased red fluorescence intensity and supports red/far-red FRET biosensing (Mo et al., 2020). By combining this stagRFP with other FRET partners, the multiplex coimaging approach was developed to comonitor Src, Akt, and ERK activities *in vivo* in the same living cells, providing an approach for elucidating complex signaling pathways (Mo et al., 2020).

In contrast to FRET-based biosensors, kinase translocation reporters (KTRs) have an entirely different design with FPs, in which phosphorylation by protein kinases causes a change in the cellular location of the reporter protein (Regot et al., 2014; Kudo et al., 2018; Figure 1A). Designing KTRs is based on the principle of phosphorylation-dependent regulation of a nuclear location signal (NLS) and/or nuclear export signal (NES) neighboring the phosphorylation sites. Thus, phosphorylation or dephosphorylation of these substrates would change the recognition status of NLS and NES motifs, resulting in phosphorylation-induced intracellular translocation. For example, prior to phosphorylation, KTR enters the nucleus due to its NLS. Following phosphorylation, the NES experiences a structural shift and expresses its activity, leading to cytoplasmic localization. As a result, phosphorylation-based translocation reporters can report protein kinase activity as a change in fluorescence intensities in the nucleus and cytoplasm. In recent years, KTRs have been developed and used for simultaneous measurements of multiple types of kinases (CDK2, JNK, p38, p53, ERK, NFAT1, NFAT4, and NFκβ) in individual live cells (Nelson et al., 2004; Tay et al., 2010; Spencer et al., 2013; Yissachar et al., 2013; Regot et al., 2014).

Furthermore, another strategy for developing protein kinase reporters with new principles was reported using single FPs that have very large dynamic ranges for reporting kinase activation. Recently, a backbone for single-fluorophore kinase activity reporters (KARs) was developed. Making use of this backbone, a suite of various colors of KARs enabled the development of biosensors for visualizing dynamics of protein kinase activities (Mehta et al., 2018). An "excitation ratiometric A kinase activity reporter," named ExRai-AKAR, is phosphorylated by PKA, which induces a conformational change modulating the fluorescence excitation spectrum of circularly permuted GFP (cpGFP) between the PKA substrate and an FHA1 domain (Mehta et al., 2018; Figure 1A). In KAR sensors, the maximum excitation peak of cpGFP shifts from ~400 to ~480 nm. The ratio of the fluorescence intensities at these two excitation wavelengths provides a readout for kinase activity. Within these newly developed KAR reporters, an "ExRai-AKAR" reporter showed a large dynamic kinase activity reporting range, and was thereby used to simultaneously track multiple distinct protein kinase activities in single living cells (Mehta et al., 2018). Subsequently, the same group further improved a second-generation reporter (ExRai-AKAR2) by the optimization of the linker sequence directly preceding and following cpEGFP. This

ExRai-AKAR2 reporter with the highest sensitivity under different experimental modalities provided another strong tool for elucidating the *in vivo* real-time analysis of PKA activity (Zhang et al., 2021).

Plant kinase sensors

Recently, a few studies have reported the development of plant protein kinase biosensors, showing their feasibility or providing templates for the design of specific sensors for the diverse classes of protein kinases found in plants. Plant scientists have often used *in-gel* kinase assays from extracts of seedlings, leaves, isolated protoplasts, and other tissue samples to monitor the activities of specific protein kinases, which have provided important experimental evidence for the roles of protein kinases involved in diverse pathways (Mori and Muto, 1997; Mustilli et al., 2002; Yoshida et al., 2002; Boudsocq et al., 2004; Yoshida et al., 2006). *In vitro* kinase assays using immunoprecipitated protein complexes with an artificial or native substrate protein have been a common method to investigate protein kinase activities in plant cells. This method may be more broadly applicable to protein kinases than *in-gel* kinase assays of extracts since protein kinase activities are assessed without protein denaturation by SDS. However, the major limitation of classical kinase assays is the inability to monitor the protein kinase activity with spatiotemporal resolution in single cells to elucidate dynamic events occurring in living cells. To overcome this limitation, genetically encoded biosensors have valuable advantages for investigating real-time protein kinase activity changes in living plant cells.

Development and applications of protein kinase biosensors in plants

Krysan and colleagues developed the first genetically encoded plant protein kinase sensor that reports MAPK activity in *A. thaliana* (Zaman et al., 2019). Since the Arabidopsis genome includes 20 *MAPK* genes that may have different and overlapping targets and physiological roles, it is a challenge to develop specific MAPK biosensors. Nevertheless, by monitoring MAP kinase activities in single-cell types and in *mapk* mutants, focused functional analyses can be pursued. Based on the previously established mammalian kinase activity sensor for MAPK, the substrate domain was replaced with an 80-amino acid Arabidopsis MKP1 (MAP kinase phosphatase) fragment (Ulm et al., 2001; Komatsu et al., 2011). This newly developed plant MAPK sensor was named sensor of MAP kinase activity (SOMA; Zaman et al., 2019). SOMA is composed of two FPs (YPet and Turquoise GL), a FHA1 phosphopeptide-binding domain 1 (FHA1), an 80 amino acid Arabidopsis MKP1 substrate domain and a 244 amino acid version of an extension for enhanced visualization linker domain (Komatsu et al., 2011; Zaman et al., 2019; Figure 1A). SOMA follows the substrate-based FRET kinase biosensor working principle: in the presence of active MAPK kinase, the MKP1 substrate domain of SOMA becomes phosphorylated, triggering an enhanced affinity of FHA1 for the substrate domain. Binding of FHA1 to the substrate domain then induces a conformational change of SOMA that brings both fluorophores "closer" and increases FRET efficiency between YPet and Turquoise GL (Figure 1, A and B; Komatsu et al., 2011; Zaman et al., 2019). The SOMA sensor was found to show reversible properties, suggesting that unknown protein phosphatases could remove the phosphate group from SOMA, which would cause the sensor to return to the lower energy transfer state. Therefore, the steady-state FRET level of SOMA would depend on MAPK activity and on reversal of SOMA phosphorylation via the activity of the unknown phosphatases. SOMA serves as a readout of MAPK activity and can be applied to plant signaling research involving MAPKs.

Live-cell imaging was pursued for monitoring MAPK activity using confocal microscopy of detached 5-day-old Arabidopsis cotyledons expressing SOMA. The mounted transgenic Arabidopsis cotyledons were placed abaxial side down in the imaging chamber and then treated with several stimuli. Using this system, several stimuli (NaCl, flg22, and chitin) all induced increases in the SOMA FRET ratio (YPet/Turquoise GL) in living plant cells. In contrast, transgenic Arabidopsis lines expressing a $SOMA^{T679A}$ mutant, in which the MAPK-dependent phosphorylation site was mutated, did not show any measurable changes in FRET ratio upon these treatments (Zaman et al., 2019). These findings demonstrated that the stress-induced gains in FRET ratio depend on the known MAPK phosphorylation site in SOMA. In addition, in a *mpk3 mpk6* P_{mpk6}*: MPK6*^{YG} genetic background (conditional loss-of-function double mutant lines in MPK3 and MPK6, in which $MPK6^{YG}$ kinase activity can be inhibited by the selective inhibitor NA-PP1; Xu et al., 2014), SOMA showed no NaCl-induced FRET gain, indicating that MPK3 and 6 are essential for the FRET increase of SOMA upon NaCl treatment. The design and application of SOMA are an exciting proof of concept and provide a quantum leap for investigating MAP kinase activity changes in live plant cells. It will be of interest to determine the extent to which SOMA can be used to study additional signaling pathways in different cell types and tissue systems. Such future work has the potential to reveal new aspects of MAPK signaling that may only be observable using living-cell imaging approaches.

Based on this first version MAPK fluorescent biosensor (SOMA), recently, Krysan and colleagues reported the development of an additional sensitive and specific MAPK biosensor in plants (Seitz and Krysan, 2020). Since SOMA mainly acts as MAPK activity biosensor for MPK3, MPK6, and possibly MPK4, additional plant-specific MAPK biosensors are needed to report the MAPK activities of different single isoforms or subclades, given that plant genomes encoded many MAP kinases (e.g. 20 isoforms are encoded in the Arabidopsis genome). Considering the potential

difficulties in designing plant biosensors, Krysan and colleagues developed an approach that would simplify and promote the procedure of creating MAPK biosensors for plants (Seitz and Krysan, 2020). Candidate protein kinase substrate domains are the most important and limiting components for the development of single protein kinase isoform biosensors, which contain both phosphoacceptor sites and a kinase docking region. To improve the efficiency of developing plant MAPK biosensors, and making use of the mammalian MAPK FRET biosensor (EKAREV; Harvey et al., 2008; Komatsu et al., 2011), a vector was generated by replacing the original docking domain, which can serve as a tool for highly efficient screening for the docking domains of plant MAPKs. In brief, the original EKAREV contains two domains (a 14 amino acid Cdc25C domain and an ERK recognition docking domain; Harvey et al., 2008; Komatsu et al., 2011; Seitz and Krysan, 2020). Next, replacing the docking domain with AP2C1 (a plant verified docking domain) within the EKAREV, the vector was used to report FRET ratio increases in response to coincubation with plant MAPKs *in vitro* (Shubchynskyy et al., 2017). Therefore, this method could be used to quickly screen for functional docking domains of Arabidopsis MAPKs and also produce MAPK biosensors at the same time (Seitz and Krysan, 2020). This strategy also could be applied to other protein kinases in plants, which might further advance protein kinase activity sensor development. In addition, these advances could broaden our knowledge of the roles of MAPKs in a wide range of plant signaling pathways affecting growth, development, and defense.

In additional research, a plant nucleus/cytoplasm translocation biosensor has also been created to investigate MAPK activity in planta. Based on the nuclear KTR (nKTR) backbone (de la Cova et al., 2017), a kinase location reporter (KLR) was designed and was shown to function in Arabidopsis (Seitz and Krysan, 2020). In *Caenorhabditis elegans*, nKTR contains both bNLS and NES, and follows the equilibrium of the competition between bNLS and NES (de la Cova et al., 2017). The nonphosphorylated biosensor localizes to the nucleus, and this biosensor localizes from the nucleus to the cytoplasm upon phosphorylation. In order to develop a plant translocation biosensor, nKTR was modified by removing the Elk1 docking domain, replacing an mClover fluorophore with an mNeonGreen, together with codon-optimization for plant systems, generating KLR in plants (Lam et al., 2012; Shaner et al., 2013; Seitz and Krysan, 2020). In Arabidopsis cells, KLR has been reported to locate to or be excluded from the nucleus, depending on phosphorylation, by mutagenizing the serine phosphorylation sites of the bNLS domain (Seitz and Krysan, 2020). Furthermore, insertions of the MAPK docking domains (MKP1 and AP2C1) into KLR generated KLR-MKP1 and KLR-AP2C1, which showed decreased stress treatment-induced fluorescence emission intensity in nuclei (Seitz and Krysan, 2020). Taken together, the approach for screening for effective docking domains of plant MAPKs and the recently reported translocation-based kinase biosensors could broaden the types of genetically encoded biosensors currently available in plants and could provide tools for the rapid creation of other biosensors.

SnRK2 activity biosensor

The SnRK2 family functions in plant stress responses. The Arabidopsis genome contains 10 members of SnRK2s genes (Boudsocq et al., 2004). The plant hormone abscisic acid rapidly activates SnRK2 kinases and plays a role in plant drought stress tolerance (Mustilli et al., 2002; Belin et al., 2006; Yoshida et al., 2006). Our group used the SOMA backbone to generate a genetically encoded biosensor for monitoring SnRK2 activation in plant cells (Zhang et al., 2020; Figure 1A). As a substrate domain that is specifically phosphorylated by SnRK2, we chose thebasic helix-loop-helix (bHLH) transcription factor ABA-RESPONSIVE KINASE SUBSTRATE1 (AKS1), which was identified to be phosphorylated by SnRK2s upon ABA exposure of guard cells (Takahashi et al., 2013, 2016). For a phosphoamino acid binding domain, we investigated the Arabidopsis 14-3-3PHI protein. AKS1 binds to 14-3-3PHI protein in a phosphorylation-dependent manner (Takahashi et al., 2013). Since the N-terminal phosphorylation sites in AKS1, Ser-30, and Ser-157 were identified as 14-3-3 binding sites (Takahashi et al., 2013, 2016), the AKS1 N-terminal region, including Ser-30, was used. This SnRK2 activity sensor (SNACS) exhibits an increased fluorescence intensity ratio (YPet to Turquoise GL) *in vitro* after the incubation with active recombinant OST1/SnRK2.6 protein, due to phosphorylation-dependent changes in FRET efficiency. In addition, ABA triggered a rapid gain in the FRET ratio in plant cells (*Nicotiana benthamiana* epidermal cells and Arabidopsis stomatal guard cells), providing the *in vivo* evidence. In negative control experiments, mutation of the SnRK2 targeted phosphorylation site of AKS1 (Ser30) disrupted the ABA-induced FRET ratio increases. In addition, a protein kinase inhibitor K252a inhibited the SNACS FRET ratio increase upon ABA treatment in Arabidopsis stomatal guard cells (Zhang et al., 2020). Furthermore, K252a decreased FRET efficiency even before abscisic acid administration, supporting the biologically relevant hypothesis of the existence of the basal SnRK2 kinase activity in stomatal guard cells (Hsu et al., 2018; Zhang et al., 2020). As other protein kinases likely have basal activities as well, kinase biosensors could shed light on real time basal activities and changes in live plant cells.

The SNACS biosensor was further applied to address recently debated stomatal signaling questions, while enabling enhanced time-resolved single cell resolution. Abscisic acid caused rapid time-resolved increases in the FRET efficiency of SNACS, showing time-dependent increases over the first 5 min after ABA application, as expected. However, both elevated CO_2 and methyljasmonate did not show any clear increases in SNACS-

mediated FRET signals (Zhang et al., 2020). Furthermore, as mentioned earlier, SNACS imaging provided an evidence for a basal activity of SnRK2 in guard cells, which itself is required for the stomatal response to CO_2 elevation (Xue et al., 2011; Zhang et al., 2020). Recent findings using SNACS enable reconciliation of diverse findings from previous studies, supporting a recent model in which a low basal SnRK2 kinase activity and basal ABA levels in guard cells are required for, or amplify, stomatal response to other non-ABA stimuli(Hsu et al., 2021; Zhang et al., 2020). Furthermore, ABA receptor quintuple and hextuple mutants that disrupt stomatal ABA responses continued to show high CO_2-induced stomatal closing, further highlighting that the CO_2 signaling pathway functions in parallel to ABA receptor signaling (Zhang et al., 2020).

These data show that SNACS provides a tool that allows dynamic monitoring of SnRK2 activation in real time in response to biological stimuli in living plant cells (Zhang et al., 2020). SNACS was further used to address stomatal signaling questions in Arabidopsis (discussed above). Future analyses of subcellular localization of SnRK2 activities, by targeting SNACS to specific cellular compartments or other tissues and cell types, would be of interest.

OUTSTANDING QUESTIONS

- How do genetically encoded protein kinase biosensors respond to endogenous protein phosphatases in plant cells?
- Protein kinase activities may exhibit distinct responses depending on the intracellular location of the kinase, the stimulus, and/or cell or tissue type; will plant kinase biosensors resolve single cell protein kinase activity changes to dissect intracellular heterogeneity of kinase signaling?
- Can plant kinase sensors be used to compare whole plant samples and single cells in time-course experiments?
- New powerful approaches discussed in this Update demonstrate how to achieve coimaging with multiple protein kinase biosensors in the same living single plant cells and tissues; what are the most important plant cell signaling networks to benefit from these characterizations?

Challenges for future plant kinase sensors

Protein kinase biosensors can provide advanced tools to investigate the dynamics of kinase activity changes in living single cells. However, there are some challenges for developing sensitive and specific protein kinase biosensors in plants. First, protein kinases normally belong to large gene families and share similar domains and functions with other family members. For example, the MAPK family contains 20 members in Arabidopsis, and closely related MPK members are likely to share identical substrates. Interestingly, SOMA seems to respond mainly to MPK3 and MPK6 (Zaman et al., 2019). Therefore, the choice of a specific substrate sequence targeted by single kinase isoforms is a crucial step for biosensor design. Alternatively, a less specific but kinase class-preferred substrate could provide information, together with kinase mutant analyses, on signal transduction mediated by subsets of protein kinases.

In addition, as mentioned above, the phosphoamino acid binding domain, which recognizes the phosphorylated residues and binds to the phosphorylated substrate domain, is also a key component in FRET-based kinase biosensors (Yaffe and Elia, 2001; Yaffe and Smerdon, 2001; Schlessinger and Lemmon, 2003). In the FRET-based PKA biosensor (AKAR1), PKA-mediated phosphorylation could promote the binding between 14-3-3τ and the substrate domain (Ting et al., 2001; Zhang et al., 2001). However, in this version, as mentioned above, the 14-3-3τ protein was found to maintain a high affinity for its substrate, which resulted in difficulty in reversing the sensor by dephosphorylation. Subsequently, the FHA domain was used in the following generations (AKAR2, AKAR3, and AKAR4) working as reversible FRET biosensors due to the lower binding affinity (Durocher et al., 2000; Allen and Zhang, 2006; Depry et al., 2011). Furthermore, in an ERK biosensor, both the FHA domain and WW domain were used (Allen and Zhang, 2006; Keyes et al., 2020). The first ERK biosensor, named Erkus, used an FHA domain (Sato et al., 2007). Subsequently, another ERK biosensor, EKAR, was designed using the WW domain (Harvey et al., 2008). In addition, the recent EKAR3 and EKAR4 used FHA and WW domains, separately (Allen and Zhang, 2006; Keyes et al., 2020).

Studies to date have shown that in the absence of stimulation, most kinase activities do not show rapid and spontaneous transient changes on the time scale of seconds. These observations indicate that kinase activities may not have a very large range in noisy basal activity, due to tight regulation within cellular networks (Ubersax and Ferrell, 2007). Many of the presently available protein kinase sensors show relatively small FRET ratio shifts (e.g. $<8\%$) upon stimulation (Sato et al., 2007; Tóth et al., 2018; Kajimoto et al., 2019). For these reasons, presently, most of the genetically engineered kinase reporters are mainly suitable for detecting stimulus or conditional changes in kinase activities during continuous real-time imaging of cells. Comparing single time point FRET ratios between different cells would require kinase sensors with larger dynamic ranges in their readouts and careful calibration experiments. The strength of kinase biosensors lies in single cell time-resolved resolution in responses to diverse stimuli of interest, which enables in depth analyses of diverse open questions.

Once a biosensor has been successfully designed and developed, and shows resolvable changes in optical properties

in response to a signal of interest, the next important step could be to test whether the sensor readout is specific to the signal or not. Responses to appropriate controls can be tested (Niwa et al., 1991; Pouvreau, 2015). As a negative control, a "dead"(nonresponsive) biosensor, in which no phosphorylation-dependent conformational change would be predicted to occur (produced by introducing a mutation at the kinase-targeted phosphorylation site in the substrate domain), is considered important (Zaman et al., 2019; Zhang et al., 2020). On the other hand, it could also be helpful to stimulate cells expressing the kinase biosensor in the presence of protein kinase inhibitors to test for phosphorylation-dependent conformation changes (Eisler et al., 2012). From a physiological perspective, plants expressing kinase sensors could be examined by testing whether these plants show normal physiological responses similar to wild-type control plants. Furthermore, blind assays, in which stimuli or mock stimuli and/or genotypes are blinded are recommended for such biosensors (Zhang et al., 2020). In Figure 1, we further provide a flow chart as guidance for protein kinase biosensor design and testing applications in plants (FRET-based biosensor as an example; Figure 1).

Concluding remarks

Currently, although only a few protein kinase activity biosensors have been reported in plants, these kinase biosensors have provided important insights into dynamic regulation and functions of signaling pathways that are associated with complex signaling networks. Furthermore, these first-generation kinase sensors provide valuable tools for time-resolved analyses of MAP kinase and SnRK2 kinase regulation in plant systems with single cell, time-resolved resolution. In this Update, we provide an overview of important aspects of the history, unique strategies, and the most recent advances in protein kinase activity sensor design developed in animal systems. The newest approaches for protein kinase biosensor design are reviewed here; these could be used for developing kinase activity sensors with spatiotemporal resolution in single plant cells, thus illuminating signaling network interactions (see Outstanding Questions). In addition, we present an overview of plant kinase reporter development and applications in the cases of MAPK and SnRK2 kinase biosensors. SOMA is the first protein kinase activity biosensor to have been developed for *in planta* visualization of MAPK activity (Zaman et al., 2019). In addition, two nucleus/cytoplasm translocation kinase biosensors (KLR-MKP1 and KLR-AP2C1) were developed and have been shown to respond to stress treatments in single plant cell analyses (Seitz and Krysan, 2020). In addition, a SnRK2 biosensor, SNACS, was developed to visualize single plant cell SnRK2 kinase regulation dynamics, and applied to clarify how the CO_2 signaling pathway merges with the abscisic acid signaling pathway downstream of basal SnRK2 kinase activity (Zhang et al., 2020). These advances, combined with the recent strategies for biosensor development described here, have strong potential for design of kinase sensors for diverse classes of protein kinases in plants, which can revolutionize our understanding of how signaling networks regulate unique functions and responses in distinct plant cell types and tissues and of how different stimuli and signaling pathways can interact. Taken together, the development and the application of genetically encoded protein kinase biosensors have much future potential.

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