

# DNA-encoded probe-based assay for profiling plant kinase activities

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## Abstract

Elucidating kinase–substrate relationships is pivotal for deciphering cellular signaling mechanisms, yet it remains challenging due to the complexity of kinase networks. Herein, we report the development of a versatile DNA-based kinase assay platform for high-throughput profiling of plant protein kinase activities and substrate preferences. Our approach employs DNA-linked peptide substrates, facilitating quantitative and specific kinase activity detection through next-generation DNA sequencing. Leveraging DNA barcodes as quantitative readouts, our approach establishes a high-throughput, sensitive, and specific platform for dissecting kinase–substrate networks in plants, representing a powerful tool for elucidating signaling mechanisms in plants.

**Keywords:** DNA-linked probe, qPCR, NGS, and kinase activity

## Introduction

Protein kinases play a crucial role in cellular growth and development in plants. Impaired kinase function frequently disrupts normal cellular processes, manifesting as growth defects and improper stress responses (1, 2). Measuring kinase activity, therefore, serves as an excellent proxy for elucidating how cells transduce signals, regulate metabolic pathways, and coordinate responses to various stimuli. As such, developing accurate, sensitive, and specific assays to measure *in vivo* and *in vitro* kinase activity is paramount.

Most current kinase assays rely on radiolabeling, fluorescence, or luminescence-based detection systems. Each of these approaches offers unique advantages but also has inherent drawbacks. Radiolabeling-based assays offer sensitivity but raise safety concerns. Luminescence-based assays (e.g. ADP-Glo assay from Promega), though popular for not requiring radiolabeled materials, indirectly measure kinase activity through ATP-to-ADP turnover. Fluorescence-based assays are sensitive to picomolar substrate detection limits but cannot easily multiplex due to the limited number of available fluorescent molecules. A new assay addressing these limitations would significantly advance plant signaling research, enabling more comprehensive studies of kinase function and its role in plant biology. In this report, we describe an approach based on DNA-linked peptide substrates to quantify and detect specific kinase activity by next-generation DNA sequencing (NGS).

## Results and discussion

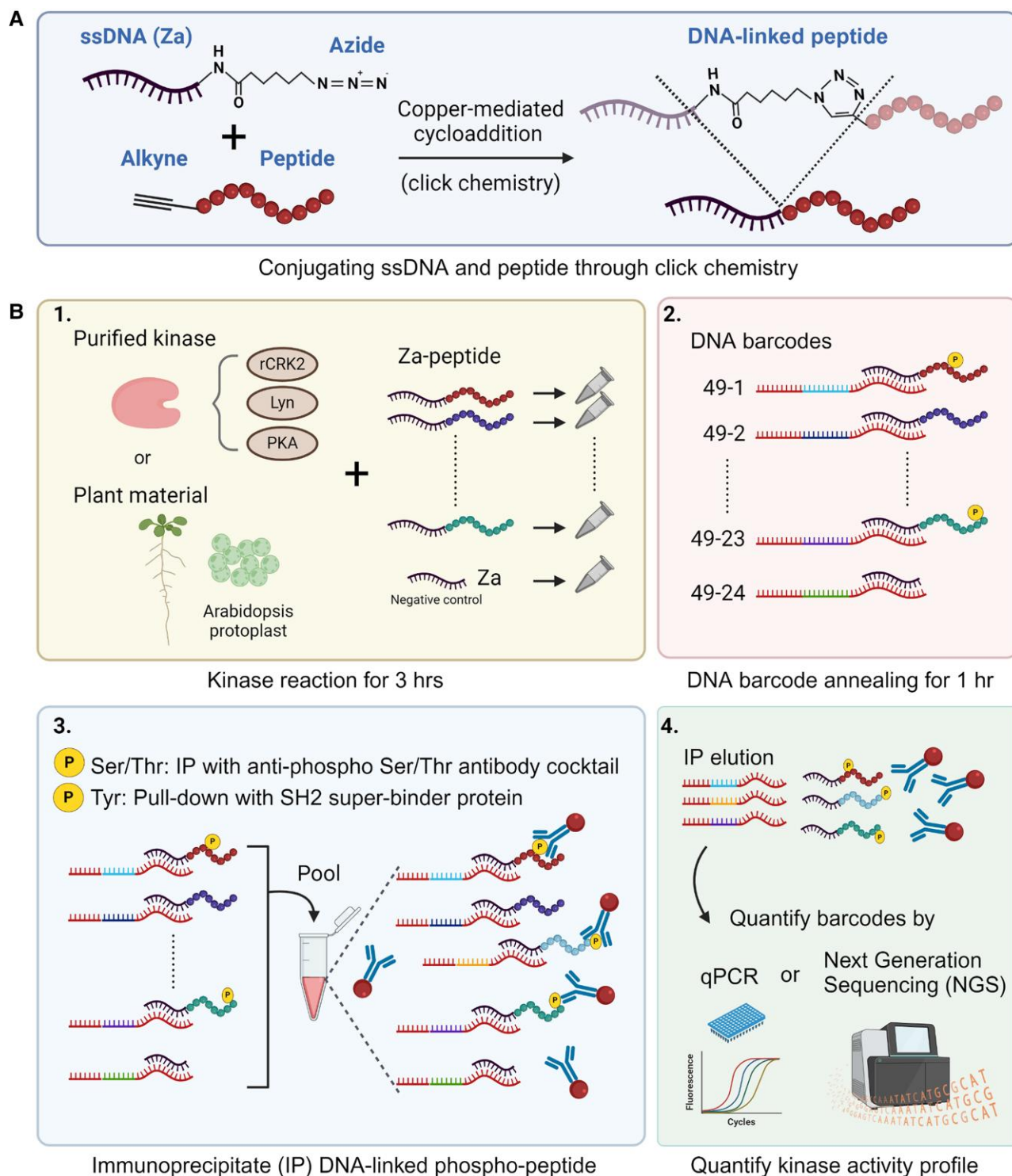
Previously, we reported the use of DNA-linked kinase–substrate probes for highly specific detection of the activities of individual kinases such as the Src family of tyrosine kinases (3, 4). The utilization of DNA as an analytical readout confers several advantages, including quantitative, precise, and specific activity detection, as well as the ability to leverage existing powerful tools for DNA sequence analysis (5). Herein, we developed a DNA-based activity profiling method for plant kinases on different peptide substrates. Specifically, we created a DNA-linked peptide array consisting of 96 Ser/Thr or Tyr-containing peptides selected from *Arabidopsis* phosphoproteomics references (Dataset S1) (6, 7). These peptides were commercially synthesized with an alkyne group and subsequently conjugated with an azide-modified short single-stranded DNA (ssDNA) termed “Za” via click chemistry (Fig. 1A), generating DNA-linked peptides. These DNA-linked peptides were then utilized for the DNA-based kinase assay as described (Fig. 1B).

To investigate the applicability of the DNA-based kinase assay for plant kinase activity profiling, we utilized CDPK/CPK-related kinase 2 (CRK2) as a representative plant tyrosine (Tyr) kinase. The CRK2 kinase possesses high Tyr-autophosphorylation activity and phosphorylates Tyr residue(s) on substrate proteins in *Arabidopsis* (8). We expressed and purified a recombinant Flag-CRK2 protein (rCRK2) from *Escherichia coli* (Fig. 2A) and assessed CRK2 activity on its known substrate peptide, the ethylene response factor 13 (ERF13) peptide (8). Our result demonstrated

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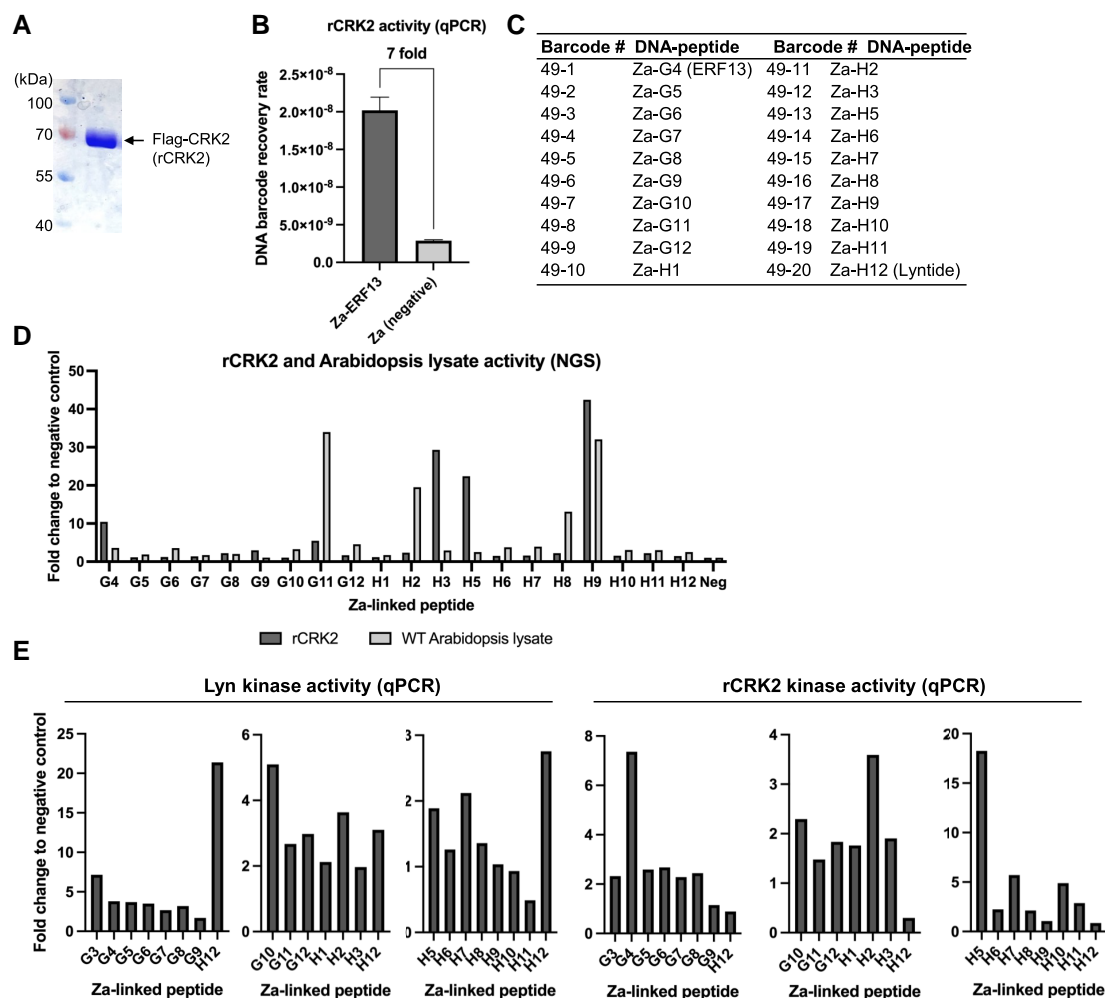
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**Fig. 1.** Methodology of DNA-based kinase assays in plants. A) Illustration for the conjugation between ssDNA and a peptide substrate via click chemistry. B) Workflow for profiling kinase activity using a DNA-based kinase assay.

that CRK2 possessed phosphorylation activity toward the ERF13 peptide, as evidenced by a 7-fold higher DNA barcode recovery rate compared to the negative control via qPCR (Fig. 2B). To comprehensively profile CRK2 activity across multiple peptides, we included twenty Tyr-containing peptides in the experiment (Fig. 2C). The experiment workflow involved an initial step, where the twenty DNA-linked peptides were reacted with rCRK2, followed by annealing to twenty distinct DNA barcodes. Subsequently, a

phospho-Tyr pull-down process was performed, and the phosphorylated DNA-linked peptides, along with their corresponding DNA barcodes, were eluted with phenyl phosphate (4) (Fig. 1B). The eluted DNA barcodes were then amplified and quantified using NGS. Strikingly, our results revealed three potential phosphorylation substrates for rCRK2: peptides H3, H5, and H9 derived from MPK kinase 6 (MPK6), MPK4, and Shaggy-like protein kinase 32, respectively, exhibiting significant enrichment alongside the known



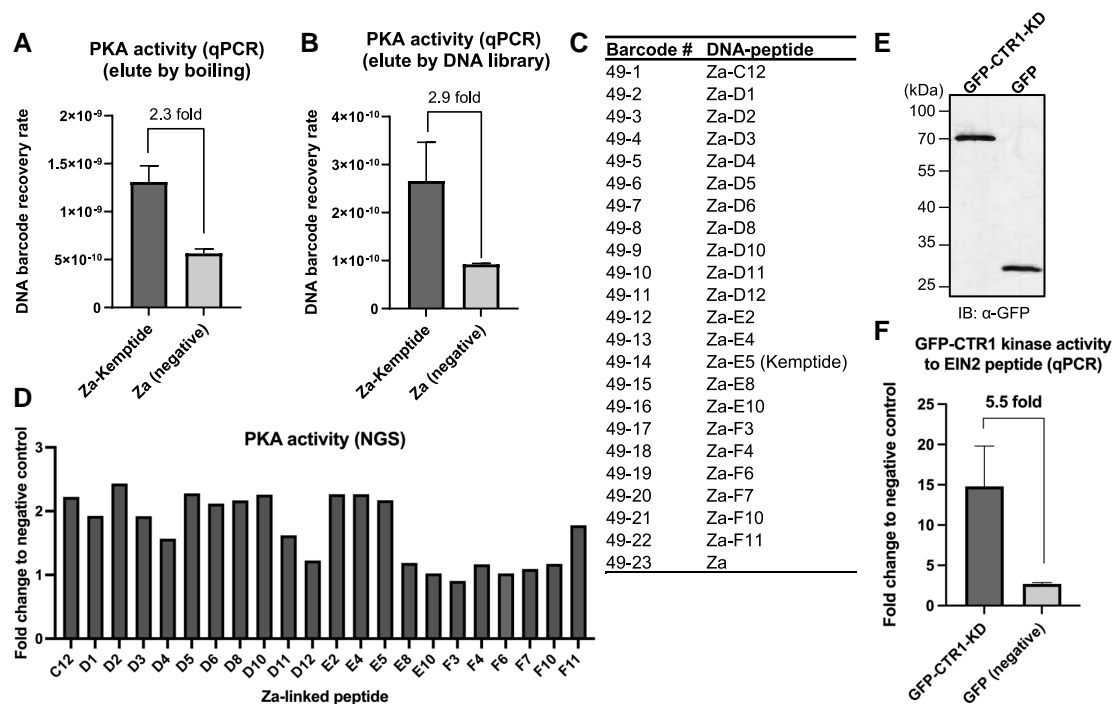
**Fig. 2.** Tyr kinase activity profiling using the DNA-based kinase assay. A) Recombinant CRK2 (rCRK2) protein purified from *Escherichia coli*. B) Kinase activity assay of rCRK2 toward the ERF13 peptide, quantified by qPCR. C) Annealing table showing the barcoded ssDNA conjugated to each peptide substrate. D) Kinase activity profiling of rCRK2 and wild-type (WT) *Arabidopsis* protein lysate against an array of 20 different peptide substrates, quantified by next-generation DNA sequencing. E) Comparative kinase activity profiling of Lyn and rCRK2 against the peptide array, quantified by qPCR. Each graph shows data from an independent immunoprecipitation, resulting in varied y-axis scaling due to differing kinase activity levels.

substrate ERF13 (G4) (Fig. 2D). Intriguingly, when incubated with *Arabidopsis* wild-type plant cell lysates for kinase reactions, the activation profiles of these 20 peptides diverged significantly from those with rCRK2 alone. This divergence likely stems from the complex kinase mixture in cell lysates beyond just native CRK2.

We next investigated the ability of the DNA-based kinase assay to distinguish substrate preferences between different kinases by comparing the DNA barcode enrichment profiles obtained from two distinct Tyr kinases, CRK2 and Lyn (Fig. 2E). Lyntide (H12), a known synthetic substrate of Lyn kinase that plays a role in mammalian cellular proliferation (9), exhibited substantial barcode enrichment in Lyn samples but not in the rCRK2 samples (Fig. 2E). Conversely, the established CRK2 substrate, ERF13 (G4), displayed significant barcode enrichment with rCRK2, but negligible enrichment with Lyn (Fig. 2E). Despite using an identical set of peptides and barcodes, the differential enrichment patterns clearly distinguished the distinct substrate specificities of these two kinases. These results highlight the ability of the assay to effectively discriminate and characterize substrate preferences across different kinases.

To extend the applicability of the DNA-based kinase assay beyond Tyr kinases, we profiled the activity of a representative

Ser/Thr kinase, protein kinase A (PKA) using its well-established synthetic substrate peptide, Kemptide (Fig. 3A–D) (10). Unlike phenyl phosphate, which specifically elutes phosphorylated Tyr-containing peptides, no known chemical can specifically elute phosphorylated Ser/Thr-containing peptides. To address this limitation, we tested two elution methods for the pull-down of phospho-Ser/Thr peptides: elution through (i) boiling and (ii) competitive elution through excessive DNAs that consist of a 105-mer random library (Fig. 3A and B). Both methods yielded a higher DNA recovery rate for the DNA barcodes associated with the Kemptide sample compared to the negative control, suggesting that the DNA-based kinase assay can be extended to profile Ser/Thr kinases. We also investigated the substrate preference of PKA by examining its activity on 22 Ser/Thr-containing peptides (Dataset S1) using NGS, with the peptides and their corresponding annealing barcodes listed in Fig. 3C. The result revealed that PKA exhibited distinct preferences among the 22 peptides tested (Fig. 3D). However, the signal-to-noise ratio for the phospho-Ser/Thr peptides was not as robust as for the Tyr peptides, likely due to nonspecific elution potentially co-eluting nonphosphorylated peptides and reducing the overall signal intensity.



**Fig. 3.** Ser/Thr kinase activity profiling using the DNA-based kinase assay. A, B) The kinase activity of the protein kinase A (PKA) on Kemptide was quantified through qPCR using two elution methods: A) boiling and B) competitive elution with the DNA library. C) Annealing table of barcodes conjugated to each DNA peptide. D) Next-generation DNA sequencing results depicted the activity profile of PKA against each peptide substrate. E) Immunoblot of affinity-purified GFP-CTR1 kinase domain and GFP control from protoplasts. F) The kinase activity of GFP-CTR1 and GFP control toward the EIN2 peptide, quantified by qPCR.

Next, we conducted DNA-linked kinase assays using kinases affinity-purified from *Arabidopsis* protoplasts, plant cells lacking cell walls, which preserve the native cellular environment by maintaining post-translational modifications and regulatory interactions (Fig. 3E and F) (11, 12). Specifically, we examined the kinase activities of Constitutive Triple Response 1 (CTR1), a Ser/Thr kinase that regulates the plant hormone ethylene signaling by phosphorylating Ethylene-Insensitive 2 (EIN2), a key positive regulator in the pathway (13). Following transient expression of GFP-CTR1 in protoplasts, the kinases were affinity-purified using antibody-coupled magnetic beads. Affinity-purified GFP-CTR1 kinases were then subjected to kinase assays using EIN2, its respective targeted DNA-linked peptides, followed by DNA barcode annealing, phospho-peptide pull-down, and qPCR. Phospho-EIN2 peptides from CTR1 were enriched with an antibody cocktail (Fig. 3E and F). Compared to controls, approximately 6-fold higher barcode recovery was observed for GFP-CTR1 samples, validating the utility of the DNA-based kinase assay for profiling the activity of affinity-purified plant kinases from a near-native context.

We established a high-throughput, sensitive, and specific platform for kinase activity detection by utilizing DNA barcodes as quantitative readouts. The method successfully profiled Tyr and Ser/Thr kinases in plants with distinct substrate preferences, demonstrating versatility and specificity. Profiling affinity-purified kinases from plant protoplasts showcased the assay's ability to investigate kinase activities in a near-native cellular context, preserving crucial modifications and regulatory interactions. This capability bridges the gap between *in vitro* and *in vivo* studies, enabling investigations of kinase signaling pathways under physiologically relevant conditions. The relatively low enrichment of

pho-Try and pho-Ser likely results from suboptimal DNA-peptide conjugation efficiency. While high performance liquid chromatography purification could improve results, this study aimed for a straightforward method accessible to labs without specialized equipment. Overall, this powerful kinase assay methodology promises to unravel complex protein kinase regulation in plants, shedding light on crucial signaling pathways that control growth and stress responses.

## Supplementary Material

Supplementary material is available at PNAS Nexus online.

## Funding

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## Author Contributions

G.M.Y. and D.K. conceived the idea and designed experiments; Y.C. conducted all experiments; H.Y.L. performed an experiment; C.A.V. analyzed the NGS data; Y.C., G.M.Y., C.A.V., and D.K. analyzed the data; Y.C. wrote the draft; Y.C., G.M.Y., C.A.V., and D.K. edited the manuscript.

## Data Availability

All data are included in the manuscript and supplementary information.

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