Moonlighting kinases with guanylate cyclase activity can tune regulatory signal networks

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Abbreviations: BRI1, BRASSINOSTEROID INSENSITIVE1; GC, guanylate cyclase; PSK, phytosulfokine; PSKR, phytosulfokine receptor; WAKL10, wall associated kinase like 10

Guanylate cyclase (GC) catalyzes the formation of cGMP and it is only recently that such enzymes have been characterized in plants. One family of plant GCs contains the GC catalytic center encapsulated within the intracellular kinase domain of leucine rich repeat receptor like kinases such as the phytosulfokine and brassinosteroid receptors. In vitro studies show that both the kinase and GC domain have catalytic activity indicating that these kinase-GCs are examples of moonlighting proteins with dual catalytic function. The natural ligands for both receptors increase intracellular cGMP levels in isolated mesophyll protoplast assays suggesting that the GC activity is functionally relevant. cGMP production may have an autoregulatory role on receptor kinase activity and/or contribute to downstream cell expansion responses. We postulate that the receptors are members of a novel class of receptor kinases that contain functional moonlighting GC domains essential for complex signaling roles.

Cyclic nucleotides form important components of signaling networks in eukaryotes and prokaryotes.¹ In fact in plants, cyclic nucleotides play integral parts in signaling cascades activated in response to biotic and abiotic stresses, and normal physiological responses during development and growth.^{2–4} Cyclic nucleotides regulate expression of the transcriptome and modulate ion channel function and thus cellular homeostasis. Therefore it was surprising that despite considerable evidence for the presence of cyclic nucleotides in plants, the key enzymes that synthesize cyclic nucleotides, adenylate cyclase or guanylate cyclase (GC), were not annotated in the *Arabidopsis thaliana* genome published in 2000.⁵

Identification of Plant Guanylate Cyclases (GCs)

Subsequent specific search strategies were based on the assumption that the catalytic center of known GCs was at least in part conserved across the different phyla. Consequently, the search motif was based on several functionally assigned amino acids in the catalytic domain of known GCs from lower eukaryotes and animals. This led to the discovery of Guanylate Cyclase 1 (GC1) in *Arabidopsis* which is a soluble protein with functional guanylate cyclase activity in vitro.⁶ A GC1 homolog involved in light regulation has since been characterized in *Pharbitis nil*.⁷ A second group of functional GCs was identified by relaxing the initial search motif.^{8,9} Surprisingly it included

several receptor kinases such as the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE1 (BRI1), phytosulfokine receptor (PSKR1), pathogen peptide1 receptor (PePR1) and wall associated kinase like 10 (WAKL10) which all share a similar kinase-GC domain structure.^{8,10–12} The GC catalytic center is found embedded within the intracellular kinase domain in the C-terminal and it is this region that has been shown to have functional activity in vitro. We have identified over 40 such kinase-GC molecules in the *Arabidopsis* genome many of which regulate plant growth and development or defense responses to pathogens.⁸ These molecules are different to animal GCs in that the GC catalytic center is nested within the kinase catalytic domain (Fig. 1).

Receptor Kinase-GCs with Dual Kinase and GC Activity

Kwezi et al.¹² expressed and purified the full-length cytoplasmic domain of PSKR1 as a soluble protein and showed that it functioned as a kinase and also had low but significant GC activity as demonstrated in an ELISA assay. Mass spectrometry has confirmed cGMP production and also indicated that no cAMP is produced.¹² The GC activity is due to the GC domain as site directed mutation of the glycine residue to lysine at position 3 of the GC catalytic center reduced cGMP production.¹² Dual

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Figure 1. Domain structures of receptor GCs. (A) Typical domain structure of animal receptor GCs such as GC-A (atrial natriuretic peptide receptor). (B) Domain structure identified in plant receptor kinase-GCs using PSKR1 as the example. GC, guanylate cyclase; TM, transmembrane spanning domain

functioning of the kinase and GC center has also been demonstrated for WAKL10.¹⁰ Kinase-GCs are highly unusual as they contain two catalytic regions in the same domain of the protein where the GC catalytic center is embedded in the kinase domain adjacent to the P activation loop (Fig. 1). A more usual scenario for an enzyme with multiple catalytic functions is to contain the catalytic functions in separate domains separated by linker regions (Fig. 1). In fact, it is thought that the receptor GC catalytic domains and kinase homology domains and the intermediary linker domain in animal receptors coevolved.¹³ Animal receptor GC domain topology (e.g., receptors to natriuretic peptide receptors) is thus distinctly different from that observed in the plant receptor kinase-GCs where the kinase domain encapsulates the GC catalytic center (Fig. 1). Even so, in the case of typical animal receptor GCs, the kinase domain is predicted to be catalytically inactive although it can regulate (by allosteric modulation) activity of the GC domain.¹³ Consequently, the very structure and the fact that we have demonstrated dual catalytic activity of this domain in two plant molecules (PSKR1¹² and WAKL10¹⁰) is both novel and significant.

Even though the GC catalytic center is encapsulated in the kinase domain, it is separate from the kinase active site and so the molecules are moonlighting proteins rather than promiscuous enzymes. Promiscuous enzymes catalyze extra reactions or use substrates in addition to those for which they have evolved, hence such enzymes can catalyze different reaction categories and are therefore considered to have a high degree of promiscuity.¹⁴ Promiscuity of the catalytic active site has evolutionary advantages as it offers starting points for additional protein functions and this is thought to contribute to the widespread nature of this phenomenon. An alternative type of promiscuity or “moonlighting function” has also been characterized in recent years where unrecognized catalytic sites or domains have been identified within proteins.¹⁵⁻¹⁷ The kinase-GCs such as PSKR1, WAKL10 and BRI are examples of moonlighting proteins with dual catalytic activity.

Natural Ligands of the Receptor Kinase-GCs Stimulate Production of cGMP In Vivo

If the GC function of the receptor kinase-GCs is functionally relevant, then it is likely that the natural ligands of the receptor kinase-GCs would stimulate cGMP production in vivo. Kwezi et al.¹² provided the first evidence demonstrating this phenomenon. When freshly isolated mesophyll protoplasts from leaf tissue were exposed to active (PSK- α) phytosulfokine peptide, cGMP production was significantly increased relative to the control protoplasts while the inactive PSK backbone does not cause a cGMP increase. To further test if this was due to the receptor kinase-GC, Kwezi et al.¹² overexpressed PSKR1 in protoplasts and observed marked increases in cGMP over basal levels. Previously the GC catalytic center of BRI (the brassinosteroid receptor) was shown to have functional activity in vitro.⁸ Here we show that epibrassinolide can stimulate increases in cGMP production in freshly isolated leaf protoplasts (Fig. 2). This is an important finding as it demonstrates that GC activity is present in vivo in two systems where functional GC catalytic centers are present in receptor kinase-GCs and supports the in vitro demonstration of GC activity. The cGMP increases occur in a time frame that is consistent with activating the GC catalytic function of the receptor kinase-GCs and so implicate cGMP in early signaling events leading to cell elongation and differentiation stimulated by these natural ligands.

Functional Roles of cGMP in PSK and Brassinosteroid Signaling

Both PSK and brassinosteroids have roles in promoting cell growth. PSK was first discovered as an essential secreted peptide necessary for cell proliferation and it also controls root and hypocotyl length by increasing cell expansion.¹⁸⁻²¹ Brassinosteroids are also critical for plant growth as indicated by the brassinosteroid receptor mutants (*bri1*) which are stunted and very slow growing.²² Cyclic GMP is also implicated in regulating

Figure 2. Effect of epibrassinolide on cGMP production in protoplasts. Freshly isolated leaf mesophyll protoplasts were treated with 10 μ M epibrassinolide for the indicated periods before halting reactions by freezing in liquid nitrogen. The control contained ethanol at the same concentration as the treatment. At 5 min, the epibrassinolide sample is significantly higher than the control ($p < 0.05$ Student's unpaired t-test). The total cellular cGMP content was measured with the Amersham cGMP enzyme immunoassay using the acetylation protocol as described in the supplier's manual (GE Healthcare, code RPN226).

cell expansion and growth. Protoplast volume increases in response to membrane permeable cGMP analogs which is presumably dependent upon activation of various ion channels.^{23,24} Therefore it is conceivable that the growth effects of PSK and brassinosteroids may in part be mediated by increases in cGMP. The kinase signaling cascade initiated by brassinosteroids is very well characterized in *Arabidopsis* and activates a small

family of transcription factors following transphosphorylation events in BRI1.²² However among the BRI1 mutants is the weak point mutation allele *bri1-301* (G989I) which has no detectable BRI1 kinase activity and relatively mild effects on phenotype which is suggestive that brassinosteroid signaling involves more than kinase activity.²⁵ This mutation is upstream of the catalytic GC center which may still contribute to BRI1 function in the mutants. Kwezi et al.¹² also showed that in the presence of cGMP the recombinant kinase domains of PSKR1 have reduced kinase activity. These results suggest that cGMP is modulating the kinase function possibly by allosterically binding to a site in the protein or remaining in the GC catalytic center. We hypothesized that the moonlighting GC domains contribute to the micro-environment of the protein in vivo and contribute to protein regulation.

In conclusion two receptors for compounds that have roles in promoting plant growth and development have also been shown to have functional GC domains embedded in their kinase domain. The natural ligands for these two receptors increase cGMP levels to similar levels to those previously reported to be necessary to obtain downstream signaling response to plant natriuretic peptides and gibberellic acid.^{24,26,27} Taken together these findings indicate that the receptors PSKR1 and BRI1 are members of a novel class of receptor kinases that contain functional moonlighting GC domains essential for complex signaling roles.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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