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Setting-up a fast and reliable cytokinin biosensor based on a plant histidine kinase receptor expressed in *Saccharomyces cerevisiae*

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Highlights

- The cytokinin biosensor is based on a yeast *sln1*Δ mutant complemented by a plant HK.

- Biosensing assay was developed to detect cytokinins from bacterial culture supernatant.
- The cytokinin biosensor is a fast, low cost, selective and highly sensitive biosystem.
- The biosensor enables identification of new steps in cytokinin biosynthesis in bacteria.

ABSTRACT

Cytokinins (CK) have been extensively studied for their roles in plant development. Recently, they also appeared to ensure crucial functions in the pathogenicity of some bacterial and fungal plant pathogens. Thus, identifying cytokinin-producing pathogens is a prerequisite to gain a better understanding of their role in pathogenicity. Taking advantage of the cytokinin perception properties of *Malus domestica* CHASE Histidine Kinase receptor 2 (MdCHK2), we thereby developed a selective and highly sensitive yeast biosensor for the application of cytokinin detection in bacterial samples. The biosensor is based on the mutated *sln1*Δ *Saccharomyces cerevisiae* strain expressing MdCHK2. The biosensor does not require any extraction or purification steps of biological samples, enabling cytokinin analysis directly from crude bacterial supernatants. For the first time, the production of cytokinin was shown in the well-known plant pathogenic bacteria *Erwinia amylovora* and was also revealed in human pathogens *Staphylococcus aureus* and *Streptococcus agalactiae*. Importantly, this biosensor was shown to be an efficient tool for unraveling certain steps in cytokinin biosynthesis by micro-organisms since this it was successfully used to unveil the role of *ygdH22*, a *LOG-like* gene, that is probably involved in cytokinin biosynthesis pathway in *Escherichia coli*. Overall, we demonstrated that our biosensor displays several advantages including time- and cost-effectiveness by allowing a rapid and specific detection of cytokinins in bacterial supernatants These results also support its scalability to high-throughput formats.

Keywords

Cytokinins, biosensor, *Saccharomyces cerevisiae*, CHASE Histidine Kinase receptor

1. Introduction

Biosensors are powerful analytical devices that are used to detect the presence or concentration of a biological analyte, such as a biocompound, a biological structure or a microorganism (Rakhi et al., 2016; Webb et al., 2016). They are becoming increasingly important because they offer the great potential of rapid screening of a large number of chemicals to many fields. They display a wide range of applications such as food analysis, drug development, medical diagnosis, industrial process control or environmental field monitoring (Schallmey et al., 2014; Eggeling et al., 2015; Vigneshvar et al., 2016; Omanovic-Miklicanin and Valzacchi 2017; Ostrov et al., 2017; Neethirajan et al., 2018). A biosensor specifically senses and transduces the presence of a bio- or chemical- molecule into detectable and measurable outputs. It consists of three parts: a component that recognizes the targeted molecule and produces a signal, a signal transducer, and a reader device. Different types of biosensors were developed based on the nature of the biological recognition element and the transducer. Biosensors provide a combination of advantages including high sensitivity and selectivity. They are inexpensive, easy to use and scalable to high-throughput formats.

Cytokinins (CKs) are a group of phytohormones that impact a wide variety of biological functions within plant growth and development, including cell division, root and shoot development, inhibition of leaf senescence, nutrient mobilization and stress responses (Werner and Schmülling, 2009; Spíchal, 2012; Kieber and Schaller, 2014). Chemically, natural CKs are adenine derivatives with distinct substitutions attached to the N_6 position of the adenine ring (Figure 1). The most common classes of CKs harbor isoprenoid side chains and occur as free bases as well as their corresponding nucleosides and nucleotides (Figure 1). In addition, they can be conjugated to sugar moieties, most commonly glucose (Figure 1), and modifications can also be found at the C_2 position with a methylthio group (2-MeS-CK) (Figure 1). CKs are synthesized with transfer of the isoprenoid moiety to adenine either in its nucleotide form or bound to RNA by isopentenyltransferase (IPT) and tRNA-isopentenyltransferase (tRNA-IPT), respectively.

CKs are not restricted to plants and they seem to be evolutionary highly conserved molecules (Spíchal, 2012), since they are increasingly reported in many organisms (i.e. bacteria, fungi, nematodes and insects) to be able to interact with plants (Table I) (Stirk and van Staden, 2010). Therefore, substantial roles of CKs in plant-host manipulation by bio-aggressor and symbiotic organisms have emerged (Table I). The CK production by some bacterial and fungal

phytopathogens, such as *Rhodococcus fascians*, *Claviceps purpurea*, *Magnaporthe oryzae* and *Ustilago maydis*, has been shown to be pivotal for their virulence (Pertry et al., 2009; Morrison et al., 2015a; Chanclud et al., 2016; Hinsch et al., 2016; Spallek et al., 2018). The plant-parasitic nematode *Heterodera schachtii* synthesizes CKs in order to manipulate the host system and establish a long-term, parasitic interaction (Siddique et al., 2015). The fly *Eurosta solidaginis* and the sawfly *Pontania* sp. are also able to synthetize CKs probably for inducing the formation of galls tissues on infected plants (Mapes and Davies, 2001; Straka et al., 2010; Yamaguchi et al., 2012). In addition to plants and organisms that interact with plants, CKs have also been detected in humans and other mammals, human pathogenic bacteria, amoebae and apicomplexa; but, CK functions in these organisms remain largely unknown (Table I). Nonetheless, Samanovic et al. (2015) revealed the secretion of several CKs by the obligate human bacterial pathogen, *Mycobacterium tuberculosis* and speculated that CKs might influence the host organism to trigger a successful infection. Moreover, Andrabi et al. (2018) clearly showed that CKs have effects on growth and cell cycle progression in the human parasite *Toxoplasma gondii*.

All the above-mentioned studies among various kingdoms thus suggest that CKs could play a broad role in pathogenic strategies of microorganisms. To clarify this hypothesis, a preliminary step obviously consists of the identification of CK-producing species. Currently, analysis of CKs requires powerful analytical tools able to detect and identify trace amounts of these compounds in biological samples, such as high performance liquid chromatography (HPLC) combined with mass spectrometry (MS) (Tarkowski et al., 2009; Farrow and Emery, 2012; Kisiala et al., 2013; Dobrev et al., 2017). Such methods are sensitive and molecule-specific, but are time-consuming owing to the pre-treatment steps including extraction, pre-concentration and purification of samples. Consequently, the requirement of such a sophisticated approach is a significant limiting factor for a high-throughput screening of CK-producing organisms. Biotechnological alternatives, especially biosensors, offer new opportunities and open new windows for the detection of a large number of compounds including CKs (Strachan et al., 2014; Tian et al., 2014; Gutiérrez et al., 2015; Teo and Chang 2015; Jarque et al., 2016). Here, we widely tested and validated a biosensor applied to the rapid screening of plant and human-pathogenic, bacteria-producing CKs. The bio-system is based on the CK-dependent growth of an engineered yeast strain expressing an apple tree CK receptor, MdCHK2 (Daudu et al., 2017). Indeed, CK perception in plants is mediated by a specific Histidine Kinase (HK) receptor sub-family containing the CHASE sensor domain (Cyclase/Histidine kinase Associated Sensory Extracellular) (Inoue et al., 2001). HK receptors

are bacterial legacy, and they are found in plants and fungi but have evolved in diverse ways to sense different types of signals (Hérivaux et al., 2017). The yeast *Saccharomyces cerevisiae* presents a unique HK receptor involved in osmosensing (Sln1) that is required to maintain yeast survival (Figure 2). In the absence of osmotic stress, Sln1 autophosphorylates and transfers the phosphate to the response regulator SSK1 *via* the phosphotransfer protein YPD1. The inactive phospho-SSK1 keeps the HOG1 pathway inactive, preventing from a lethal over-accumulation of glycerol in absence of osmotic stress. Since the signal transduction pathway is conserved in eukaryotic cells, plant CHASE HK receptors (CHK) can advantageously replace the yeast Sln1 for initiating the phosphotransfer and inhibiting the HOG1 pathway to allow yeast growth without osmotic stress conditions. Nevertheless, the system is functional only if the CHK receptors are activated by their specific ligands, namely CKs. Therefore, the conditional survival of the recombinant yeast strain *sln1*Δ-MdCHK strictly depends on CKs perception (Figure 2). In regards of some characterized CHK among plants, MdCHK2 perceives the widest range of CKs, including free-bases, ribosides, glucosides and methylthio cytokinins, with a substantial sensitivity towards nanomolar concentrations (Daudu et al., 2017) and was, therefore, the best candidate to develop the CK biosensor.

In this work, the yeast strain *sln1*Δ lacking *SLN1* and complemented with MdCHK2 (Figure 2; Daudu et al., 2017) is exploited as CK biosensor. We demonstrate the ability of our engineered biosensor to specifically detect CKs directly from bacterial crude supernatants thus allowing (i) the efficient identification of new CK-producing species. The new biosensor can also be used (ii) to identify new genes involved in the CK biosynthesis/metabolism pathway in bacteria. This biosensor, based on a CK-dependent yeast growth assay, provides a simple, rapid, selective, sensitive and cost-effective analytical method for a high-throughput screening for CK-producing bacteria.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this study are summarized in Table II. We employed the pathogenic *Erwinia amylovora* wild-type strain (CFBP1430) (Paulin and Samson, 1973). The wild-type *Escherichia coli* strain (CGSC number: 7636; BW25113) as well as the *miaA*Δ (CGSC number: 10972; JW4129-2), *miaB*Δ (CGSC number: 8749; JW0658-1) and *ygdH22*Δ (CGSC number: 10169; JW2766-5) derivative mutant strains were obtained from the Coli Genetic Stock Center (Yale). Concerning the identification of *ygdH22* gene in *E. coli* (WP_069916468.1), we used the *LOG*-like *Rv1205* sequence from *M. tuberculosis* to perform

a BLAST search. Two gram positive strains, *Staphylococcus aureus* ATCC25923 and *Streptococcus agalactiae* NEM316 were investigated (Glaser et al., 2002).

2.2. Bacterial growth conditions

The strains were first streaked directly onto LB agar plates and incubated at 37°C for 16 h, except for *E. amylovora* at 26°C. Liquid culture, started by suspending a single colony in 4 mL of LB, were grown for 16 h on a rotary shaker (250 rpm) at 26°C or 37°C. This culture was centrifuged for 1 minute at 11,000g and washed/rinsed twice with 2 mL of M9 medium supplemented with glucose or King B medium, depending on the experiment (King et al., 1954; Cesbron et al., 2006). Then, 250 mL of these culture media were inoculated with the rinsed cells and grown at 26°C or 37°C for 16 h on a rotary shaker (150 rpm). The bacterial cultures were stopped at their maximum growth rate, as determined by measuring the OD_{630}, centrifuged for 15 minutes at 5000rpm, and finally the supernatants were sterilized by using 0.22 µm filters. Four replicates were analyzed for all samples.

2.3. Yeast bioassays

*Saccharomyces cerevisiae sln1*Δ carrying the plasmid pYES2-MdCHK2 (Daudu et al., 2017) was maintained at 30°C on selective solid medium MMAS (0.7 g/L YNB (WA), 0.06 g/L DOB-LEU-HIS-ARG-URA, 20 g/L galactose, 0.2 g/L G418, 0.06 g/L L-Canavanine) supplemented with isopentenyladenine. For the bioassays, yeasts were suspended in 5 mL YCGal medium (7 g/L YNB, 0.8 g/L CSM-URA, 20 g/L galactose); aliquots of 10 µL were added to a 96-well plate. To test the CK activity of bacterial samples, 50 µL of sterilized supernatants were added to each well. A technical triplicate was performed for each bacterial supernatant. As a negative control, free media (King B or M9) were tested, and as a positive control, 10 µM isopentenyladenine diluted in the free media (OlChemim, Olomouc, Czech Republic). The plates were incubated at 30°C for 48 h, then the growth of the yeast cells was measured at OD_{630} (iMark^{TM} Microplate Absorbance Reader, BioRad).

2.4. LC-MS/MS cytokinin analysis

Bacterial CKs were isolated from 15 mL of sterilized supernatants and purified according to Kisiala et al. (2013). The profiles of 25 CK forms were analyzed, including free bases, riboside and nucleotide derivatives, as well as glucoside and methylthiol conjugates as described in Kisiala et al. (2013). Purified CK were identified and quantified by LC-MS/MS (Agilent 1100 series HPLC connected to a Sciex Applied Biosystem 5500 API mass

spectrometer), according to the conditions outlined in Farrow and Emery (2012). An aliquot was injected on a Luna C18 reversed-phase column (3 µm, 150 × 2.0 mm; Phenomenex, Torrance, Canada), and the CK were eluted with an increasing gradient of 0.08% CH_3COOH in CH_3CN (A) mixed with 0.08% CH_3COOH in ddH2O (B), at a flow rate of 0.2 ml/min. The initial conditions were 5% A and 95% B, changing linearly in 17 minutes to 95% A and 5% B. Conditions remained constant for 5 minutes, and then, immediately returned back to initial conditions for 18 minutes. The effluent was introduced into the electrospray source using conditions specific for each CK, and quantification was obtained by multiple reaction monitoring of the protonated intact CK molecule $[M+H]^+$ and the specific product ion (Farrow and Emery, 2012).

3. Results and discussion

3.1. Design of the biosensor assay to detect CKs in bacterial crude supernatant

While current sophisticated methods for CK analysis are highly sensitive and molecule-specific, they remain a time-consuming, laborious and expensive process. Here, we designed a biotechnological alternative, especially dedicated to the rapid identification of CK-producing pathogenic bacteria, exploiting the yeast biosensor system previously described in Daudu et al., 2017. We developed a standardized protocol as schematized in the Figure 3. It consists in measuring the yeast growth in presence of bacterial crude supernatants. The yeast growth being strictly dependent on the presence of cytokinins, the production of cytokinins by bacterial strains can be easily highlighted by our biosystem. Proofs-of-concept were attempted using the plant pathogen *Erwinia amylovora* and the human pathogens *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli*, respectively responsible for agricultural damage and major human diseases (Table II). As a first step, we wanted to ensure that the King B bacterial rich medium could be used for the yeast assays (Figure 4). Both negative (without iP) and positive (with iP) controls were included and correspond to the growth of the recombinant yeast strain *sln1*Δ-MdCHK2 in absence or presence of pure isopentenyladenine (iP), a classical free-base CK form. These controls allow the validation of the bioassay through the observation of a strictly CK-dependent yeast growth. Surprisingly the free King B medium stimulated the yeast growth probably indicating the presence of CK-like molecules in this rich medium (Figure 4). This observation was confirmed by HPLC-MS/MS analysis which revealed that free King B medium contains high levels of iPR (33.5 pmol/mL), iPNT (11.8 pmol/mL), iP (3.71 pmol/mL), *t*Z (2.25 pmol/mL) and to a lesser extent 2MeSZ (0.85 pmol/mL) and DZNT (0.88 pmol/mL) (Table III). Since the only non-mineral source in the medium composition is peptone,

we can surmise that this element naturally contains CKs that are detected by our system. Consequently, we tested a minimal medium, not containing any organic components, by using M9 medium (Figure 4). The bioassays revealed that the free M9 medium did not induce the yeast growth and HPLC-MS/MS analysis confirmed the absence of CKs in this medium (Table III). Therefore, in order to prevent any CKs contamination, we opted for the use of minimal M9 medium for our bacterial cultures.

3.2. Application of the biosensor to detect CK production by plant pathogens

The necrogen pathogen *Erwinia amylovora* is a Gram negative enterobacteria responsible for fire blight of members of the *Malinae* tribe of the *Rosaceae* family including apple and pear, and which causes severe damages in orchards (Vanneste, 2000). The pathogenicity of *E. amylovora* relies upon its ability to inject protein effectors into the plant cell through a type III secretion system (Boureau et al., 2006). After infection through flowers or lesions, the bacteria trigger an oxidative burst and cell membrane disruption leading to the progressive necrosis of succulent shoots (Venisse et al., 2001). To date, a CK secretion by *E. amylovora,* which could be involved in the infection process, has never been described hitherto. Interestingly, we took advantage of the yeast biosensor to test this exciting hypothesis. We performed the bioassay with crude bacterial supernatants directly derived from *E. amylovora* cultures in M9 medium (Figure 5). Classically, both negative and positive controls were included allowing the validation of the bioassay. Concerning the *E. amylovora* cultures, the bacterial supernatants clearly induced yeast growth. This result strongly suggests that *E. amylovora* secretes CKs. Advanced analysis using HPLC-MS/MS clearly confirmed the production of CKs by *E. amylovora*. The iP-derivatives including iPNT (4.20 pmol/mL), iPR (0.11 pmol/mL), iP (3.41 pmol/mL) and 2MeSiP (1.63 pmol/mL) were the main forms synthetized (Table III). Previous studies showed that the secretion of high amount of CKs by *Pantoea agglomerans, Pseudomonas savastanoi* and *Rhodococcus fascians* facilitated or initiated the symptoms development (Sisto et al., 2004; Barash and Manulis-Sasson, 2007; Pertry et al., 2010). On this basis, we can hypothesize that CK production by *E. amylovora* might participate to the disease development; however, functional studies will be needed.

3.3. Application of the biosensor to detect CK production in mammal pathogens

The two Gram positive bacteria *S. aureus* and *S. agalactiae* are commonly responsible for various infections in humans. *S. agalactiae,* a Group B *Streptococcus* species, is a commensal bacterium of the human intestinal and vaginal tracts in 15–30% of healthy adults,

but remains one of the most important invasive pathogens in newborn babies and elderly persons (Lanotte et al., 2013; Landwehr-Kenzel and Henneke, 2014). *S. aureus*, both a commensal and a human pathogen, is responsible for various skin infections and currently is a concern to the medicine community because of the emergence of antibiotic-resistant isolates (Tong et al., 2015). To date, the production of CK by both bacterial species is unknown. Thus, as previously performed with *E. amylovora*, crude supernatants derived from bacterial cultures in minimal medium M9 were tested through the biosensor. Both *S. aureus* and *S. agalactiae* triggered a yeast growth signal output highlighting the secretion of CKs by both pathogenic strains (Figure 6). The confirmation by HPLC-MS/MS analysis led to the accurate identification of the CKs pattern. While *S. aureus* significantly produces iP (0.22 pmol/mL) and 2MeSZ (0.73 pmol/mL), *S. agalactiae* mostly produces iPNT (0. 63 pmol/mL), iP (0.19 pmol/mL), iPR (0.05 pmol/mL), 2MeSZ (0.58 pmol/mL) and 2MeSZR (0.05 pmol/mL) (Table III). The role of CKs in *S. aureus* and *S. agalactiae* infection processes remains to be elucidated. Recently, Samanovic et al. (2015) showed, for the first time, a CK production by the obligate human pathogen *M. tuberculosis* and speculated that CKs might be used as signaling molecules to communicate among mycobacteria and/or act on the host as a tool for invasion in order to establish a successful infection. The discovery of CK secretion by *S. aureus* and *S. agalactiae* reinforces the notion that CKs might be involved in their infection strategies in humans.

3.4. Application of the biosensor to characterize CK biosynthesis pathways in pathogenic microorganisms

Escherichia coli is one of the most diverse bacterial species with various pathogenic strains able to induce different intestinal or extraintestinal disorders. This bacterium is known for producing CKs (Burrows et al., 1969) and the first enzymes involved in the CK metabolism have been identified. The tRNA-IPT encoded by the *miaA* gene is responsible for the first step of CK synthesis by the formation of prenyl-tRNA. The tRNA-methylthiotransferase encoded by *miaB* gene performs the methylthiolation (Persson et al., 1994). We took advantage of this knowledge to develop an additional application of the biosensor dedicated to the screening of CK-deficient mutants in order to elucidate biosynthetic pathways. To this aim, we used the *E. coli miaA*Δ and *miaB*Δ mutants to bring some additional controls in order to reinforce the robustness of the system. As previously, bacterial cultures were performed in minimal M9 medium. The wild type (WT) strain was used as control and consistently triggered yeast growth indicating the bacterial CK secretion, confirmed by HPLC-MS/MS analysis (Figure 7; Table IV). As expected, no yeast growth was observed in presence of *miaA*Δ mutant supernatant

(Figure 7) since disruption in *miaA* is known to abolish CK production (Gray et al., 1996; Podlešáková et al., 2013). Analytical analysis clearly strengthened the drastic suppression of CK biosynthesis in *miaA* mutant (0.31 pmol/mL) compared to the WT strain (16.63 pmol/mL) (Table IV). These results clearly support the reliability of the biosensor. With respect to the *miaB*Δ mutant, the supernatant induced yeast growth, but it was slightly weaker than wild-type supernatant (Figure 7). Since *miaB* is required for methylthiolation, other CK forms can still be produced and detected. HPLC-MS/MS confirmed the absence of any methylthiolated CKs (Table IV). Nevertheless, more free-base and nucleotide CKs were secreted, resulting in a higher amount of total pool of CKs (Table IV). The lower yeast growth can probably be explained by the 2MeSiP, which is the most active ligand for MdCHK2 (Daudu et al., 2017); thus, converging toward a less-pronounced activation in presence of the *miaB*Δ supernatant.

Recently, a homolog of the plant Lonely Guy (*log*) gene involved in the direct conversion from nucleotide- to free base CKs was identified for the first time in *Mycobacterium tuberculosis* (Samanovic et al., 2015). By running a BLAST approach, we identified in the *E. coli* genome a sequence matching with the *M. tuberculosis log* gene that is currently annotated lysine decarboxylase (*ygdH22*). A bioassay revealed that the bacterial supernatant, from the *ygdH22*Δ mutant culture, induces less yeast growth compared to wild type, emphasizing that *ygdH22* is likely involved in CK metabolism (Figure 6). Indeed, CK analysis showed slightly lower free-base CK levels compared to the WT profile, while contents of nucleotide-type CKs were almost identical between *ygdh22*Δ mutant and wild type strain (Table IV). Thus, we can surmise that the annotated *ygdh22* corresponds to a *log* gene in *E. coli*. However, its involvement in CK activation seems to be minor. Some genes still need to be identified, such as 5'-ribonucleotide phosphohydrolase or adenosine nucleosidase, in order to shed more light on the CK biosynthesis pathway in bacteria. However, we firmly demonstrated that a rapid screening of mutants can efficiently be performed with our CK biosensor.

4. Conclusion

This work demonstrates the exploitation of the recombinant yeast strain *sln1*Δ-MdCHK2 as a CK biosensor. This was applied successfully to detect CKs directly from bacterial crude supernatants without any pre-treatment steps including extraction, purification and concentration of CK metabolites. The systematic HPLC-MS/MS analyses run for validation of our results, confirms the strong performance and robustness of our yeast system in its ability to sense low, nanomolar CK amounts from a crude biological sample. The sets of data allow us to propose a standardized protocol, schematized in Figure 3, dedicated to (i) the high-

throughput screening of CK-producing bacteria and (ii) the identification of missing components pertinent to CK metabolism in bacteria. Finally, besides the proof-of-concept, this simple, rapid, selective and highly sensitive bio-system revealed for the first time that there is CK production by the plant pathogenic bacterium *E. amylovora* and the human bacterial pathogens *S. aureus* and *S. agalactiae*, raising questions about the role of CK compounds in the infection processes of pathogens. In addition, our system allowed the identification of a *log* gene probably involved in CK biosynthesis in *E. coli*. The ease-of-use of this biosensor greatly facilitates the identification of CK-producing bacteria opening the way to a renewed interest for the role of CKs in pathogenic microorganisms.

The proposed biosensor evidently opens a new field for the high-throughput screening of CK-producing bacteria. Moreover, our system can be expanded to the detection of CKs in other microorganisms such as pathogenic fungi, since fungal virulence can be associated with production of these hormones (Chanclud et al., 2016; Hinsch et al., 2016; Trdá et al., 2017; Kind et al., 2018). Recently, Samanovic et al. (2018) showed that CK can be used as signaling molecules outside of plants which broadens the repertoire of small molecules that can affect gene expression in all domains of life. This reinforces the development of our system. Now, the biosensor will be also of great interest for screening CK analogs that are biologically more active than natural CKs since CKs have been implicated in therapeutic processes. Specifically CKs are shown to be involves in: anticancer effects (Vermeulen et al., 2002; Voller et al., 2010, Casati et al., 2011), neuroprotectants (Fei et al., 2009; Lee et al., 2012), antioxidant effects (Othman et al., 2016), antiparasitic processes (Andrabi et al., 2018) or immunomodulating functions (Ciaglia et al., 2013; Lappas, 2015). Furthermore, the vast availability of sensory domains in nature provides a great diversity of other potential molecules for screening. With the rise of synthetic biology, novel biosensors based on the engineering of hybrid receptors are more widely in development (Ward et al., 2002; Salis et al., 2009; Reyes-Darias et al., 2015; Bi et al., 2016). Based on this approach, alternative sensory domains might be fused with the cytoplasmic signaling domain of MdCHK2 in order to develop the monitoring of novel compounds.

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6. Figure legend

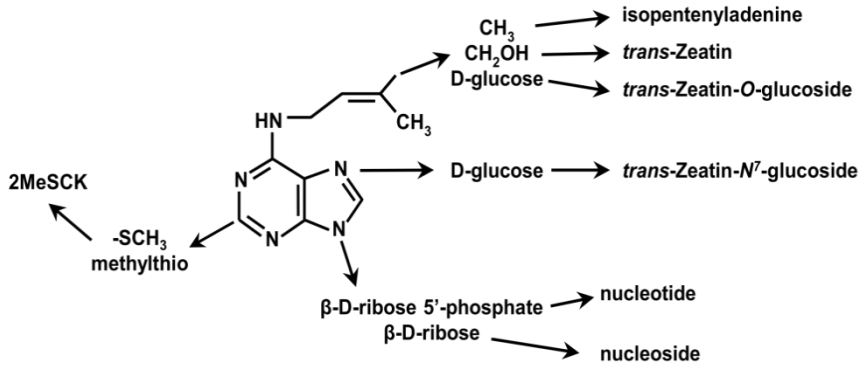

Figure 1. **Structure of common isoprenoid CKs.** CKs are adenine derivatives that can be prenylated at the N^6 position. Various conjugations on the adenine base as well as the lateral isoprenoid chain lead to multiple forms of CKs.

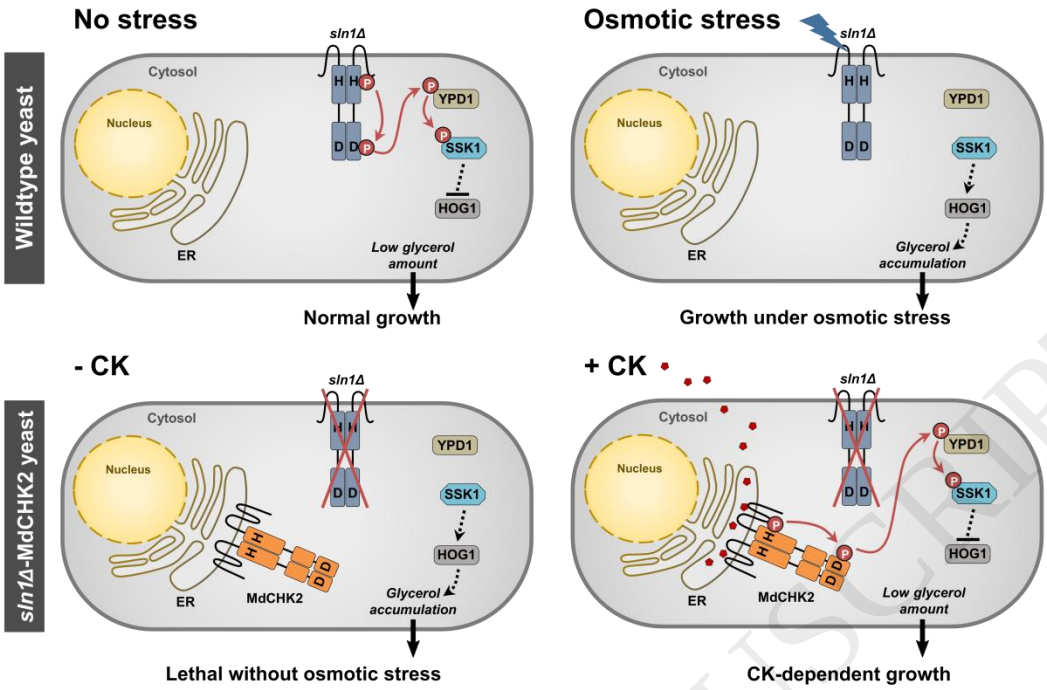

Figure 2. Using the *sln1*Δ-MdCHK2 yeast system as a tool for CK biosensing. Yeast complementation assays were previously performed taking advantage of the SLN1 signaling pathway in yeast (*S. cerevisiae*) (Daudu et al., 2017). Only MdCHK-complemented yeast displays a growth phenotype in presence of pure CKs. We exploited this system to develop a sensitive CK biosensor and to use it in order to detect CK in bacterial supernatants. MdCHK2 is located in the endoplasmic reticulum of *S. cerevisiae* according to Daudu *et al.*, 2017.

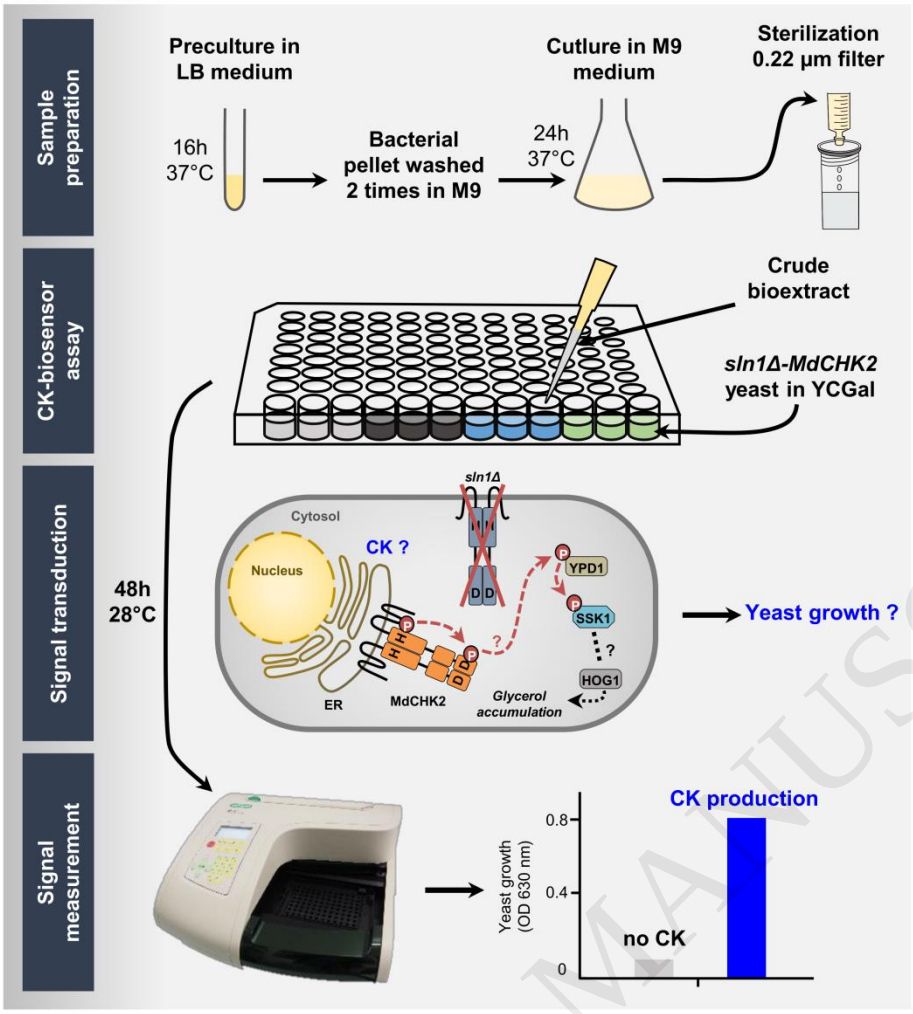

Figure 3. Standardized protocol for CK detection using the *sln1*Δ-MdCHK2 yeast biosensor. The bacterial strains are cultivated in M9 liquid medium for 48 hours. After centrifugation, the supernatants are filter-sterilized and added in the yeast culture medium (YCGal medium). After 48 hours at 30°C, the yeast growth is measured at OD_{630nm}. While an absence of yeast growth indicates an absence of cytokinins in the bacterial supernatant, the presence of a yeast growth exhibits the presence of cytokinins, thus revealing the secretion of cytokinins by the bacterial strains.

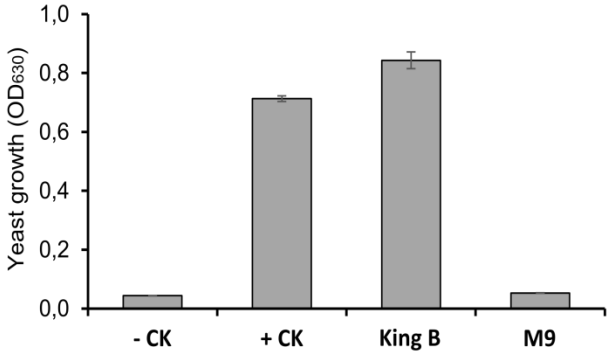

Figure 4. CK biosensing assay with bacteria-free media. The growth rate of *sln1*Δ-MdCHK2 yeast cells is given as turbidity measured at OD_{630}. Both minimal (M9 supplemented with glucose) and rich (King B) media have been tested. Positive (+CK) and negative (-CK) controls represent biosensing assays with YCGal medium supplemented or not supplemented with exogenous isopentenyladenine, respectively.

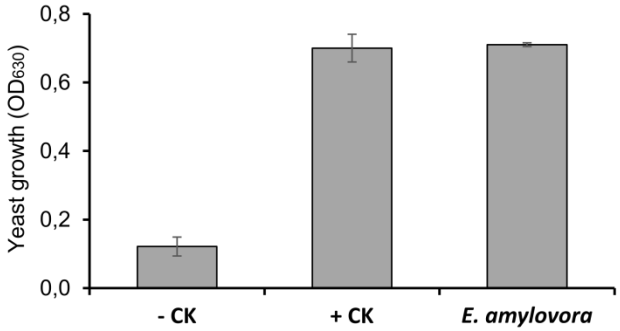

Figure 5. CK biosensing assay in response to *Erwinia amylovora* cultures. The growth rate of *sln1*Δ-MdCHK2 yeast cells is given as turbidity measured at OD_{630}. M9 medium has been used for *E. amylovora* cultures and crude supernatants were tested on yeast CK biosensor. Positive (+CK) and negative (-CK) controls were included corresponding to M9 medium+Glucose supplemented or not supplemented with isopentenyladenine, respectively.

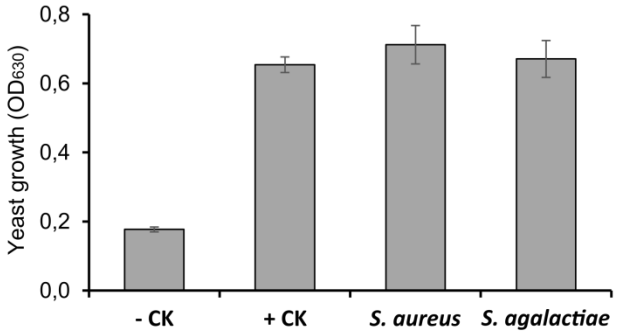

Figure 6. CK biosensing assay in response to *Staphylococcus aureus* and *Streptococcus agalactiae* cultures. The growth rate of *sln1*Δ-MdCHK2 yeast cells is given as turbidity measured at OD_{630}. M9 medium has been used for the bacterial cultures and crude supernatants were then used for the CK biosensing assay. Positive (+CK) and negative (-CK) controls were included corresponding to M9 medium+Glucose supplemented or not supplemented with isopentenyladenine, respectively.

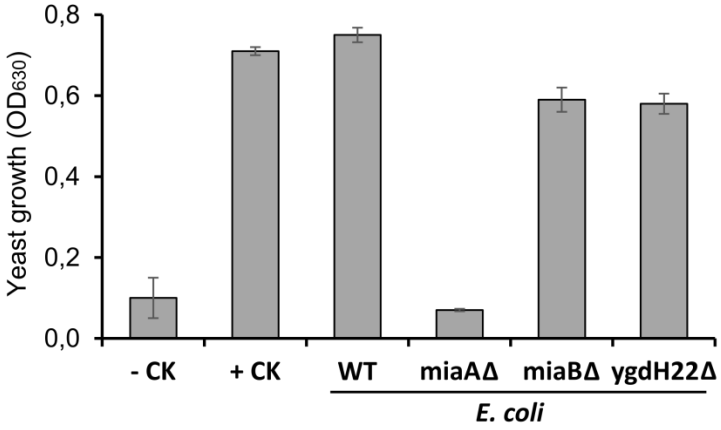

Figure 7. CK biosensing assay in response to culture of *Escherichia coli* mutants. The growth rate of *sln1*Δ-MdCHK2 yeast cells is given as turbidity measured at OD_{630}. M9 medium has been used for the bacterial cultures. Crude supernatants were then used for the CK biosensing assay. The wild-type (WT) *E. coli* strain serves as control. *E. coli miaA***Δ** and *miaB***Δ** mutants are strains known for being affected in their CK content, whereas the *E. coli ygdh22*Δ mutant strain had not been previously characterized. Positive (+CK) and negative (-CK) controls were included corresponding to M9 medium+Glucose supplemented or not supplemented with isopentenyladenine, respectively.

7. Tables

Table I. Examples of non-plant organisms known for producing CKs and their associated roles.

Species	Roles	References
Plant-pathogenic bacteria		
Agrobacterium tumefasciens	Virulence	Hwang et al., 2013
Erwinia herbicola	Gall formation	Lichter et al., 1995
Pseudomonas syringae	Unknown	Akiyoshi et al., 1987
Rhodococcus fascians	Virulence	Pertry et al., 2009
Plant-symbiotic bacteria		
Bacillus subtilis	Root growth and plant defense	Arkhipova et al., 2005
Sinorhizobium meliloti	Nodulation	Frugier et al., 2008
Mesorhizobium loti		Lohar et al., 2004
Methylobacterium extorquens	Unknown	Koenig et al., 2002
Pseudomonas fluorescens G20-18	CK-induced defenses (biocontrol)	Groβkinski et al., 2016
Plant-pathogenic fungi		
Magnaporthe oryzae	Virulence	Chanclud et al., 2016
Claviceps purpurea	Virulence	Hinsch et al., 2015
Amanita bisporigera	Unknown	Morrison et al., 2015a
Phellinus igniarius	Unknown	Morrison et al., 2015a
Lycoperdon perlatum	Unknown	Morrison et al., 2015a
Ustilago maydis	Virulence	Morrison et al., 2015b
Venturia inaequalis	Unknown	Cooper and Ashby, 1998
Plant-mutualistic fungi		
Thelephora terrestris	Unknown	Kraigher et al., 1991
Plant-parasitic nematod		
Heterodera schachtii	Infection	Siddique et al., 2011
Plant-galling insects		
Eurosta solidaginis	Gall formation	Mapes and Davies, 2001
Pontania sp.		Yamaguchi et al., 2012

Pachypsylla celtidis		Straka et al., 2010
Human-pathogenic bacteria		
Escherichia coli	Unknown	Skoog et al., 1966; Persson et al., 1997
Pseudomonas aeruginosa AK2	Unknown	Karnwal and Kaushik, 2011
Mycobacterium tuberculosis	Unknown	Samanovic et al., 2015
Amoeba		
Dictyostelium discoideum	Self-spore germination inhibitor	Mik et al., 2017
Apicomplexa		
Toxoplasma gondii	Regulation of the growth and cell cycle	Andrabi et al., 2018
Plasmodium berghei	Unknown	Andrabi et al., 2018
Other species		
Canis familiaris	Unknown	Seegobin et al., 2018
Homo sapiens	Unknown	Barciszewski et al., 2000

Table II. Pathogenic or control bacterial strains used for the CK screening using the *sln1Δ*-MdCHK2 yeast CK biosensor. Different human and plant pathogens including Gram positive and Gram negative strains were used.

Host	Gram	Strain		Name	Reference	CK production
Plant	Negative	*Erwinia amylovora*		CFBP 1430	Paulin and Samson, 1973	Unknown (this study)
Human	Negative	*Escherichia coli* (K-12)	WT	BW25113	Persson *et al*., 1994	Known
			*miaA*Δ	JW4129-2		
			*miaB*Δ	JW0658-1		
			*ygdH22*Δ	JW2766-5	Kurakawa *et al*., 2007	
	Positive	*Staphylococcus aureus*		ATCC25923	Treangen *et al*., 2014	Unknown (this study)
		Streptococcus agalactiae		NEM316	Glaser *et al*., 2002	

Table III. CK concentration in M9 and King B media, *E. amylovora, S. aureus* and *S. agalactiae* cultures (pmol/mL). M9 medium have been used for bacterial cultures and the CK analysis was performed from the supernatants, using LC-MS/MS. NT, nucleotides ; RB, riboside ; FB, free base ; MET, Methylthiols ; *t*ZNT, *trans*-Zeatin riboside-5′-monophosphate ; DZNT, dihydrozeatin riboside-5′-monophosphate ; *c*ZNT, *cis*-Zeatin riboside-5′-monophosphate ; iPNT, isopentenyladenosine-5′-monophosphate ; iPR, isopentenyladenosine ; *t*Z, *trans*-Zeatin ; *c*Z, *cis*-Zeatin ; iP, isopentenyladenine ; 2MeSZR, 2-Methylthio-Zeatin Riboside ; 2MeSiPA, 2-Methylthio-isopentenyladenosine ; 2MeSZ, 2-Methylthio-Zeatin ; 2MeSiP, 2-Methylthio-isopentenyladenine; nd, not detected.

	M9 Medium	**King B Medium**	***E. amylovora***	***S. aureus***	***S. agalactiae***
NT					
*t*ZNT	nd	nd	0.026 ± 0.025	nd	nd
DZNT	nd	0.88 ± 0.05	nd	nd	nd
*c*ZNT	nd	nd	nd	nd	0.01 ± 0.00
iPNT	nd	11.8 ± 1.39	4.20 ± 0.33	0.11 ± 0.25	0.63 ± 0.10
Total NT	**nd**	**12.68 ± 1.44**	**4.23± 0.36**	**0.11 ± 0.25**	**0.64 ± 0.10**
RB					
iPR	nd	33.5 ± 3.59	0.11 ± 0.03	0.01 ± 0.01	0.05 ± 0.01
Total RB	**nd**	**33.5 ± 3.59**	**0.11 ± 0.03**	**0.01 ± 0.01**	**0.05 ± 0.01**
FB					
*t*Z	nd	2.25 ± 0.19	nd	nd	nd
*c*Z	nd	nd	nd	nd	0.03 ± 0.02
iP	nd	3.71 ± 0.46	3.41 ± 0.39	0.22 ± 0.03	0.19 ± 0.02
Total FB	**nd**	**5.96 ± 0.65**	**3.41 ± 0.39**	**0.22 ± 0.03**	**0.22 ± 0.02**
MET					
2MeSZR	nd	nd	nd	0.05 ± 0.03	0.05 ± 0.01
2MeSiPA	nd	0.02 ± 0.002	0.18 ± 0.05	nd	nd
2MeSZ	0.37 ± 0.03	0.85 ± 0.06	0.96 ± 0.06	0.73 ± 0.15	0.58 ± 0.40
2MeSiP	nd	nd	1.63 ± 0.17	nd	nd
Total MET	**0.37 ± 0.03**	**0.87 ±0.062**	**2.77 ± 0.28**	**0.77 ± 0.13**	**0.63 ± 0.40**
Total CK	**0.37 ± 0.03**	**53.01 ± 5.74**	**10.52 ± 1.06**	**1.11 ± 0.16**	**1.54 ± 0.47**

Table IV. **CK concentration in wild type and mutant *Escherichia coli* strains (pmol/mL)**. Bacterial cells were grown in M9 medium and the CK analysis was performed from the supernatants. NT, nucleotides ; RB, riboside ; FB, free base ; MET, Methylthiols ; *t*ZNT, *trans*-Zeatin riboside-5′-monophosphate ; iPNT, isopentenyladenosine-5′-monophosphate ; iPR, isopentenyladenosine ; *c*Z, *cis*-Zeatin ; iP, isopentenyladenine ; 2MeSiPA, 2-Methylthio-isopentenyladenosine ; 2MeSiP, 2-Methylthio-isopentenyladenine; nd, not detected.

	WT	***miaA*Δ**	***miaB*Δ**	***ygdH22*Δ**
NT				
tZNT	0.014 ± 0.00	0,016 ± 0.00	0.018 ± 0.01	0,015 ± 0.00
iPNT	0.87 ± 0.01	0,015 ± 0.00	2.47 ± 0.24	0.80 ± 0.02
Total NT	**0.89 ± 0.01**	**0.031 ± 0.00**	**2.49 ± 0.25**	**0.82 ± 0.02**
RB				
iPR	0.38 ± 0.002	nd	0.42 ± 0.03	0.43 ± 0.17
Total RB	**0.38 ± 0.002**	**nd**	**0.42 ± 0.03**	**0.43 ± 0.17**
FB				
*c*Z	0.31 ± 0.004	0.28 ± 0.05	0.20 ± 0.01	0.30 ± 0.06
iP	12.8 ± 0.28	nd	14.9 ± 0.26	11.3 ± 0.17
Total FB	**13.1 ± 0.29**	**0.28 ± 0.05**	**15.0 ± 0.27**	**11.6 ± 0.23**
MET				
2MeSiPA	0.09 ± 0.00	nd	nd	0.15 ± 0.02
2MeSiP	2.17 ± 0.02	nd	nd	2.47 ± 0.23
Total MET	**2.26 ± 0.02**	**nd**	**nd**	**2.62 ± 0.25**
Total CK	**16.63 ± 0.32**	**0.31 ± 0.05**	**17.91 ± 0.55**	**15.47 ± 0.65**