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RESEARCH PAPER

Diphenylurea-derived cytokinin oxidase/dehydrogenase inhibitors for biotechnology and agriculture

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Abstract

Increasing crop productivity is our major challenge if we are to meet global needs for food, fodder and fuel. Controlling the content of the plant hormone cytokinin is a method of improving plant productivity. Cytokinin oxidase/dehydrogenase (CKO/CKX) is a major target in this regard because it degrades cytokinins. Here, we describe the synthesis and biological activities of new CKX inhibitors derived mainly from diphenylurea. They were tested on four CKX isoforms from maize and Arabidopsis, where the best compounds showed IC_{50} values in the 10^{-8} M concentration range. The binding mode of the most efficient inhibitors was characterized from high-resolution crystal complexed structures. Although these compounds do not possess intrinsic cytokinin activity, we have demonstrated their tremendous potential for use in the plant tissue culture industry as well as in agriculture. We have identified a key substance, compound 19, which not only increases stress resistance and seed yield in Arabidopsis, but also improves the yield of wheat, barley and rapeseed grains under field conditions. Our findings reveal that modulation of cytokinin

Abbreviations: CKX, cytokinin oxidase/dehydrogenase; iP, N^6-isopentenyladenine; PGR, plant growth regulators; SP, seeds per silique; TFM, trifluoromethoxy group; TGW, thousand grain weight.

levels via CKX inhibition can positively affect plant growth, development and yield, and prove that CKX inhibitors can be an attractive target in plant biotechnology and agriculture.

Keywords: Agriculture, biotechnology, CKX inhibitor, crystal structure, cytokinin, cytokinin oxidase/dehydrogenase, diphenylurea, plant tissue culture, stress, yield.

Introduction

The global population and its need for food, fodder and bioenergy fuel are rapidly increasing. Seeds are the major source of the world's food calories. However, recent increases in meat consumption, together with the use of grain for biofuel production have placed new pressures on global grain supplies (Edgerton, 2009). One way to meet this challenge is to increase crop productivity on existing farmland. Cytokinins are key plant hormones, and numerous improvements in seed yield have been achieved by increasing cytokinin content in plants (e.g. Ashikari *et al*., 2005; Zalewski *et al*., 2010; Bartrina *et al*., 2011).

Cytokinins regulate essential plant processes, including cell division, development of shoot, root and reproductive organs, seed fill and senescence (Kieber and Schaller, 2014). Cytokinins are degraded by cytokinin oxidase/dehydrogenase (CKO or CKX, EC 1.5.99.12, Chatfield and Armstrong, 1986; Hare and Van Staden, 1994), which catalyzes their irreversible oxidative breakdown to adenine/adenosine and the corresponding aldehyde (Whitty and Hall, 1974; Brownlee *et al*., 1975). *Arabidopsis thaliana* contains seven CKXs (Werner *et al*., 2003), of which AtCKX2 is the most active and well-studied isoform (Galuszka *et al*., 2007). *Zea mays* has 13 *CKX* genes, of which *ZmCKX1* plays a crucial role in cytokinin degradation (Houba-Hérin *et al*., 1999; Morris *et al*., 1999). Each CKX isoform usually differs in substrate specificity, spatial and temporal expression patterns and sub-cellular localization (e.g. Šmehilová *et al*., 2009; Vyroubalová *et al*., 2009).

Cytokinins gained interest in the search for potential agrochemicals due to their positive effects on tillering, flower and seed setting, delaying of senescence, and mitigation of stress consequences (Koprna *et al*., 2016). Nonetheless, exogenous application of cytokinins has not found a place in agricultural practice, most probably due to the complexity and variability of their effects. However, it has been shown that when levels of endogenous cytokinins are slightly increased throughout certain periods, an improvement in agricultural traits can be achieved. For example, a reduction or loss of function of *OsCKX2* in rice (*Oryza sativa*) led to accumulation of cytokinins in inflorescence meristems, resulting in an increased number of reproductive organs. The total grain number per plant was enhanced by ~30% and the grain size was not affected (Ashikari *et al*., 2005). The key role of CKX in the formation of inflorescence meristems was also confirmed in Arabidopsis, where double *ckx* knock-out plants showed 55% yield increase as a result of higher seed number per silique (Bartrina *et al*., 2011). Silencing of *HvCKX1* expression in barley (*Hordeum vulgare*) was accompanied by 31–43% higher grain number. The thousand grain weight (TGW) was approximately 20% higher and total grain yield per plant was 50–65% higher than those of control plants (Zalewski *et al*., 2010).

Before genetically modified (GM) plants were engineered, chemical inhibitors of CKX were already known. They were discovered in the 1980s among synthetic cytokinins; these include diphenylurea, thidiazuron (TDZ), 1-(2-chloropyridin-4-yl)-3-phenylurea (CPPU) and 1-(2,6-dichloropyridin-4-yl)-3-phenylurea (DCPPU). These compounds inhibit the degradation of natural cytokinins catalyzed by CKX (Chatfield and Armstrong, 1986; Burch and Horgan, 1989; Kopečný *et al*., 2010). These ureas are, however, classified as cytokinins because their cytokinin properties clearly outweigh their CKX inhibitory activities. For example, TDZ inhibits CKX only at high micromolar concentrations, but exhibits strong cytokinin effects in the low nanomolar range (Nisler *et al*., 2016). Nowadays, TDZ and CPPU are used as important growth promoters for various horticultural crops, such as kiwi fruit and melon (Arima *et al*., 1995). Furthermore, TDZ is a component in a large number of agrochemical products used for cotton defoliation in many countries. TDZ also has an irreplaceable role in the plant tissue culture industry for its ability to induce adventitious shoot formation and to promote axillary shoot proliferation (Lu, 1993; Guo *et al*., 2011).

Anilinopurine derivatives have been shown to be potent CKX inhibitors (Zatloukal *et al*., 2008) that promote *in vitro* organogenesis (Aremu *et al*., 2015), and increase plant stress tolerance toward salinity (Aremu *et al*., 2014) and cadmium (Gemrotová *et al*., 2013). A positive effect of F-INCYDE [the name of the most active inhibitor, 2-fluoro-6-(3-methoxyphenyl)aminopurine] on heat stress tolerance and grain yield of rice in field conditions was reported by Syngenta in patent applications. Their data obtained in India during the years 2012–2016 demonstrated that F-INCYDE improved the tolerance of rice seedlings to stresses of heat and transplanting. Treated plants also had a grain yield increase of up to 8.8% (Camblin and Pingel, 2017; Camblin *et al*., 2017; Kon *et al*., 2017). Multi-year analysis for 2015–2016 showed that F-INCYDE increased rice grain yield by up to 6.5%, mainly due to a reduction in the number of empty spikelets and a 2% increase in TGW. A clear beneficial effect of the treatment was

observed when the maximum temperature was above 36 °C on at least one day after application (Camblin *et al.*, 2017). These results, together with the commercial interest in F-INCYDE, prove that the development of CKX inhibitors offers promise in agriculture.

Here, we present a comprehensive developmental study of the biological properties of much more potent CKX inhibitors derived from CPPU, DCPPU and diphenylurea. We performed structure-function analysis using four CKX enzymes—AtCKX2 from *Arabidopsis thaliana*, and maize ZmCKX1, ZmCKX4a, and ZmCKX8. The binding mode of the two best inhibitors with ZmCKX4a and ZmCKX8 isoforms was analyzed by X-ray crystallography. Furthermore, we demonstrated the effects of selected compounds in cytokinin bioassays, and their applications in plant tissue culture. Key compounds were further tested for alleviation of stress responses and for yield enhancement in Arabidopsis. Finally, we evaluated the effect of one compound on yield and yield-forming parameters in rapeseed, barley and wheat, in field trials.

Materials and methods

Cytokinins and chemicals

N^6-benzyladenine (BA), *trans*-zeatin, CPPU, DCPPU, and TDZ were gifts from Olchemim, Czech Republic (https://www.olchemim.cz/). The compound 2,6-dichloro-4-isocyanatopyridine was from Atlantic Research Chemicals, UK (https://atlantic-chemicals.com/). Compounds 2-chloropyridin-4-amine, 1,3-dichloro-5-isocyanatobenzene, aniline, 2-(2-aminophenyl)ethan-1-ol, 2-(trifluoromethoxy)aniline, 3-(trifluoromethoxy)aniline, 4-(trifluoromethoxy)aniline, 2-methoxyaniline, (2-aminophenyl)methanol, 2-aminobenzoic acid, triphenylphosphine (PPh_3), triethylamine (TEA), hexachloroethane, phthalimide, potassium phthalimide, *tert*-butyldimethylsilyl chloride, hydrazine, diethyl azodicarboxylate (DIAD), carbonyldiimidazole (CDI) and 2-aminoethan-1-ol, were purchased from Sigma Aldrich, USA (https://www.sigmaaldrich.com). Compounds 2-(2-aminophenyl) acetic acid, 1-(2-aminophenyl)ethanol, 3-chloro-5-(trifluoromethoxy) aniline, 3-bromo-5-(trifluoromethoxy)aniline, methyl 3-amino-5-chlorobenzoate, 3-chloro-5-methoxyaniline, 3-(2,2,2-trifluoroethoxy) phenylamine and 1-isothiocyanato-3-(trifluoromethoxy)benzene were from Fluorochem, UK (http://www.fluorochem.co.uk/). Finally, 3-(trifluoromethylthio)aniline was from abcr GmbH, Germany (https://www.abcr.de).

General synthesis

Compounds 1–32 (listed in the Supplementary data at *JXB* online) were synthesized by nucleophilic addition of substituted aniline (or aniline) to substituted 4-isocyanatopyridine, isocyanatobenzene, or to isothiocyanatobenzene (Goldschmidt and Bardach, 1892). If the isocyanate was not commercially available it was synthesized from amine and diphosgene according to Kurita *et al.* (1976) with minor modifications. Briefly, starting amine (0.5 mmol) was dissolved in dry tetrahydrofuran (THF, 3 ml) and triethylamine (1.2 mmol) was added. This solution was added dropwise to a solution of diphosgene (0.26 mmol) in dry THF (5 ml) or dichloromethane (DCM, 5 ml) at –20 °C, then the white suspension was allowed to warm up to laboratory temperature, 25 °C and stirred for 30 min. If the conversion to isocyanate was not complete (monitored by TLC analysis), the solution was warmed up to 50 °C for another 30 min. After cooling to 25 °C, a white precipitate of triethylamine hydrochloride was filtered off and the remaining solution was evaporated. Crude isocyanates were used without analysis, directly in further synthesis reactions. This procedure led to synthesis of these isocyanates: 2-chloro-4-isocyanatopyridine, 1-isocyanato-3-(trifluoromethoxy)benzene, 1-chloro-3-isocyanato-5-(trifluoromethoxy)benzene, 1-bromo-3-isocyanato-5-(trifluoromethoxy) benzene, 1-chloro-3-isocyanato-5-methoxybenzene, 1-isocyanato-2-(trifluoromethoxy)benzene, 1-isocyanato-4-(trifluoromethoxy)benzene, 1-isocyanato-3-(trifluoromethylsulfanyl)benzene and 1-isocyanato-3-(2,2,2-trifluoroethoxy)benzene. The final compounds **1–32** were prepared by stirring a solution containing the isocyanate (0.5 mmol) and the corresponding amine (0.5 mmol) in THF, DCM, or acetone, at either 25°C or higher (details are provided below). The formation of urea derivative was monitored using TLC analysis (mobile phase usually chloroform:methanol, 9:1). If the (substituted)-aniline to be coupled with isocyanate contained a free hydroxyl group, this was protected by *tert*-butylchlorodimethylsilane (TBDMS), according to common protocols (Wuts and Greene, 1991), prior to the condensation reaction. Then the urea derivative formed was deprotected with 2-propanolic HCl (Wuts and Greene, 1991). All compounds were characterized by MS-ESI, ^{1}H NMR and melting point analysis. Details of synthesis, purification and analysis are provided in the Supplementary data available at *JXB* online.

General experimental procedures

Thin-layer chromatography (TLC) was performed using aluminum sheets with silica gel F_{254} from Merck (Germany). Column chromatography (CC) was performed using Merck silica gel Kieselgel 60 (230–400 mesh). The chromatographic purity was analyzed using HPLC with UV and PDA detectors (Beckman Gold System, CA, USA). The retention time (R_t) of the compounds synthesized was also scored. Compounds (1 mg) were dissolved in 1 ml of 10% methanol and injected (10 µl) onto a reverse-phase column (Symmetry C18, 5 µm, 150 mm×2.1 mm; Waters, Milford, MA, USA) held at 25 °C. Solvent (A) consisted of 15 mM ammonium formate adjusted to pH 4.0. Solvent (B) was methanol. With a flow-rate of 200 µl min^{-1}, the following binary gradient was used: 0 min, 10% B; 0–24 min; linear gradient to 90% B; 25–34 min, isocratic elution with 90% B; 35–45 min, linear gradient to 10% B. Eluting compounds were detected by scanning the UV absorbance of the eluate between 240 and 320 nm. The effluent was then introduced to a PDA detector. The purity of compounds (%) was recorded at a compound absorption maximum [λ(max)]. Melting points were determined on a Kofler block and were uncorrected. Mass spectra were determined using a Waters Micromass ZMD (USA) mass spectrometer (solution in MeOH, direct inlet, coin voltage 20 V, trace amounts of formic acid or aqueous ammonia were used to influence ionization and formation of clusters). The major peaks being reported with intensities were quoted as percentage of the base peak. NMR spectra were recorded on a JEOL ECA-500 spectrometer (Japan) operating at a frequency of 500.16 MHz (^{1}H). ^{1}H NMR chemical shifts were referenced to the solvent signals.

CKX inhibition measurements

The ability of the synthesized compounds to inhibit CKXs was determined based on their IC_{50} values in the 2,6-dichlorophenolindophenol (DCPIP) assay (Frébort *et al.*, 2002). For each compound a concentration-dependent curve of CKX residual activity was constructed, and IC_{50} determined using GraphPad Prism. The assays were performed at 37 °C in 100 mM potassium phosphate buffer, pH 7.4 using the substrate N^6-isopentenyladenine (iP) at a saturating concentration (45 µM for AtCKX2 and ZmCKX4a, 10 µM for ZmCKX1, and 12 µM for ZmCKX8). The compounds were tested with two replicates and the entire test was repeated at least twice.

Pure enzymes were used in the case of ZmCKX1, ZmCKX4a and ZmCKX8. ZmCKX1 was expressed in the yeast *Yarrowia lipolytica* and purified according to a published protocol (Kopečný *et al.*, 2005). *Escherichia coli* BL21 STAR (DE3) cells carrying pTYB12-*ZmCKX4a* and *ZmCKX8* plasmids were grown at 18 °C overnight and the enzymes were purified as previously described (Zalabák *et al.*, 2014). In the case of AtCKX2, the enzyme produced in the growth media from *Saccharomyces cerevisiae* was used (Frébortová *et al.*, 2007).

Crystallization and structure refinement of ZmCKX4a and ZmCKX8 complexes

ZmCKX4a (15 mg ml^{-1}, 50 mM Tris-HCl, pH 8.0) was co-crystallized with 2 mM compound 19 or 20 in drops containing 100 mM HEPES buffer, pH 7.5 and 50% of 2-methyl-2,4-pentanediol. Crystals were immediately plunged into liquid nitrogen. ZmCKX8 (17 mg ml^{-1}, 50 mM Tris-HCl, pH 8.0) was crystallized in 100 mM HEPES buffer, pH 7.5, 15% polyethylene glycol and 15% isopropanol. Crystals were soaked with 4 mM compound 20 for 40 s and then cryoprotected using 15% glycerol. Diffraction data were collected at 100 K on the Proxima 1 beamline at the SOLEIL synchrotron (Saint-Aubin, France), at 1.95 Å resolution. Intensities were integrated using the XDS program (Kabsch, 2010). Data quality was assessed using the correlation coefficient $CC_{1/2}$ (Karplus and Diederichs 2012). The crystal structure of ZmCKX8 was determined by performing molecular replacement with Phaser (University of Cambridge, freeware) (Storoni *et al.*, 2004) using a monomer of ZmCKX4a (PDB ID 4O95) as a search model (Kopečný *et al.*, 2016). Model refinement was achieved with BUSTER-TNT software (Global Phasing Limited, UK (Bricogne *et al.*, 2011), and electron density maps were evaluated using COOT (Crystallographic Object-Oriented Toolkit, freeware) (Emsley and Cowtan, 2004). Structure quality was validated using MolProbity (Duke University, freeware) (Chen *et al.*, 2010). Molecular graphics images (Fig. 2B-E) were generated using PyMOL software (Schrödinger, Inc., USA).

Cytokinin bioassays

The Arabidopsis response regulator 5 *(ARR5::GUS)* reporter gene assay was generally performed as described previously (Romanov *et al.*, 2002), and details are in Nisler *et al.* (2016). The CYTOKININ RESPONSE 1/ ARABIDOPSIS HISTIDINE KINASE 4 (CRE1/AHK4) cytokinin receptor activation assay was done as described by Spíchal *et al.* (2004). The wheat leaf senescence assay was performed as described by Holub *et al.* (1998).

Quantitative real-time PCR

Arabidopsis thaliana (ecotype Col-0) was grown on agar for one week and subsequently, plants seedlings were spray-treated with water (control), 5 μM N^6-benzyladenine (BA, positive control), or 5 μM and 0.1 μM compound 19. RNA was collected at 1, 3, 5, and 24 h after spraying. Total RNA was isolated from the entire seedling using TRIzol reagent (100 mg sample ml^{-1} reagent, Invitrogen, USA). The RNA pellet was air-dried on ice for 5 min, resuspended in water and treated twice with a Turbo DNase-free kit (Invitrogen, USA). First-strand cDNA was synthesized by SuperScript III reverse transcriptase and oligo(dT) primers (Thermo Fisher Scientific, USA). The RNA was reverse transcribed from three biological replicates (each replicate represents 15 seedlings plantlets) and qRT–PCR reactions were performed in quadruplicate on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) using Luna universal probe qRT–PCR master mix (New England Biolabs, USA); both primers were used at 400 nM with a 200 nM dual-labeled TaqMan probe (FAM/TAM or FAM/MGB-NFQ). Primers and TaqMan probes were designed using Primer Express 3.0 software (Life Technologies, USA) and are shown in Supplementary Table S1. Cycle threshold values were normalized for genes of the elongation factor 1-alpha (*AtEF1α*) and actin-8 (*AtACT8*) and amplification efficiency. Expression values were obtained from four independent replicates, each consisting of 15 plants. Expression values were determined and statistically evaluated using the DataAssist v3.0 Software package (Life Technologies). Before performing a *t*-test, data groups were verified by an *F*-test to determine whether they possess the same variance. Gene names, dual probes and primer pairs used for qRT–PCR are given in Supplementary Table S1.

Shoot regeneration on tobacco leaf disks and poplar explants

Tobacco (*Nicotiana tabacum*) leaf discs (ac. 0.5 cm) were placed in Petri dishes with 10 ml Murashige and Skoog (MS) medium, including 30 g l^{-1}sucrose and 7 g l^{-1} plant agar (pH 5.8). After autoclaving, this medium was supplemented with 0, 0.01, 0.1 or 1 μM compound **19** or **21** combined with 0, 0.01, 0.1 or 1 μM iP. For each hormone, three replicates each of five leaf discs were used. The experiment was evaluated after three weeks and repeated three times.

Shoot regeneration in poplar (*Populus* × *canadensis* 'Robusta') involved two steps. In the first phase, callus induction was initiated for two weeks on a callus induction medium consisting of MS salts and vitamins supplemented with 30 g l^{-1} sucrose, 7 g l^{-1} agar and 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid. The explants were then transferred to a shoot induction medium consisting of the same basal medium, but containing a combination of 0 or 1 μM iP with 0 or 1 μM compound 19 or 20. Leaf disks (0.5–1 cm^2) were punched out from *in vitro* plantlets. Fifteen explants in each of three replicates per treatment were used in the experiment. The experiment was evaluated after four weeks.

Alleviation of stress consequences in Arabidopsis seedlings

The assay was performed as described in detail by Ugena *et al.* (2018). *Arabidopsis thaliana* (ecotype Col-0) seeds were germinated on full MS medium for three days without any treatment. They were then transferred to the same medium containing 10 nM compound 19 in the absence or presence of the abiotic stress factor (100 mM NaCl or 100 mM mannitol, the latter representing osmotic stress). DMSO (0.1%) was used as a solvent control in all variants. The dynamics of rosette growth and the relative growth rate were evaluated by recording the green area using RGB imaging, twice a day during the 6 d after seedling transfer, in the PlantScreen™ XYZ system (Photon Systems Instruments, Brno, Czechia).

Effects of compound 19 on phenotype and seed yield of A. thaliana

Arabidopsis thaliana (ecotype Col-0) was grown in a growth chamber on soil at 24 °C under long-day conditions (16 h light/8 h dark), at a light intensity of 100 μmol $m^{-2}s^{-1}$, and 65% humidity. Compound 19 was dissolved in water (with 0.1% DMSO and 0.01% Silwet) and sprayed on plants 12 days after seed stratification at 4 °C (in the dark for 72 h). In the first experiment, 25 plants were grown for each treatment (control, 0.1, 1, and 10 μM compound 19) and for all sets of plants, the number of siliques per plant and total grain yield were determined. The number of seeds per silique (SP) parameter was evaluated using 10 randomly selected plants from each treatment, and from six siliques of each plant. The weight of 100 seeds was determined three times for each treatment from the combined sample. In a second experiment, we used the same approach with 18 plants. In addition, their rosette leaf size was determined following 25 days of growth using Adobe Photoshop Elements 9 (Adobe, USA), where the rosette area was selected manually using the magic wand tool.

Field trials

Field experiments were done with spring barley (*Hordeum vulgare*), winter wheat (*Triticum aestivum*), and winter rapeseed (*Brassica napus*), according to the criteria for Good Agricultural Practices (six randomized plots of 10 m^2 were used for one variant). The plots were treated solely with compound 19.

Results and discussion

Design and chemistry of novel CKX inhibitors

In previous studies (Kopečný *et al.*, 2010, Nisler *et al.*, 2016), we analyzed several CPPU and TDZ derivatives and their inhibitory strength towards CKXs. Our results shed light on the reactive loci of the urea compounds, essential for their binding to the active site of CKX, and allow the targeted design of new compounds. Insertion of 2-hydroxyethyl at the *ortho*-position of the TDZ phenyl ring (the name of the compound was abbreviated to 2HE-TDZ; Fig. 1A) enhanced the inhibitory strength of the ligand (Nisler *et al.*, 2016). We therefore synthesized the corresponding derivatives of CPPU and DCPPU (compounds 1 and 4, Fig. 1B and Table 1). Moreover, we found that 1-(3,5-dichlorophenyl)-3-phenylurea (compound 6) exhibits slightly better inhibitory potency than DCPPU (Table 1). This diphenylurea was therefore selected for a larger screening study, in which its empty phenyl ring received one or two carbon-containing substituents in the *ortho*-position to evaluate their effect on CKX inhibition (compounds 6–15, Fig. 1C and Table 1).

Another strong incentive for further development was the fact that compound 3TFM-TDZ (Fig. 1A) binds at the active site of ZmCKX4a in the opposite orientation to that of 2HE-TDZ (Nisler *et al.*, 2016). The compound 1-(2-(2-hydroxyethyl) phenyl)-3-(3-(trifluoromethoxy)phenyl)urea (compound 19, 3TFM-2HE, Fig. 1A) was therefore synthesized and analyzed (Tables 2 and 3). Among the four compounds 16–19 (Fig. 1D), compound 19 exhibited the highest CKX inhibitory potency, comparable to that of compound 12. It became obvious that CKX isoforms had space to accommodate not only the trifluoromethoxy group (TFM) but also two chlorine atoms, as found in the structure of compound 12. Thus compounds 20, 21 and 22 were synthesized (Fig. 1D and Table 2). Compounds 23–31 (Fig. 1E and Table 2) were produced to explore the structure-activity relationships of compounds modified on the ring bearing the 3TFM group. The thiourea derivative compound 32 was designed analogous to the active urea compound 19. Finally, compounds 2 and 5 were prepared to analyze the effect of a 3-methoxyphenyl group, which is present in the structure of F-INCYDE (Zatloukal *et al.*, 2008).

Compounds 1–32 were prepared according to common protocols for the synthesis of diphenylurea derivatives (Goldschmidt and Bardach, 1892). Many derivatives of diphenylurea, CPPU, and DCPPU have been prepared previously (Bruce and Zwar, 1966; Takahashi *et al.*, 1978; Okamoto *et al.*, 1981), but only Kopečný *et al.* (2010) analyzed their CKX-inhibitory activity.

Inhibition of AtCKX2 and ZmCKX1

AtCKX2 and ZmCKX1 are among the most active isoforms of CKXs in their species (Galuszka *et al.*, 2007; Zalabák *et al.*, 2014). It is known that *ZmCKX1* is expressed in maize roots, tassels and kernels (Vyroubalová *et al.*, 2009, Zalabák *et al.*, 2014). Expression of the *AtCKX2* gene was detected in the shoot apex, in stipules, and occasionally in the most apical parts of inflorescence stems in Arabidopsis (Werner *et al.*, 2003). ZmCKX1 is is a glycosylated and an extracellular protein (Kopečný *et al.*, 2005; 2006). AtCKX2 may also be an extracellular protein (Werner *et al.*, 2003). Recently, a homology model of AtCKX2 revealed that the amino acid composition of its active site is almost identical to that of ZmCKX1 (Nisler *et al.*, 2016).

The attachment of a 2-hydroxyethyl group to the *ortho* position of the phenyl ring of CPPU and DCPPU led to improvements in the CKX-inhibitory strength of both molecules (compounds 1 and 4**, Table 4**). The attachment of a methoxy group to the *meta* position had the opposite effect (compounds 2 and 5). Interestingly, a diphenylurea derivative 3,5 DCl-Ph-U (compound 6) displayed better inhibitory activity than the pyridyl-phenylurea derivative, DCPPU. This means that the presence of *endo*-cyclic nitrogen is not essential for CKX inhibition. This result was also confirmed in the case of compound 12, which displayed approximately seven times lower IC_{50} values than compound 4. Among all other substitutions tested on the 3,5 DCl-Ph-U backbone, only hydroxymethyl (compound 8) and 2-hydroxyethyl (compound 12) groups increased the inhibitory activity of the compound. An overview of the IC_{50} values is given in Table 1.

Compounds 16–22 are 1-phenyl-3-(3-trifluoromethoxyphenyl)urea (3TFM-Ph-U) derivatives (Fig. 1D, Table 2). The compounds 16 and 19 exhibit very similar IC_{50} values to those of compounds 8 and 12, which contain a 3,5-dichlorophenyl ring (Fig. 1C, Table 2). This demonstrates that the TFM group has an effect on IC_{50} values comparable to that of two chlorines. If the compound contains, on one phenyl ring, a combination of a TFM group with chlorine (compound 20), bromine (compound 21) or 2-hydroxyethylamine (compound 22), the IC_{50} value decreases to 10^{-8} M. These compounds are the strongest inhibitors of AtCKX2 and ZmCKX1 among all the derivatives developed so far (Tables 2 and 3).

Compounds 23–32 are 1-(2-(2-hydroxyethyl)phenyl)-3-phenylurea (Ph-2HE-U) derivatives (Fig. 1E, Table 2) with modifications on the TFM-bearing ring. We were interested to compare the inhibitory activity of compounds 20 and 24, the latter being distinguished by missing three fluorine atoms from the methoxy group. This inhibitor showed an almost 8-fold loss of inhibitory activity, compared with compound

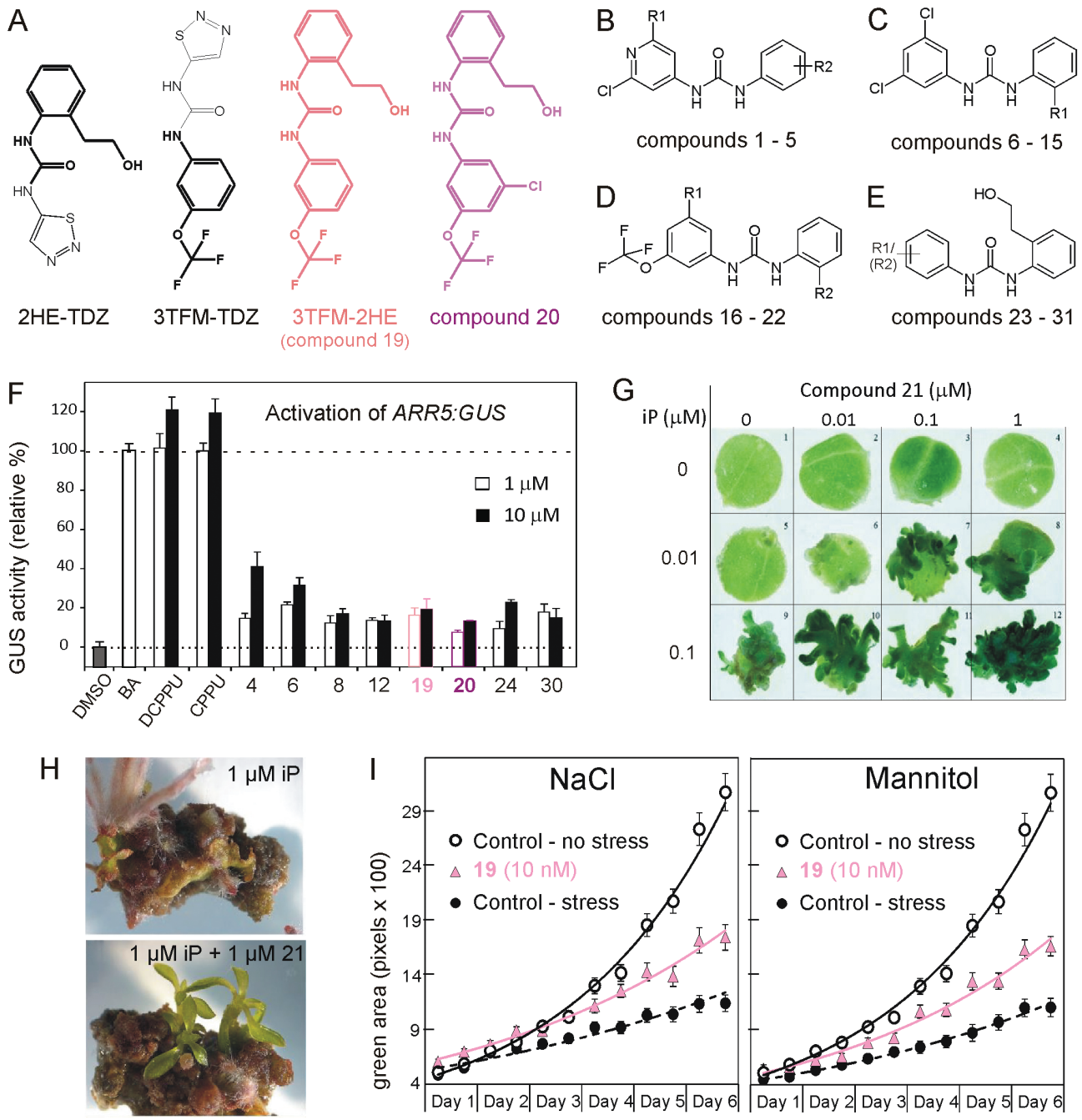

Fig. 1. (**A**) Structures of two previously described CKX inhibitors (black, Nisler *et al.*, 2016) and two new CKX inhibitors (pink and purple) from this work. A combination of parts of molecules that are shown in black and bold gave rise to compound 19. (**B-E**) General structures of prepared derivatives 1–31. (**F**) Quantitative evaluation of the cytokinin activities of the compounds in *ARR5*:GUS assays. Their activity is compared to that of 1μM N^6-benzyladenine (BA), which was set as 100% activation (dashed line). DMSO (0.1%) was used as solvent control (dotted line). Error bars show SD of two parallel assays, each consisting of two replicates. (**G**) The effect of iP, compound 21 and combinations thereof, on adventitious shoot induction from tobacco leaf discs. The image corresponds with data in Table 4. Last line for 1 μM iP is omitted from the picture. (**H**) *De novo* organogenesis on poplar leaf explant four weeks after incubation. (**I**) The effect of compound 19 on growth (presented as green area) of Arabidopsis seedlings grown under stress. Error bars show SD of the mean for five replicate determinations.

20. Compounds 28 and 29, having the TFM group in *ortho* and *para*-positions, respectively, exhibited much weaker inhibitory potency than compound 19. Conversely, the exchange of the TFM group in the structure of compound 19 for a trifluoromethylsulfanyl group (compound 30) resulted in a lower IC_{50} value, as shown in Table 2. Finally, results obtained

Table 1. Structures of CPPU, DCPPU and 3,5DCl-Ph-U (1-(3,5-dichlorophenyl)-3-phenylurea) derivatives prepared, and overview of their IC_{50} values measured with four CKX isoforms.

CPPU and DCPPU derivatives		IC_{50} **(μM)**			
Compound	**R substituents**	**AtCKX2**	**ZmCKX1**	**ZmCKX4a**	**ZmCKX8**
CPPU	R1—H, R2—H	56±3	20.5±1.1	7.6±0.6	1.6±0.1
1	R1—H, R2 - 2-CH_2CH_2OH	4.7±0.2	1.3±0.2	9±0.5	1.8±0.02
2	R1—H, R2 - 3-OCH_3	64±4	7.0 ±0.2	-	-
3	R1—H, R2 - 3Cl (pyridin-4-yl)	31±2	6.6±0.1	-	-
DCPPU	R1—Cl, R2—H	2.7±0.21	4.5±0.1	2.2±0.2	0.25±0.01
4	R1—Cl, R2 - 2-CH_2CH_2OH	0.8±0.1	0.43±0.06	3.9±0.3	0.49±0.02
5	R1—Cl, R2 - 3-OCH_3	17±2	0.68±0.03	3.7±0.1	2.2±0.2
3,5DCl-Ph-U derivatives					
6	R1—H	2.1±0.14	0.84±0.06	1.3±0.2	0.54±0.01
7	R1 - CH_2NH_2	8.4±0.07	8.1±0.7	-	-
8	R1 - CH_2OH	0.23±0.01	0.40±0.03	1.7±0.1	1.2±0.023
9	R1—COOH	28±1.4	1.4±0.3	-	-
10	R1 - CH_2CH_2Cl	4.2±0.5	5.6±0.3	-	-
11	R1 - $CH_2CH_2NH_2$	20±2	1.5±0.28	-	-
12	R1 - CH_2CH_2OH	0.11±0.01	0.18±0.01	2.5±0.5	0.33±0.014
13	R1 - CH_2COOH	175±25	16±1	-	-
14	R1 - CH_2CONH_2	30±4.2	0.69±0.01	-	-
15	R1 - -CH_2CH_2-	5.1±0.35	0.74±0.01	-	-

Structural formulas and positions of substituents within each urea compound are shown in Fig 1B and C, respectively. Errors show SD of at least two parallel assays, each consisting of two replicates.

Table 2. Structures of 3TFM-Ph-U (1-phenyl-3-(3-(trifluoromethoxy)phenyl)urea) and Ph-2HE-U (1-(2-(2-hydroxyethyl)phenyl)-3-phenylurea) derivatives prepared, and overview of their IC_{50} values measured with four CKX isoforms.

3TFM-Ph-U derivatives		IC_{50} **(μM)**			
Comp.	**R substituents**	**AtCKX2**	**ZmCKX1**	**ZmCKX4a**	**ZmCKX8**
16	R1—H, R2 - CH_2OH	0.2±0.01	1.1±0.07	8.1±0.3	4.1±0.1
17	R1—H, R2—$CH(OH)CH_3$	13±0.7	1.7±0.1	-	-
18	R1—H, R2 - CH_2OCH_3	3.5±0.5	5.5±0.7	-	-
19	**R1—H, R2 - CH_2CH_2OH**	**0.23±0.01**	**0.10±0.01**	**1.5±0.07**	**0.19±0.01**
20	**R1—Cl, R2 - CH_2CH_2OH**	**0.025±0.004**	**0.058±0.002**	**0.37±0.08**	**0.059±0.004**
21	**R1—Br, R2 - CH_2CH_2OH**	**0.033±0.003**	**0.035±0.003**	**0.25±0.07**	**0.030±0.002**
22	R1 - $NHCH_2CH_2OH$ R2 - CH_2CH_2OH	0.064±0.007	0.029±0.005	1.5±0.04	0.61±0.08
Ph-2HE-U derivatives					
23	R1 - 3-COOH R2 - 5-Cl	2.4±0.2	11±1	34.3±6.7	-
24	R1 - 3-OCH_3 R2 - 5-Cl	0.21±0. 1	0.18±0.01	10.8±9	0.78±0.04
25	R1 - 3-OCH_3 R2 - 5-OCH_3	1.4±0.03	1.0±0.06	-	-
26	R1- 3-CH_2OH R2- 5-Cl	0.69±0.05	0.73±0.01	14.5±0.6	-
27	R1- 2-CH_2OH R2- 5-Cl	1.5±0.07	0.6±0.03	-	-
28	R1 - 2-OCF_3	80±4	16±3	-	-
29	R1 - 4-OCF_3	16±1.5	5.1±0.4	-	-
30	R1 - 3-SCF_3	0.13±0.01	0.074±0.001	0.82±0.18	0.54±0.008
31	R1 - 3-CH_2OCF_3	1.0±0.03	1.0±0.01	3.5±0.2	-
32	R1 - 3-OCF_3, thiourea	-	80±15	-	-

Structural formulas and positions of substituents within each urea compound are shown in Fig 1D and E respectively. Errors show SD of at least two parallel assays, each consisting of two replicates.

Table 3. Overview of the inhibitory strengths of known CKX inhibitors and comparison with the most potent CKX inhibitors from this work.

	IC_{50} (μM)			
Compound	**AtCKX2**	**ZmCKX1**	**ZmCKX4a**	**ZmCKX8**
TDZ	62±6	44.3±2.7	223±25	4.6±0.1
CPPU	56±3	20.5±1.1	7.6±0.6	1.6±0.1
DCPPU	2.7±0.2	4.5±0.2	2.2±0.1	0.25±0.01
F-INCYDE	1.0±0.2	0.6±0.1	17±4	0.18±0.02
2HE-TDZ	3.9±0.6	2.8±0.5	136±8	0.50±0.02
3TFM-TDZ	5.5±0.6	4.8±0.3	39.4±1.4	0.32±0.02
3TFM-2HE (19)	**0.23±0.01**	**0.10±0.01**	**1.5±0.07**	**0.19±0.01**
20	**0.025±0.004**	**0.058±0.002**	**0.37±0.08**	**0.059±0.004**
21	**0.033±0.003**	**0.035±0.003**	**0.25±0.07**	**0.030±0.002**
22	0.064±0.007	0.029±0.005	1.5±0.13	0.61±0.08

Errors show SD of at least two parallel assays, each consisting of two replicates.

with compound 32 (the thiourea analogue of 19) revealed that this modification is not a promising way to produce efficient CKX inhibitors.

In summary, most of the compounds studied displayed the same structure-inhibitory relationship towards both AtCKX2 and ZmCKX1. However, some differences can be observed. ZmCKX1 is somewhat more sensitive to inhibition and less selective than AtCKX2. For example, compounds 2, 5, 9, 11, 14, and 17 are better inhibitors of ZmCKX1 (Table 1). Such information could be used to develop CKX inhibitors with increased selectivity, with respect to monocotyledonous and dicotyledonous plant species.

Inhibition of ZmCKX8

ZmCKX8 transcripts are abundant in maize roots and stems (Vyroubalová *et al.*, 2009; Zalabák *et al.*, 2014). The ZmCKX8 binding pocket is similar to that of ZmCKX1. The presence of a non-conserved glutamate (E372 in ZmCKX8 and E381 in ZmCKX1) favors binding of smaller substrates such as cytokinin bases. Despite this similarity with ZmCKX1, ZmCKX8 showed markedly different sensitivity towards each inhibitor.

A significant difference between ZmCKX8 and ZmCKX1 is the stronger binding of CPPU and DCPPU to ZmCKX8, and the fact that neither an additional 2-(2-hydroxyethyl) nor 3-methoxy group improved the IC_{50} values of these derivatives (Table 1). The substitution of the pyridine ring with phenyl (DCPPU versus compound 6) did not improve the IC_{50} values either. Addition of a hydroxymethyl group (compound 8) or 2-hydroxyethyl group (compound 12) on compound 6 had a negative or no effect, respectively. Only further substitution of compound 19 with chlorine or bromine led to a significant improvement, as observed for compounds 20 and 21, which exhibited IC_{50} values in the 10^{-8} M concentration range (Table 2).

Inhibition of ZmCKX4a

ZmCKX4a represents the second group of CKX isoforms that differ in the residue located at the entrance to the active site. ZmCKX4a has a small non-polar residue, A373, which clearly allows the binding of bulkier substrates, such as cytokinin glucosides or monophosphates (Kopečný *et al.*, 2016). For this reason, selected inhibitors were evaluated against CKX isoforms from both groups. *ZmCKX4a* is expressed in immature tassels. The ability of the compounds studied to inhibit ZmCKX4a was generally weaker, compared to other isoforms (Tables 1 and 2). However, the inhibition pattern was similar to that of ZmCKX8. For example, addition of a 2-hydroxyethyl group did not strengthen the inhibitory activity, as can be seen from a comparison of compound 1 versus CPPU, compound 4 versus DCPPU, and compound 6 versus 12. On the other hand, substitution of the pyridine ring by phenyl (DCPPU versus compound 6 and compound 4 versus compound 12) improved IC_{50} values by about 50% (Table 1). Compound 19 showed an average IC_{50} value and only the presence of an additional substituent resulted in a marked increase in inhibitory strength, as can be seen for compounds 20 and 21. No other compounds showed improvement (Table 2).

Summary of CKX inhibition

We compared the inhibitory potency of key derivatives identified in this study with those of the best known CKX inhibitors (Fig. 2A and Table 3). It is obvious that known compounds exhibited IC_{50} values spanning high nanomolar and low micromolar concentrations, with F-INCYDE being the best. However, compound 19 studied here exhibited 10-fold lower IC_{50} values, and further improvement was achieved by the introduction of another halogen atom. This development led to compounds 20 and 21, which are the best inhibitors of all substances studied to date, and which inhibit all three CKXs at 10^{-8} M concentrations.

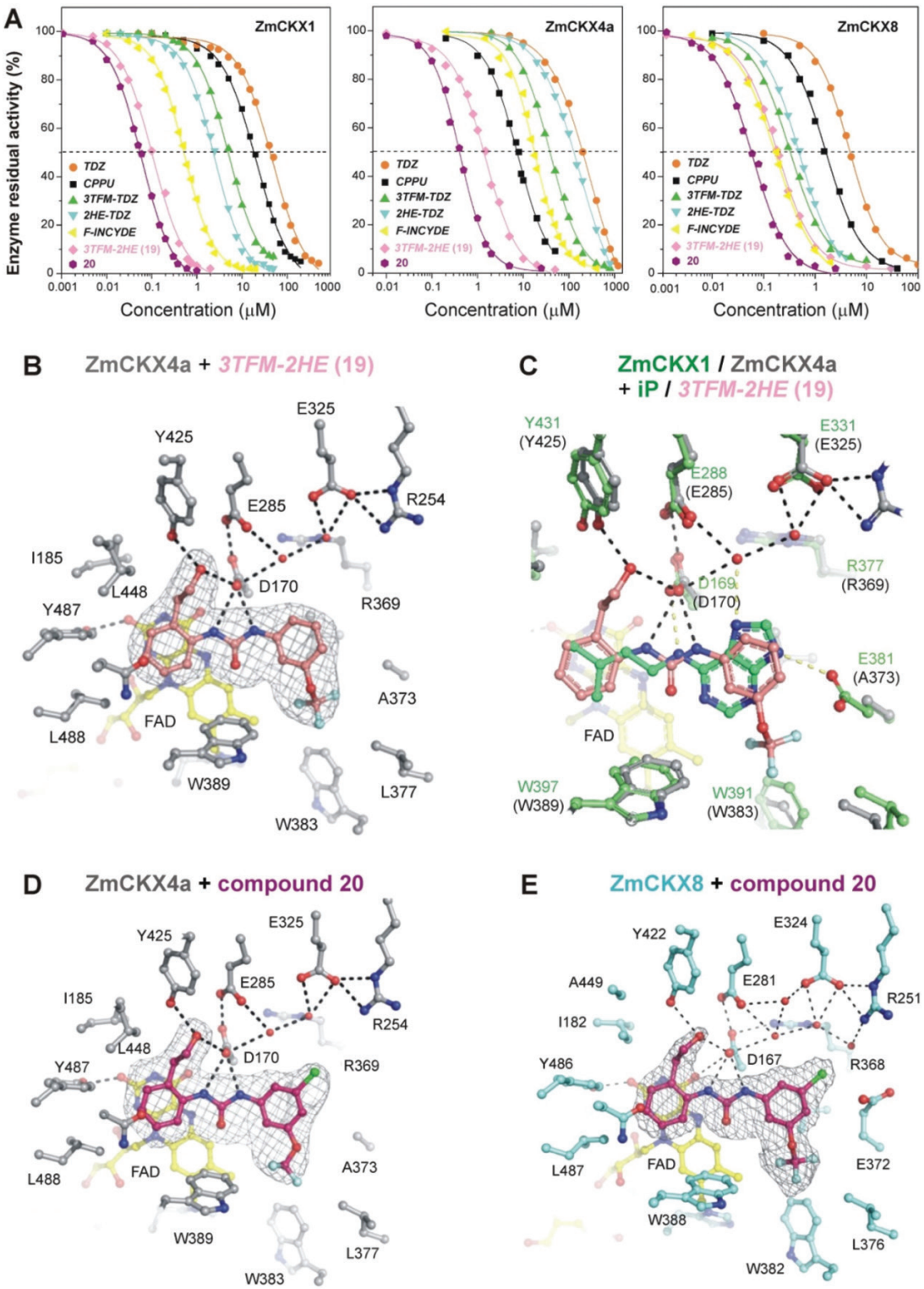

Fig. 2. (**A**) Concentration-dependent curves of residual CKX activity for known CKX inhibitors, and for inhibitors 19 and 20. (**B**-**E**) Binding of inhibitors 19 and 20 at the active site of CKX. (**B**) Omit electron density map for inhibitor 19 (3TFM-2HE, pink color) in ZmCKX4a. The FAD molecule is shown in yellow and other atoms are color-coded. (**C**) Superposition of bound substrate iP (green, Malito *et al.*, 2004) and inhibitor 19 (pink) on ZmCKX1 (green) and ZmCKX4a (grey), respectively. (**D, E**) Omit electron density evidence for bound inhibitor 20 (violet) in ZmCKX4a and ZmCKX8. The mesh represents a simulated annealing F_o-F_c omit map contoured at 3σ.

Mode of binding of inhibitors 19 and 20 at the active site of CKX

The binding mode of compounds 19 and 20 was characterized using three high-resolution crystal structures of ZmCKX4a and ZmCKX8 complexes (Table S2). While the structure of ZmCKX4a itself, as well as its complex with CPPU and TDZ-derived inhibitors, has been published (Kopečný *et al.*, 2016; Nisler *et al.*, 2016), that of ZmCKX8 has not been described. The structure of ZmCKX8 is very similar to that of ZmCKX1 and ZmCKX4a with root-mean-square deviation (RMSD) values of 1.13 Å (471 Cα atoms) and 0.92 Å (436 Cα atoms), respectively (Fig. S1). ZmCKX8 exhibits the typical two-domain topology of the FAD-binding domain (residues 32–242 and 487–530) and the substrate-binding domain (residues 243–486). In general, the structures comprise 19 helices and 17 strands like in the case of ZmCKX1, and as deduced by define secondary structure of proteins (DSSP) algorithm (Kabsch and Sander, 1983). A significant difference is the presence of two helices (α10 and α11) above the entrance to the substrate channel, which is similar to the structure of ZmCKX1. On the contrary, the equivalent region is highly disordered in ZmCKX4. Both ZmCKX1 and ZmCKX8 are more ordered at the N-terminus and carry an additional α-helix (Fig. S1).

The crystal structure complexes indicated that inhibitors bind to the CKX active site similar to cytokinin substrates (Fig. 2C). Both compounds interact with one oxygen atom of the catalytic aspartate (D169/170/167 in ZmCKX1/ZmCKX4a/ZmCKX8, respectively) via their nitrogen atoms. These urea nitrogen atoms establish a bidentate hydrogen bond with distances of between 2.9 Å and 3.1 Å. Both inhibitors adopt a planar conformation parallel to the plane of the isoalloxazine ring of the FAD co-factor, allowing favorable "parallel-displaced" π-π electrostatic interactions. The oxygen atom of the 2-hydroxyethyl side chain replaces a water molecule observed in cytokinin-bound structures, and it makes H-bonds with the hydroxyl group of Tyr425 and the oxygen atom of Asp170 in ZmCKX4a (Tyr422 and Asp167 in ZmCKX8; Fig. 2B-E). The 3-TFM-phenyl ring of compound 19 in ZmCKX4a points toward the hydrophobic pocket composed of the side chains of Trp389, Trp383, Phe57, Leu377, and His387 (Fig. 2B). The 3-chloro-5-TFM-phenyl ring of compound 20 overlaps with the 3-TFM-phenyl ring of compound 19 when comparing the ZmCKX4a and ZmCKX8 structures (Fig. 2D, E). The chlorine atom points outside the binding cavity. Neither the chlorine nor the 3TFM group makes any direct hydrogen bond with neighboring residues.

Activation of the cytokinin primary response regulator and interaction with cytokinin perception

Since the diphenylurea derivatives display cytokinin activity and binding features common to purine-based cytokinins (Bruce and Zwar, 1966), the most promising compounds from this work were tested for cytokinin responses *in planta*. We used transgenic Arabidopsis plants harboring the *ARR5:GUS* reporter gene (D'Agostino *et al.*, 2000). Arabidopsis response regulator 5 (*ARR5*) is a primary response gene with a cytokinin-dependent promoter, which integrates the responses of cytokinin signaling pathways, including those linked to the cytokinin receptors ARABIDOPSIS HISTIDINE KINASE2, 3 and 4 (AHK2, AHK3 and AHK4). The activity of the compounds was compared with that of 1 μM N^6-benzyladenine (a positive control, Fig. 1F).

Compounds CPPU and DCPPU both strongly activated the expression of *ARR5*. Compounds 4 and 6 reached only ~30% of the activity of DCPPU, which means that the 2-(2-hydroxyethyl) group in compound 6 and substitution of a nitrogen atom with a carbon atom in compound 4, both decrease the ability to activate *ARR5* expression. All other compounds tested showed only limited ability to trigger *ARR5* expression (mostly <20% of 1 μM N^6-benzyladenine activity).

The compounds were further tested for their ability to directly activate the AHK4 receptor. All compounds tested, 1–6, 10–14, 19, 20 and 21, were totally inactive towards this receptor (Fig. S2). Thus it is possible that the observed negligible activity of some derivatives in the ARR5 assay (Fig. 1F) is not a result of their intrinsic cytokinin activity, but can instead be attributed to an increased level of endogenous cytokinins caused by inhibition of AtCKXs. It is also well known that the rapid turnover (within hours) of cytokinins is controlled by CKXs (Terrine and Laloue, 1980; Redig *et al.*, 1996; Schmülling *et al.*, 2003). In these experiments, Arabidopsis seedlings were grown in the presence of the tested compounds for 16 h, giving enough time for endogenous hormone levels to be altered as a result of the inhibitory action of the compounds.

The effect of compound 19 on CKX and ARR gene expression in Arabidopsis

The above conclusion is consistent with our results obtained in gene expression analysis of the cytokinin primary response genes belonging to the type-A *ARR* gene family being rapidly elevated by cytokinins (D'Agostino *et al.*, 2000). Compound 19 downregulated *ARR5* expression during the first 5 h after treatment, but a two-fold upregulation of *ARR5* was observed 24 h after the treatment. The other *ARR* genes analyzed (*ARR7*, *ARR9* and *ARR16*) behaved similarly; their expression was either not significantly altered or it was downregulated during the first 5 h after treatment, but all genes became gradually upregulated 24 h after treatment (Fig. S3A). The course of expression of *ARRs* after administration of cytokinin BA was completely different. All *ARRs* analyzed were significantly upregulated ($P<0.05$, *t*-test) at all time points and, to a higher extent, when compared with the effect of compound 19. The expression of the type-A *ARR* and cytokinin levels are undoubtedly correlated (e.g. Kiba *et al.*, 2019). Our results thus indicate that during the first hours after application of

compound 19, the concentration of active cytokinins is decreased and starts steadily increasing after that. Such an increase of active cytokinins 1 d after treatment is most likely responsible for the biological activity of the CKX inhibitors.

Regarding the expression of the *AtCKX* gene family, both treatments (BA and compound 19) upregulated most of the analyzed *AtCKXs* at all time points, and the extent of upregulation intensified over time. This response was generally stronger to BA application than to treatment with compound 19 (Fig. S3B). These results indicate that compound 19 disrupts the homeostasis of cytokinin metabolism less than BA, and even though *CKX* genes become upregulated, compound 19, with a slight delay, stimulates the cytokinin signaling pathway.

The effect of selected compounds on retention of chlorophyll in wheat leaves

Cytokinins are known to delay leaf senescence processes including chlorophyll degradation, as measured in a wheat leaf senescence assay (Gan and Amasino, 1995, 1997). In the present study, leaves treated with TDZ and *t*Z, the two strongest cytokinins, retained approximately 80% of their chlorophyll after five days in the dark. DCPPU only retained about 40%, and CPPU was almost inactive in this assay. Among the new urea compounds, those behaving as weak CKX inhibitors were inactive, or retained only up to 10% of chlorophyll (compared to the DMSO control), while the strongest CKX inhibitors (compounds 4, 12, 19, 20, and 21) retained ~20% more chlorophyll than the DMSO controls (Fig. S4). This slight anti-senescence activity of the compounds is lower than the activity of classic cytokinins (as seen in the ARR5::GUS assay, Fig. 1F), and again, is likely to be due to inhibition of CKX and maintenance of endogenous cytokinins in the leaves.

Shoot regeneration on tobacco leaf discs

Tobacco is very responsive to the shoot-inducing effects of cytokinins. After three weeks of incubating with tobacco leaf discs, it was clear that iP alone was able to induce adventitious shoots only when 0.1 μM or higher concentrations were used. Compounds 19 and 21 failed to produce shoots at any concentration, when applied alone (Fig. 1G and Table 4). This demonstrates that these compounds do not exhibit shoot-forming (cytokinin) activity in tobacco explants, on their own. However, an interesting synergistic effect of both iP and the inhibitor was observed (Table 4). A minimal level of 0.01 μM compound 21 was sufficient to boost the shoot-inducing ability of 0.01 μM iP. For compound 19, 0.1 μM was needed to show this effect. Furthermore, when higher concentrations of iP were combined with inhibitors, their stimulatory effect could be observed (Fig. 1G). Cytokinin iP has a double bond in its chemical structure which makes it vulnerable to the action of cytokinin oxidases (Kamínek, 1992). These findings demonstrate that the compounds 19 and 21 block the breakdown of iP in *in vitro* plantlets, and enhance its morphogenetic activity. Compound 21 performed better than compound 19 in this bioassay, which correlates well with their IC_{50} CKX values (Table 2).

Indirect shoot regeneration on poplar leaf explants

In the widely planted tree Canadian poplar, induction of adventitious shoots is uncommon (Kutsokon *et al.*, 2013). Compounds 19 and 21 were tested alone or in combination with iP for their ability to enhance the effect of a sub-optimal concentration of iP on *de novo* shoot formation on poplar leaf explants. When applied alone, neither 1 μM iP nor compounds 19 and 21 induced shoots after four weeks of incubation. On the other hand, *de novo* shoot formation occurred when iP was combined with compounds 19 or 21 (Fig. 1H and Fig. S5). The highest average number of regenerated shoots per explant (1.3) was observed for 1 μM iP+1 μM compound 21, followed by the combination of iP with compound 19 (0.3 of regenerated shoots per explant). The results demonstrate again that compounds 19 and 21 do not show cytokinin activity but do protect iP against degradation *in planta*. This result has never

Table 4. Number of adventitious shoots regenerated on tobacco leaf disks as a result of iP interaction with CKX inhibitors 19 and 21.

	Number of adventitious shoots regenerated			
Concentration (μM)	0	0.01	0.1	1.0
iP	*Inhibitor 19*			
0	0	0	0	0
0.01	0	0	12.2±1.0 a	16.6±1.9 ab
0.1	17.7±3.1 ab	24.0±1.7 bcd	22.9±1.7 bcd	27.3±2.7 cde
1.0	30.8±3.2 de	21.6±2.8 bc	18.8±4 ab	30.2±4 e
iP	*Inhibitor 21*			
0	0	0	0	0
0.01	0	0.8±0.4 a	2.7 ±1.4 a	8.2 ±2.1 ab
0.1	10.2 ±2.5 ab	13 ±1.7 abc	26.2±5.4 cd	20.6±8.5 bcd
1.0	18.3±1.9 bc	18±2.0 bc	21.5±3.2 bcd	33.3±2.7 d

Mean values (± SD) followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$).

been shown previously in *Populus x canadensis* and certainly not for iP, which is generally considered to be a rather weak cytokinin.

Alleviation of stress consequences in Arabidopsis seedlings

Compound 19 was tested for its ability to alleviate the effects of abiotic stress on Arabidopsis seedlings, represented by application of 100 mM NaCl as salinity stress or 100 mM mannitol as osmotic stress. The abiotic stress conditions caused severe penalties in terms of shoot biomass formation and relative growth rate. Salt and osmotic stress reduced the shoot green area by 63% and 64%, respectively, whereas treating the seedlings with compound 19 alleviated the penalty by almost 20% (Fig. 1I). The same trend was observed for average relative growth rate, which was lowered to 51% and 54% by salt and osmotic stress, respectively, whereas the presence of compound 19 reduced this negative effect by ~11% (Fig. 1I; Table S3). The growth of Arabidopsis seedlings under optimal conditions was not affected by 10 nM compound 19 alone during the experiment.

Effect on phenotype and seed yield of A. thaliana

Arabidopsis seedlings were sprayed once with compound 19 at the stage of four true leaves (up to 14 days after planting). Treated plants produced a higher number of seeds per silique (SP) without affecting the total number of siliques per plant. The control plants had 25.6±6.1 SP, while plants treated with 0.1, 1, and 10 μM compound 19 had 36.0±6.7, 33.2±7.3, and 32.4±6.1 SP (an increase by 41%, 30% and 27%, respectively, from the controls), respectively. The results were significantly different at P=0.03 (t-test). Total grain yield of treated plants was increased over the grain yield of control plants by 21% and 17% after application of, 0.1 μM and 1 μM compound 19, respectively,. The highest concentration, 10 μM, was not effective. The weight of 100 seeds of treated plants was lowered by 5–8% compared with non-treated plants. Van Daele *et al.* (2012) reported a negative correlation between seed number produced per plant and seed size in many Arabidopsis mutants altering seed production.

In a second independent experiment, plants treated with 0.1, 1, and 10 μM compound 19 displayed an increase in SP of 18%, 21% and 14%, respectively over the control plants (P=0.05, t-test). Again, the weight of 100 seeds of treated plants was lower by 4–7%; however, total grain yield was significantly higher (at P=0.05, t-test) by 19% and 15% after 0.1 μM and 1 μM application of compound 19, respectively. The seed yield of the plants treated with 10 μM compound 19 was 6% lower, indicating that this concentration, as in the first experiment, was indeed too high. The treated plants (0.1 μM compound 19) had up to 38% larger leaf rosettes than control plants 25 days after sowing (Fig. S6 and Table S4). This finding indicates higher growth rate in the treated plants. Furthermore, we observed that siliques of controls ripened earlier than siliques of treated plants (Fig S7. The lifespan of all treated plants was extended by approximately 10 days compared to control plants. This is consistent with the observation of Gan and Amasino (1995), who demonstrated that endogenously produced cytokinins can delay senescence in transgenic tobacco plants. A longer photosynthetically-active lifetime could also contribute to a higher yield, as the plants have more time for seed filling.

Discussion on cytokinin activity versus CKX inhibition

Currently, three types of compounds are available that induce cytokinin effects in plants. These substances have different relationships to the CKX enzyme. The published data, together with results in this article, indicate that there are at least three possible scenarios for cytokinin action in plants.

1) Natural cytokinins (*cis*/*trans*-zeatin, iP, N^6-benzyladenine or *meta*-topolin) exhibit strong cytokinin effects, but they are also excellent CKX substrates. They are usually applied on plants in a single dose at one time point. Thus, it is possible that the concentration of cytokinin at the time of administration is too high, but after a few days it is too low, depending on the rate of inactivation and degradation. This may initially cause hormonal imbalances and stress, while having no long-term positive effects. For these reasons, the results of field experiments with cytokinins are very variable and difficult to reproduce, contributing to the fact that natural cytokinins have not found an assured role among commercialized plant growth regulators (PGR; reviewed by Koprna *et al.*, 2016).

2) Some strong synthetic cytokinins (TDZ, CPPU) have little ability to inhibit CKXs. Their cytokinin effect may last for a longer period of time, because of their higher resistance to CKX degradation. However, their use can overdose plants with a cytokinin signal that results from a combination of their exogenous application and increased endogenous cytokinin levels due to CKX inhibition. Again, this can cause negative and/or side effects. For example, in some plant species like cotton, TDZ application leads to an overproduction of ethylene, which is used for defoliation (Suttle, 1985, 1986). Except for this application, synthetic cytokinins are commercially used only in the horticultural and floricultural sectors (reviewed by Nisler, 2018).

3) Conversely, there are strong CKX inhibitors with no or very limited cytokinin activity. F-INCYDE (according to Zatloukal *et al.*, 2008) and key compounds from this work (compounds 19, 20 and 21) belong to this group, as indicated by the results presented here. These compounds can moderately, and over the long-term, increase the level of endogenous cytokinins, which seems to be a considerable advantage over their exogenous application (Gemrotová *et al.*, 2013; Kon *et al.*, 2017).

Field trials with spring barley, winter wheat and winter oilseed rape

Finally, we investigated the effect of compound 19 on the yield and yield-forming parameters in field trials of important crop species (according to Good Agricultural Practices criteria).

Spring barley

In 2016, barley seeds were primed with compound 19 at 10 or 50 μM concentrations. The resulting plants had higher seed yields by 3% and 0.7%, respectively, compared with controls (Table 5), and TGW for the 10 μM treatment was 1.8 % higher than that of control plants. Phenotypic analysis further showed that the higher yield (for 10 μM treatment) could be due to a greater number of strong and medium tillers in the treated plants (9.8% more) than than controls. In 2017, the effect of foliar application of a 5 μM concentration of compound 19 at two different growth stages was investigated. Treatments at growth phases BBCH 21–23 (tillering) and BBCH 51 (beginning earing) increased seed yield by 6.3% and 6.4% respectively, compared with untreated plants.

Winter wheat

In 2017, winter wheat seeds were primed with 50 μM compound 19. The mature treated plants had a 4% higher seed yield and 28% increase in strong and medium tillers, than the control plants (Table 5). The same experiment, repeated in 2018, resulted in a 6.6% increase in seed yield; however, the number of strong and medium tillers decreased by 4.7%. In 2017, the foliar application of 5 μM compound 19 at BBCH 25 and at BBCH 51 growth stages resulted in seed yields greater by 5.9% and 5% respectively, compared with control plants.

Winter rapeseed

In 2017, the foliar application of a 5 μM solution of compound 19 at BBCH 30 and BBCH 33–35 growth stages resulted in a higher seed yield by 5.7 % and 7.5 % respectively, compared with controls (Table 5). In addition, the seeds of the treated plants were slightly heavier; TGW of controls was 5.51 g, while for treated plants it was 5.7 g (BBCH 30) and 5.79 g (BBCH 33–35). This increased seed weight of the treated plants contributed to an increase in the overall seed yield.

In field trials, compound 19 was applied at 0.51 g ha^{-1} (300 l of 5 μM solution per hectare). This is a very small amount when compared to typical doses of other commercially-applied agrochemicals that increase cereal yield. The PGRs thus used to date can be divided into two groups. The first group includes chemicals that block gibberellin biosynthesis and thus reduce stem growth and lodging. If the seed yield is higher, this is due to the fact that more stalks are harvestable. Growth retardants, such as trinexapac ethyl, paclobutrazol, etc., which belong to this group, are the most widely used PGRs (Rademacher, 2017). The second group includes triazole and strobilurin fungicides that improve disease control and enhance green leaf area (Rolston *et al.*, 2004). Again, if the seed yield is higher, it is mainly due to increasing plant vitality rather than controlling flower or seed setting. The application rate for the above-mentioned chemicals is between 100 and 200 g ha^{-1} (Rolston *et al.*, 2004). According to the Grains Research and Development Corporation of the Australian government, which evaluated the results of field trials from two to three seasons, the PGRs tested slightly increased seed yield in barley, but not in wheat (Damian, 2014).

It is well-documented that cytokinins help plants to cope with environmental stresses (Hare *et al.*, 1997; Cortleven *et al.*, 2019). Indeed, the CKX inhibitor F-INCYDE improved the

Table 5. The effect of foliar and seed application of compound **19** on yield parameters in spring barley, winter wheat and winter rapeseed.

Year / type of application	Yield (% of control)[a]	TGW (% of control)	Number of tillers (% of control)[b]
Barley			
2016 / seed coating 10 μM	+3.0	+1.8	+9.8
2016 / seed coating 50 μM	+0.7	-4.0	0.0
2017 / foliar application at BBCH 21–23	+6.3	-	-10.0
2017 / foliar application at BBCH 51	+6.4	-	
Wheat			
2017 / seed coating 50 μM	+4.0	-	+28.0
2018 / seed coating 50 μM	+6.6	-	-4.7
2017 / foliar application at BBCH 25	+5.9	-	+4.4
2017 / foliar application at BBCH 51	+5.0	-	-
Oilseed Rape			
2017 / foliar application at BBCH 30	+5.7	+3.5	-
2017 / foliar application at BBCH 33–35	+7.5	+5.1	-

[a] Yield in tons per hectare was determined at a grain moisture level of 14%. Grain yield is average from six experimental plots.
[b] Number of tillers represents strong and medium tillers combined and is an average from 50–60 plants. Foliar application was performed with 5 μM solution. BBCH stands for the BBCH-scale, which describes phenological growth stages of individual crops.

tolerance of both rice seedlings and mature plants to heat stress in India (Camblin *et al.*, 2017; Kon *et al.*, 2017). Plants treated with F-INCYDE also had a 6-8.8% higher grain yield. The effects observed in barley and wheat (grain yield increase of 3-6.6%) with compound 19 were obtained in central Europe with an almost optimal climate for these crops. It is thus possible that application of compound 19 could have even better effects on crops grown in areas with sub-optimal conditions. The CKX inhibitors may also show synergistic effects with fertilizers and other agrochemicals used in agriculture because of their different mode of action. However, this remains to be elucidated in future studies.

Conclusion

Here, we have presented the results of our lab-to-field approach for the development of novel PGRs for use in agriculture and biotechnology. Our platform is based on compatible technologies, from molecular targeting of organic synthesis, through bioassays and *in planta* testing, to field trials. The compounds reported here are very potent urea-based inhibitors of cytokinin oxidase/dehydrogenase (CKX), a key enzyme of cytokinin degradation in plants. The exceptional efficacy of several substances has been shown for four CKX isoforms from two different plant species: Arabidopsis and maize. The ability of compounds 19 and 21 to inhibit the degradation of cytokinin iP *in vivo* has been demonstrated in Canadian poplar and tobacco plant tissue cultures. From the results we propose that such compounds could find interesting applications in plant biotechnology, for example in the regeneration of species for which this is not yet possible. We further show that CKX inhibitor compound 19 alleviates stress-induced retardation of seedling growth in Arabidopsis and increases its seed yield. Compound 19 also increased the yield of wheat, barley and rapeseed under field conditions.

It has been shown that GM plants with down-regulated CKX activity provide a significantly higher yield. Furthermore, Ashikari *et al.* (2005) found that one of the quantitative trait loci for grain yield in rice is *OsCKX2*. The aim of this work was to provide an alternative to these GM plants, i.e. a chemical tool capable of gently and constantly increasing endogenous cytokinin levels in plants. In addition to avoiding legislative obstacles associated with the use of GM organisms, CKX inhibitors offer a number of other benefits. They can be applied to a wide range of plant species and allow flexibility of application with respect to dose and time. Here we have presented the development of novel CKX inhibitors which can function in a range of plant species.

Supplementary data

The following supplementary data are available at *JXB* online.

Table 1. Custom TaqMan probes and primers used for gene expression screening of *AtCKXs* and *ARRs*.

Table 2. Data collection and refinement statistics.

Table 3. Effect of compound 19 in alleviating abiotic stress consequences in Arabidopsis seedlings.

Table 4. Effect of compound 19 on the size of rosette leaves of Arabidopsis plants.

Figure S1. Superposition of ZmCKX8 structure with that of ZmCKX1 and ZmCKX4a.

Figure S2. The ability of selected compounds to activate cytokinin receptor CRE1/AHK4.

Figure S3. Effect of compound 19 on the relative expression of four selected *ARR* (A) and six *AtCKX* (B) genes in Arabidopsis seedlings.

Figure S4. The effect of selected compounds on retention of chlorophyll in a wheat leaf senescence assay.

Figure S5. Quantitative evaluation of *de novo* shoot organogenesis in *Populus x canadensis*.

Figure S6. Effect of compound 19 on the size of rosette leaves of Arabidopsis plants.

Figure S7. Effect of compound 19 on senescence and silique ripening in Arabidopsis plants.

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Author contributions

JN, LS and MS designed the research; JN designed and synthesized all compounds; LH and ZW assisted with chemical synthesis; RK, ZP, and DZ contributed to enzyme kinetics; DK, PB, and SM performed the X-ray crystallographic study; LS, ZP, and ND performed the cytokinin bioassays and stress alleviation assays; NM and SW performed the plant tissue culture assays; RK performed the field trials; MK and RK performed gene expression analysis; JN evaluated the Arabidopsis phenotype and wrote the paper with contributions by co-authors.

Conflict of interest

Authors of this manuscript (JN, DK, LS, and MS) have filed a Czech patent application (PV 2020–144), which claims the protection of several compounds described in this paper and their use as plant growth regulators.

Data availability

Data reporting compound structures are publicly accessible in the Protein Data Bank archive. The atomic coordinates and structure factors of ZmCKX8 with inhibitor 20 have been deposited in the Protein Data Bank (www.rcsb.org) under accession code 6YAQ (https://www.rcsb.

org/structure/unreleased/6YAQ), and the complexes of ZmCKX4a with 3TFM-2HE (19) and compound 20 are under accession codes 6YAO (https://www.rcsb.org/structure/unreleased/6YAO) and 6YAP (https://www.rcsb.org/structure/unreleased/6YAP).

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