Role of Cytokinin Oxidase/Dehydrogenase in Crop Improvement

Abstract

The phytohormone cytokinin regulates various crucial functions of plant growth and development such as shoot apical meristem activity, flower development, vascular development etc. and also has negative role in lateral root development and root apical meristem activity. Cytokinin is degraded by cytokinin oxidase/dehydrogenase (*CKX*) irreversibly and reversible inactivation by glucosylation. Various homologous genes of *CKX* have been identified in Arabidopsis and in many other crop plants. The root specific expression of *CKX* showed positive results for larger root with higher lateral root numbers and higher root to shoot biomass. The site-specific manipulation of *CKX* gene has been done in crops like wheat, barley, maize, rice, legumes and horticultural crops to regulate the cytokinin levels in the particular tissues to enhance the yield, tolerance to various abiotic stresses (drought, salt, lower soil fertility), biofortification for many micronutrients (Zn, Fe) in the seed, propagation, etc. And the use of modern techniques in manipulation of *CKX* have been started to improve the crop plant and the outcome is very promising for the future application in commercial agricultural activities.

Key words: Cytokinin, cytokinin oxidase/dehydrogenase, drought, lateral root, yield

1. Introduction

The demand for the agricultural crop is increasing day by day as the global population increases (Godfay *et al.,* 2010). The current situation of the world is about 815 million people were undernourished reported by United Nations Food and Agriculture Organizations (FAO, 2016). The main cause for this undernourishment is the protein-energy malnutrition and micronutrient deficiency in food (UNICEF, 2015). In the current situation where the land is decreasing due to population outbreak and food demand is increasing, the more intense use of cropland could meet the demands but due to unpredictable and changing environment condition the problem is becoming worse (Godfray *et al.,* 2010). Natural hazards like drought, flood and biotic stresses are limiting the crop production (FAO, 2019).

In the current scenario of human population, the improvement in the majorly grown crop like cereals, legumes, oilseeds etc., faces numerous abiotic stresses during their life cycle, is the solution for feeding the growing population (Swaminathan and Bhawani, 2013).The plants with larger root system can withstand in the drought and low nutrient soil conditions (Brown *et al.,* 1989; Saxena *et al.,* 1996; Kondo *et al.,* 1999; Steel *et al.,* 2006; Henry, 2012). For this, the cytokinin oxidase/dehydrogenase enzyme can be an important player and can be exploited to improve the crops productivity. This enzyme degrades the phytohormone cytokinin in specific tissue at specific time in the plant. It has been reported by several scientists that cytokinin negatively regulated the lateral root formation (Goodwin and Morris, 1979; Wightman *et al.*, 1980) and the mutant plant of Arabidopsis defective response for cytokinin showed the high branching of roots (Mason *et al.*, 2005; Reifler *et al.*, 2006; To *et al.*, 2008).

Cytokinin level in plants is regulated by various processes like reversible glycosylation, action of adenine phosphoribosyl transferase and also degradation by the cytokinin oxidase/dehydrogenase (*CKXs*) irreversibly (Sakakibara, 2006). The manipulation of level of cytokine in the barley, *Populus* and rape was used to improve the agronomic value by using the variants of cytokinin dehydrogenase (Ramireddy *et al.,* 2018a; Nehnevajova *et al.,* 2019) and lead to improve root growth and drought tolerance. Recently Khandal *et al.* (2020) reported that the increased activity of *CKX* in chickpea roots enhances the drought tolerance, seed yield and also concentration of micronutrients like zinc, iron, potassium and copper without compromising with the protein level in seed. The cytokinin is necessary for seed development (Letham, 1963) and it was also reported by Ashikari *et al.* (2005) in rice. In wheat the concentration of endogenous cytokinin was found high at the time of rapid endosperm nuclear and cell division in developing seed (Bennet *et al.,* 1973). The overexpression of *AtCKX3* reduces the formation of primordium in floral meristem leading lower number of flowers (Werner *et al.,* 2003).

In this review article, the information regarding cytokinin and its regulation through *CKX* at different stages and in different crops has collected and compiled for better understanding of *CKX* functions which can help in developing the crops with higher nutrition quality and can grow under limited water condition and low fertile soil without compromising the seed yield.

1.1 Cytokinin and its metabolism

The plant hormone cytokinin is synthesized by two processes either by an isopentenyltransferase (IPT) attaching on isoprenoid side chain of adenosine triphosphate or adenosine diphosphate (ATP/ADP) synthesizing nucleotide of isopentenyl adenine (iP) and *tans-zeatin* (tZ) or by a tRNA-IPT leading, indirectly to the *cisZ*-type cytokinin. Cytokinin is activated by the LONELY GUY (LOG) by releasing the free base from the nucleotide forms. Cytokinin oxidase/dehydrogenase are the key player in destruction of cytokinin. O-glucosylation or N-glucosylation both can inactivate the cytokinins (Jameson, 2017; Kieber and Schaller, 2018; Worthen *et al.,* 2019; Chen *et al.,* 2020).

1.2 Role of cytokinin in growth and development

1.2.1 In the shoot tissues

In the shoots, the development of shoot apical meristem (SAM) is controlled by various factors like transcriptional factors, external signals and phytohormons specially cytokinins (Skoog and Miller, 1957). Werner *et al.*(2001; 2003) and Wybouw and De Rybel(2019) reported the highly decreased SAM in the plant deficient in cytokinin and suggested that cytokine is crucially required by the plant for developing and maintaining SAM. Similar observations were reported by various workers (Miyawaki *et al.,* 2006; Nishimura *et al.,* 2004; Higuchi *et al.,* 2004; Riefler *et al.,* 2006) in their experiments by mutating the multiple receptor or *IPT*gene which suggested that the cytokinin positively regulate the SAM activity (Waidmann *et al.,* 2019).

The growth of vascular cambium and radial growth of plant is also controlled by the cytokinin (Nieminen *et al.,* 2008; Matsumoto *et al.,* 2008). Experiment of Nieminen *et al.* (2008) in poplar and birch trees found that the genes of receptors of cytokinin and other cytokinin signaling genes were expressed in cambial zone, and also found reduced cambial activity in the transgenic plant made by targeted expression of *CKX* genes in the cambial cells.

Cytokinin has an antagonistic activity to the auxin in shoot by promoting the growth of axillary buds, in *Physcomitrella* iPR and extracellular iP are the common cytokinin required to induce bud formation (Schwartzenberg *et al.,* 2007).

The sink-source relations and leaf senescence is also regulated by cytokinin concentration in plant (Werner and Schmulling, 2009). This was reported by Werner *et al.* (2008) in tobacco plant deficit in cytokinin leads to reduction in chlorophyll synthesis and lower in sugar content and increased amount of starch in the shoot and also reduction in invertase activity in vacuole.

1.2.2 In the root tissues

Cytokinin positively regulate the vascular differentiation in the root meristem. The work on characterization of *wooden leg* (*wol*) allele of *CRE1/AHK4 (*a dominant-negative allele), Mahonen *et al.* (2000) found that cytokinin is necessary for procambial cell division during embryogenesis to ensure vascular differentiation.

Cytokinin play a role in root apical meristem (RAM) unlike to SAM development, it negatively regulate the activity of RAM, this was proven by various scientist as their results suggest that plant develop a large RAM and rapid growth of roots in the condition where cytokinin deficit or lower signal output (Werner *et al.,* 2003; Mason *et al.,* 2005; Miyawaki *et al.,* 2006; Reiflier *et al.,* 2006; Heyl *et al.,* 2008; Zou *et al.,* 2018).

The lateral root formation is negatively regulated by cytokine; this was observed in several experiments by making transgenic and mutant plants (Werner *et al.,* 2003; Lohar *et al.,* 2004; To *et al.,* 2004; Mason *et al.,* 2005; Reflier *et al.,* 2006). The lateral root formation was inhibited by cytokinin by blocking the first division of xylem-pole pericycle cells only (Laplaze *et al.,* 2007; Li *et al.,* 2006).

2. Characterization and utilization of cytokinin oxidase/dehydrogenase

Cytokinin oxidase/dehydrogenase is a key enzyme which regulates the cytokinin level in the plant, this was reported by various researchers in different crops- *Arabidopsis thaliana* (Werener *e tal.,* 2003), maize (Brugiere *et al.,* 2003), rice (Ashikari *et al.,* 2005), pea (Gaudinova *et al.,* 2005) barley (Zalewski *et al.,* 2014), foxtail millet (Wang *et al.,* 2014), *Fragaria vesca* (Jiang *et al.,* 2016), and wheat (Ogonowska *et al.,* 2019). Cytokinin oxidase/dehydrogenase is encoded by a small gene family, in *Arabidopsis thaliana* seven homologous genes are present (Mameaux *et al.,* 2012). Till date it has not been reported in chickpea, only *CaCKX6* was characterized by Khandal *et al.*(2020). Summary of some crop improvement works by utilizing the *CKX* manipulation is presented in table 1.

2.1 In model plant *Arabidopsis thaliana*

The Arabidopsis plant has seven homologous of *CKX* gene and the tissue specific expression of them is different, Werner *et al.,* (2003) reported that *AtCKX1* and *AtCKX2* expressed in young tissues like shoot apex, *AtCKX4* expressed in stomatal precursor cells, root caps and in cells of trichomes, *AtCKX5* expressed in the procambial region of root meristem, *AtCKX6* expressed in vascular tissues and *AtCKX3* expression was associated with cell cycle.

The *CKX7* was reported as localized in cytosol (Schmulling *et al.,* 2003) this was experimentally demonstrated by Kollmer *et al.* (2014), they fused the green fluorescent protein (GFP) in *CKX7* at c-terminus and this construct was expressed under control of Cauliflower mosaic virus 35S promoter in Arabidopsis plant. The fluorescence signal was detected under confocal microscopy and reported the expression was found in cytosol.

Overexpression of *AtCKX* under control of 35S promoter in Arabidopsis was analysed and found that rate of root elongation was 70-90% more than wild type in 35S: *AtCKX1* and 35S:*AtCKX3*expressing seedling and the lateral root formation was also higher than the wild type (Werner *et al.,* 2003).

Overexpression of *CKX* in Arabidopsis plant was examined for the stress tolerance and reported a higher salt stress tolerance in the plant overexpressing *CKX1* among all other *CKX*. And the drought stress tolerance was also observed in these mutant plant and found more drought tolerant than wild type (Nishiyama *et al.,* 2011).

2.2 In monocot plants

Five different type of *ZmCKX* genes have been characterized in maize (Morris *et al.,* 1999; Bilveu *et al.,* 2003). In another experiment, the maximum expression of *ZmCKX* was found in kernels, tassels and ears as detected by semi-quantitative real time PCR reported by Massonneau *et al.*(2004).

In barley, the real time PCR data analysis for *HvCKX* showed that the *HvCKX1* expressed in mature kernels, roots and leaves, while *HvCKX2* expression was found in roots and leaves but the *HvCKX3* was predominantly expressed in mature kernels (Galuszka *et al.,* 2004).The over expression of *HvCKX* under constitutive expressed 35S promoter was carried out in Arabidopsis and tobacco plant and found shorter internode and dwarf habit along with larger root system (Galuszka *et al.,* 2004). Root specific expression of *CKX1* or *CKX2* under control of root specific promoter pEPP, pPEP and pRET by *Agrobacterium* mediated transformation in barley was done and observed that these mutant line showing tolerance under drought condition and also accumulate more minerals (Ramireddy *et al.,* 2018a). The analysis of transgenic plants of barley expressing *CKX* specifically in root accumulate higher Zn concentration having larger root system (Ramireddy *et al.,* 2018b).*HvCKX1* and *HvCKX3* knockout mutants of barley plant was analysed and reported that lower number of grains and grain weight observed in *CKX3* mutant lines (Gasparis *et al.,* 2019).

In wheat, the RNA interference mediated gene silencing done for *TaCKX2.2.1-3A*and the mutant plants were screened for yield traits. The results showed that grain number per spike was enhanced as the expression of *TaCKX2.2.1-3A* reduces in T3 (Li *et al.,* 2018) and the grain weight was found associated with *TaCKX2.2.1-3D* (Zhang *et al.,* 2012b). The crown root growth was found associated with the expression of *TaCKX* in the limited water environment (Ogonowska *et al.,* 2019)

Ashikari *et al.* (2005) reported in rice that the Gn1a QTL is associated with the increase in yield which is a gene for *CKX2.* Overexpression of this gene produces more yield but downregulation has opposite impact on yield. Li *et al.* (2016) used gene editing tool to change the form of *OsCKX2* gene resulted in increased number of flowers and panicle size. Enhanced expression of *OsCKX4* in a enhancer mutant line of rice showed that crown root initiation required the activity of *OsCKX4* as recorded more crown roots, more root growth and strong root gravitropic response. Over expression of *OsCKX4* mutant lines showed more crown root growth and RNAi mutant lines of *OsCKX4* produced lesser crown root growth (Gao *et al.,* 2014).

2.3 In dicot plants

In tobacco plants the *CKX1* was overexpressed under root specific promoter (Werner *et al.,* 2010) and reported an enlarged root system which provided tolerance to drought stress. In tomato plants, the accumulation of hydrogen peroxide (H_2O_2) is regulated by cytokinin, the H_2O_2involved in the degradation of chlorophyll. The protein gel blot analysis represent that the *CKX35* was found in chlorotic leaves and reduces the cytokinin levels and hence increased amount of H_2O_2 and in the green leaves the *CKX37* was predominantly present which is associated with active cytokinin concentration and normal hydroperoxide level in the leaves (Cueno *et al.,* 2012).

In strawberry (*Fragaria vesca*),the real time PCR analysis showed that the expression of *FvCKXs* was higher under drought, salt stress, heat stress and ABA treatment in roots, leaves and young fruits, this suggests that cytokinin has a negative role in growth and development under the stress conditions (Jiang *et al.,* 2016).

Liu *et al.* (2013) reported 12 *BrCKX* and 13 *BrIPT* genes in chinese cabbage and the stress related stimuli were found in the promoter region of these genes deteceted through transcription level analysis and they confirmed that these *BrCKX* and *BrIPT* genes have role in drought and salinity stress. In oilseed rape23 *BnCKX* genes were identified (Liu *et al.,* 2018) and real time PCR results suggests that *BnCKX5-1, 5-2,6-1* and *7-1*could be associated with the pod development and length of silique length. The analysis of transgenic oilseed rape plants with overexpression of *CKX2* represent the higher root-shoot biomass and accumulation of higher concentration of various micro and macro nutrients like P, Ca, Mg, S, Zn. Cu, Mo and Mn. The transgenic plant showed enhanced growth of chlorophyll under S and Mg deficiency and plants were able to extract more amount of Cd and Zn from contaminated medium and soil (Nehnevajova *et al.,* 2019).

For horticultural and silvicultural crops, grafting is an important technique for propagation but a problem during grafting is very common that undesirable lateral bud emergence reduces the grafting efficiency. To overcome of this problem Li *et al.* (2017) develop a transgenic with expression of tryptophan-2-monooxygenase (iaaM) under root specific promoter (SbUGT), this arrangement solved the problem of success of grafting but the root elongation biomass were reduced. They develop one more transgenic using *CKX* under same promoter, the root biomass was found higher. They made the cross of these two and reported that the negative effect due to iaaM was neutralized by activity of *CKX* and this made the grafting successful without compromising root development. The promoter *SbUGT* was used to express the *AtCKX2* in *Populus* which reduces the root sprouts in field condition resolving the problem of root-sprout mediated transgene spread (Li *et al.,* 2019).

2.4 In legumes

It was reported by Lohar *et al.* (2004) that the *AtCKX3* under constitutive expression positively regulates the nodulation in legumes demonstrated in *Lotus japonicus*. In *Medicago tuncatula* Jardinaud *et al.* (2016) expressed *AtCKX3* under epidermal promoter pEPI in medicago and found upregulation of nodulation factor when inoculated with *S. meliloti* and when it expressed with cortex-specific promoter (pCO) the infection was reduced (Rival *et al.,* 2012).

The cellular localization of *CKX3* was reported in growing nodules and cortical cell division of the nodule primordium for this the promoter of *CKX3* was fused with YFP (p*CKX::YFP*) and the fluorescence signal was recorded in *Lotus japonicum.* Phenotypic evaluation of *CKX3* mutant plants indicates that *CKX3* activity positively regulates the nodulation and the activity of cytokinin dose opposite function (Reid *et al.,* 2016).

Le *et al.* (2012) reported 14 *GmIPT* and 17 *GmCKX* genes in soybean and comparative analysis of promoter sequence of *GmCKX* genes with Arabidopsis genome and suggested that *GmCKX* were related with abiotic stress like drought.

Recentaly Khandal *et al.* (2020) used the chickpea root specific promoter (*CaWRKY31*) to express the chickpea cytokinin oxidase/dehydrogenase 6 (*CaCKX6*) in *Arabidiopsis thaliana* and chickpea. Analysis of the transgenic plants represent increase in lateral root number and root to shoot biomass, and these transgenic lines withstand in long term drought condition without compromising with nodulation and nitrogen fixation. Their results also clearly indicates that those line produced upto 25% more yield and also having more Zn, Fe, K and Cu in the seeds with similar protein content.

3. Conclusion and future prospective

In the past the understanding related to cytokinin oxidase/dehydrogenase (*CKX*) has been significanctly improved. From the above mentioned data it is clear that *CKX* enzyme has a key role in crop growth and development, it can regulate cytokinin level which play very crucial role in meristematic activity, root development, seed formation, chlorophyll concentration etc. Targeted manipulation of *CKX* provides the energy to the plant to withstand under stress conditions and enhancement in the yield with more nutrition values. In future the use of *CKX* manipulation through breeding programme or through editing tool can be done for the crop improvement for the majorly grown crops like cereals, pulses, oilseeds to feed the growing population and *CKX* can contribute to “ new green revolution” (Ashikari *et al.*, 2005). Now the future challenge for researcher is to find out natural electron acceptors of *CKX* enzyme.

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List of abbreviation

ABA: Abscisic Acid
CKX: Cytokinin Oxidase/Dehydrogenase
H_2O_2: Hydrogen Peroxide
iP: Isopentyl Adenine
PCR: Polymerase Chain Reaction
QTL: Quantitative Trait Locus
RAM: Root Apical Meristem
SAM: Shoot Apical Meristem
YFP: Yellow Florescence Protein

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Table 1: Some important crop improvement works using *CKX* manipulation.

Plant	***CKX* variant**	**Action**	**Effect**	**Source**
Arabidopsis	*CKX1*, *CKX2*, *CKX3*, *CKX4*	Overexpression	Retarded shoot growth and enhanced root growth	Nishiyama *et al.* (2011)
			Tolerant to salt stress Tolerant to drought stress	
	CKX7	Overexpression	Root growth and xylem differentiation	Kollmer *et al.* (2013)

Rice	*CKX2* (Gn1a)	*CKX* mutant	High in panicle branches	Ashikari *et al.* (2005)
			High in grains/panicle	
			High in grain number	
		RNAi	High in grain number	
		Overexpression	Low in grain number	
	CKX2	RNAi	High in tiller number	Yeh *et al.* (2015)
			High in grain number	
			High in grain weight	
			No response for grain/panicle	
	CKX2 (Gn1a)	CRISPR/Cas9	High in panicle size	Li *et al.* (2016)
			High in flower number	
	*CKX*9	CRISPR/Cas9	High in tiller number	Duan *et al.* (2019)
		Overexpression	Low in panicle size	
			Low in grain number	
Barley	*HvCKX1*	RNAi	High in spike number in T4	Zalewski *et al.* (20014)
			High in grain number in T4	
	HvCKX9	RNAi	No effect at T4	
	HvCKX1	RNAi	High in spike number	Holubova *et al.* (2018)
			High in grain number	
			Low in 1000 grain weight	
			High in yield	
		CRISPR/Cas9	No yield data provided	
	HvCKX1	CRISPR/Cas9	Limited effect	Gasparis *et al.* (2019)
	HvCKX3	CRISPR/Cas9	Low in grain number	
			Low in grain weight	
	CKX2	Targeted overexpression in root	Tolerant in long term drought	Ramireddy *et al.* (2018)
			Biofortification of Zn, Fe and other micronutrients	Ramireddy *et al.* (2018b)
Wheat	*Ta2.2.1-3A*	RNAi	High in grain weight	Li *et al.* (2018)
			No change in spikelet number	
	GW2	CRISPR/Cas9 + TILLING;	High in grain size High in 1000 grain	Wang *et al.* (2018)

		triple mutant on A, B, D sub-genome	weight	
	CKX2.2.1-3D	Mutation association analysis	High in 1000 grain weight	Zhang *et al.* (2012b)
	CKX2.1-3D	Mutation associated analysis	High in grain size	Lu *et al.* (2015)
			High in grain weight High in grain filling	
	CKX4-3A, 3D	Variant association analysis	High in grain weight	Chang *et al.* (2015)
Tobacco	*CKX1*	Root specific overexpression	Tolerant to drought stress Higher accumulation of minerals Improved leaf chlorophyll content	Werner *et al.* (2010)
Medicago sativa	*MsCKX*	Overexpression	Tolerant to salt stress	Li *et al.* (2019)
Lotus	*CKX3*	qRTanlysis	Helping in nodule development	Reid *et al.* (2016)
Brassica	*CKX5-1, 5-2,6-1, 7-1*	qRT analysis	Development of siliques	Liu *et al.* (2018)
	CKX2	Overexpression	Enhanced root growth	Nehnevajova *et al.* (2019)
			Enhanced chlorophyll content Accumulation of higher amount of Cd and Zn	
Populus	*CKX2*	Root specific overexpression	Inhibition of sprouts development	Li *et al.* (2019)