Global View on the Cytokinin Regulatory System in Potato

Sergey N. Lomin, Yulia A. Myakushina, Oksana O. Kolachevskaya, Irina A. Getman, Ekaterina M. Savelieva, Dmitry V. Arkhipov, Svetlana V. Deigraf and Georgy A. Romanov*

Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia

Cytokinins (CKs) were earlier shown to promote potato tuberization. Our study aimed to identify and characterize CK-related genes which constitute CK regulatory system in the core potato (*Solanum tuberosum*) genome. For that, CK-related genes were retrieved from the sequenced genome of the *S. tuberosum* doubled monoploid (DM) Phureja group, classified and compared with Arabidopsis orthologs. Analysis of selected gene expression was performed with a transcriptome database for the *S. tuberosum* heterozygous diploid line RH89-039-16. Genes responsible for CK signaling, biosynthesis, transport, and metabolism were categorized in an organ-specific fashion. According to this database, CK receptors StHK2/3 predominate in leaves and flowers, StHK4 in roots. Among phosphotransmitters, StHP1a expression largely predominates. Surprisingly, two pseudo-phosphotransmitters intended to suppress CK effects are hardly expressed in studied organs. Among B-type RR genes, StRR1b, StRR11, and StRR18a are actively expressed, with StRR1b expressing most uniformly in all organs and StRR11 exhibiting the highest expression in roots. By cluster analysis four types of prevailing CK-signaling chains were identified in (1) leaves and flowers, StHK2/3→StHP1a→StRR1b/4+; (2) shoot apical meristems, stolons, and mature tubers, StHK2/4→StHP1a→StRR1b/+; (3) stems and young tubers, StHK2/4→StHP1a→StRR1b/11/18a; and (4) roots and tuber sprouts, StHK4→StHP1a→StRR11/18a. CK synthesis genes StIPT3/5 and StCYP735A are expressed mainly in roots followed by tuber sprouts, but rather weakly in stolons and tubers. By contrast, CK-activation genes StLOGs are active in stolons, and StLOG3b expression is even stolon-confined. Apparently, the main CK effects on tuber initiation are realized via activity of StLOG1/3a/3b/7c/8a genes in stolons. Current advances and future directions in potato research are discussed.

Keywords: potato, cytokinin, two-component system, receptor, response regulator, gene expression, signaling chains, multistep phosphorelay

INTRODUCTION

Potato tubers (*Solanum tuberosum* L.) are well known and widespread sources of food, feed, and technical substances (starches). Cytokinins (CKs), classical plant hormones, are known to promote potato tuber formation, at least in conventional *in vitro* systems (Palmer and Smith, 1970; Aksenova et al., 2000; Romanov et al., 2000; Cheng et al., 2020). CKs are also involved in the formation of artificial "tuberoids" on tobacco and tomato shoots (Guivarch et al., 2002; Eviatar-Ribak et al., 2013)
as well as tuber-shaped rounded tumors on agrobacteria-infected plants (Dodueva et al., 2020). In addition to stimulating the formation of tubers, CKs contribute to their sprouting (Hartmann et al., 2011; Aksenova et al., 2013). The morphogenic effect of CKs certainly exploits their ability to induce cell divisions (Miller et al., 1955; Sakakibara, 2006; Romanov, 2009; Kieber and Schaller, 2014), although is not limited to this. All the above emphasizes the importance of CK regulatory system for such a valuable tuber-producing crop as potatoes.

Over the past decade, a prominent progress has been achieved in potato research. First of all, this concerns the sequencing of the complete genome of *S. tuberosum* group Phureja doubled monoploid DM1-3 516 R44 (DM) by the Potato Genome Sequencing Consortium [PGSC] (2011). Thereafter, a set of genes/proteins controlling tuberization was uncovered (Dutt et al., 2017; Hannapel et al., 2017). It became clear that the regulation of tuberization is based on a complex crosstalk between numerous hormonal and non-hormonal factors (Aksenova et al., 2012, 2014). In our research, we focused on the hormonal part of this regulatory network. On the basis of our experimental data (Kolachevskaya et al., 2015, 2017, 2018, 2019a) and data from recent literature, an updated hypothesis of hormonal regulation of potato tuberization was advanced (Kolachevskaya et al., 2017, 2019b), where CKs play an important role, especially at the tuber induction and initiation stages. Furthermore, the DNA sequence coding for CK receptors (sensor histidine kinase) and basic CK-signaling machinery were identified in the sequenced DM genome and analyzed by means of bioinformatics tools (Lomin et al., 2018b). In parallel, a suite of genes encoding sensor histidine kinases from tetraploid cultivar “Désirée” were cloned and expressed, giving rise to individual CK receptors. These receptors were studied in-depth, including their phylogenetics, conserved domains, 3D-structures, ligand-binding properties, organ-specificity of expression and responsiveness to CKs, regulatory cis-elements in their promoters, subcellular localization, homo- and heterodimerization, and mode of interaction with phosphotransmitters (Lomin et al., 2018b, 2020; Arkhipov et al., 2019).

Receptors are considered key proteins in hormone signaling, but they cannot signal by themselves. Regulation by any hormone *in planta* requires dozens of genes/proteins that function in various aspects of the given hormonal system. Like any other hormonal system, the CK regulatory system can be conventionally divided into a relatively conserved central part (CK signaling, synthesis, metabolism, and transport) and a more variable periphery—mainly CK-responsive regulatory genes (Bhargava et al., 2013; Brenner and Schmülling, 2015). For example, the central part of the model Arabidopsis plant distinguished by small genome size comprises, according to the latest estimates, some 80 genes (Supplementary Figure 1). Among them, 34 genes belong to the so-called two-component system (TCS) and constitute a signaling pathway termed multistep phosphorelay (MSP) from transmembrane receptors to primary response genes in the nucleus (Heyl and Schmülling, 2003; Mizuno, 2005; Kieber and Schaller, 2014, 2018; Pekárová et al., 2016, 2018; Arkhipov et al., 2019; Hallmark and Rashotte, 2019). Other involved genes have been classified as genes for CK synthesis (20 genes in total) (Kamada-Nobusada and Sakakibara, 2009), catabolism, and reversed inactivation (12 genes) (Schmülling et al., 2003; Hoyerová and Hošek, 2020), as well a transport (eight genes) (Liu et al., 2019). The CK regulatory system in potatoes is far less studied than in Arabidopsis. Here we intend to gain insight into the genome-wide composition and functioning of the central part of CK regulatory system in potatoes, with a particular focus on tuber formation. The prospects for using current knowledge to improve potato yield are discussed below.

**CK-RELATED GENES IN POTATOES**

Current molecular studies of potatoes are based on the sequenced DM genome representing the core genome of this widespread crop. Nevertheless, the size of even this minimal genome (844 Mb) is many times larger than the genome of the model Arabidopsis plant (135 Mb). However, this difference essentially disappears when we compare the numbers of protein-coding genes: 27,029 in Arabidopsis thaliana "Columbia" (Swarbreck et al., 2008) and 39,031 in DM potato (Potato Genome Sequencing Consortium [PGSC], 2011). In this case, the size of the one genome is now only reduced to only 1.44 times that of the other. Consequently, a large dissimilarity in numbers of genes of CK regulatory systems between these two species seems unlikely. Table 1 demonstrates that the core potato genome encodes orthologs of nearly all gene families involved in the central part of the CK system in Arabidopsis. And indeed, the sizes of orthologous gene families are rather close in Arabidopsis and potato. In Arabidopsis, the hormonal part of this regulatory network. On the basis of our experimental data (Kolachevskaya et al., 2015, 2017, 2018, 2019a) and data from recent literature, an updated hypothesis of hormonal regulation of potato tuberization was advanced (Kolachevskaya et al., 2017, 2019b), where CKs play an important role, especially at the tuber induction and initiation stages. Furthermore, the DNA sequence coding for CK receptors (sensor histidine kinase) and basic CK-signaling machinery were identified in the sequenced DM genome and analyzed by means of bioinformatics tools (Lomin et al., 2018b). In parallel, a suite of genes encoding sensor histidine kinases from tetraploid cultivar “Désirée” were cloned and expressed, giving rise to individual CK receptors. These receptors were studied in-depth, including their phylogenetics, conserved domains, 3D-structures, ligand-binding properties, organ-specificity of expression and responsiveness to CKs, regulatory cis-elements in their promoters, subcellular localization, homo- and heterodimerization, and mode of interaction with phosphotransmitters (Lomin et al., 2018b, 2020; Arkhipov et al., 2019).

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### TABLE 1 | Proven or high-probable components of the CK regulatory system in DM potatoes.

| Gene | GenBank | Amino acids | Primary transcript | Location |
|------|---------|-------------|--------------------|----------|
| CHK  |         |             | PGSC0003DMT400015729 | ST4.03ch07:44284533.44287254 R |
|      |         |             | PGSC0003DMT4000084727 | ST4.03ch05:14385273.14361236 F |
|      |         |             | PGSC0003DMT4000077575 | ST4.03ch04:2774233.2781847 R |
| HK   |         |             | PGSC0003DMT400011818 | ST4.03ch12:60313883.60322789 F |
|      |         |             | PGSC0003DMT400020285 | ST4.03ch12:1112062.111265 R |
| HPT  |         |             | PGSC0003DMT4000071077 | ST4.03ch04:1543966.1544627 F |
|      |         |             | PGSC0003DMT4000051875 | ST4.03ch05:50113044.5011958 F |
|      |         |             | PGSC0003DMT4000090747 | ST4.03ch12:60090199.6009910 R |
|      |         |             | PGSC0003DMT400012600 | ST4.03ch11:15411271.1545411 F |
|      |         |             | PGSC0003DMT4000075907 | ST4.03ch04:2709257.271448 F |
|      |         |             | PGSC0003DMT400008290 | ST4.03ch07:402301.406809 F |
|      |         |             | PGSC0003DMT4000020233 | ST4.03ch12:2023372.202870 F |
| RR-B I |     |             | PGSC0003DMT400083064 | ST4.03ch05:6752023.6754796 R |
|      |         |             | PGSC0003DMT4000209623 | ST4.03ch02:3040961.3040963 R |
|      |         |             | PGSC0003DMT4000071618 | ST4.03ch04:24951499.2495497 F |
|      |         |             | PGSC0003DMT4000076726 | ST4.03ch10:5809466.5809664 R |
|      |         |             | PGSC0003DMT4000063187 | ST4.03ch03:5371704.5371289 F |
|      |         |             | PGSC0003DMT400007964 | ST4.03ch06:3495418.3495637 F |
|      |         |             | PGSC0003DMT400042922 | ST4.03ch06:3424763.3424993 F |
|      |         |             | PGSC0003DMT400092899 | ST4.03ch03:23672034.2367254 R |
|      |         |             | PGSC0003DMT400098603 | ST4.03ch03:37709010.3770947 R |
| CRF  |         |             | PGSC0003DMT400020509 | ST4.03ch05:5561709.5561803 F |
|      |         |             | PGSC0003DMT400006041 | ST4.03ch06:38128680.38129840 F |
|      |         |             | PGSC0003DMT400086922 | ST4.03ch03:44865282.4486641 F |
|      |         |             | PGSC0003DMT400015411 | ST4.03ch04:1541524.1542378 F |
|      |         |             | PGSC0003DMT400037749 | ST4.03ch05:2800989.2801840 R |
|      |         |             | PGSC0003DMT400025099 | ST4.03ch09:46852324.4685283 R |
|      |         |             | PGSC0003DMT400083203 | ST4.03ch01:61398842.6139083 R |
| ATP/ADP-IPT |   |             | PGSC0003DMT40009795 | ST4.03ch12:270799.276885 F |
|      |         |             | PGSC0003DMT400068271 | ST4.03ch11:3983873.39844810 R |
|      |         |             | PGSC0003DMT400032989 | ST4.03ch02:41114766.41117388 F |
|      |         |             | PGSC0003DMT400020890 | ST4.03ch11:41500955.4150580 F |

(Continued)
TABLE 1 | continued

| Gene ID | Protein | Amino acids | Primary transcript | Location |
|---------|---------|-------------|-------------------|----------|
| StLOG3a/LOG1 | LOC102581470 XP_006339070.1 | 220 | PGSC0003DMT400027157 ST4.03ch10:54947968.54951688 F |
| StLOG3b | LOC102592821 XP_006354329.1 | 218 | PGSC0003DMT400055525 ST4.03ch09:5646817.5650774 F |
| StLOG7a/LOG2 | LOC102583076 XP_006348482.1 | 218 | PGSC0003DMT400042349 ST4.03ch01:3283634.3289271 F |
| StLOG7b | LOC102587326 XP_006359221.1 | 217 | PGSC0003DMT400072345 ST4.03ch04:70714467.70717363 F |
| StLOG7c/LOG3 | LOC102592408 XP_006342033.1 | 225 | PGSC0003DMT400009551 ST4.03ch01:1308627.1312950 F |
| StLOG8a | LOC102597227 XP_006351690.1 | 213 | PGSC0003DMT400021223 ST4.03ch08:35250415.35253629 R |
| StLOG8b | LOC102595783 XP_015167145.1/XP_006354132.1 | 206/205 | PGSC0003DMT400081828 ST4.03ch01:1308627.1312950 R |

Gene families (left column) are marked bold. Slash separated and underlined end numbers in the Protein column corresponds to different splice forms. 1This family contains non-expressing genes in the displayed organ set (StRR22a) or in the overall organism (StRR22b).

receiver (REC-) domains (Supplementary Figure 2B), which most likely render these proteins inactive. Other potato non-canonical genes are listed in the Supplementary Table 1 as they hardly contribute to CK action. Notably, the potato genome contains genes encoding type C pseudo RRs (PRR type C) which are lacking in Arabidopsis. At least an essential portion of Arabidopsis PRR orthologs harbor CCT motif and take part in the photoperiodic flowering control unrelated to the CK system and to being subject to circadian rhythms (Mizuno, 2005). Any other role of the remaining PRRs (Supplementary Table 1) in CK action cannot be completely excluded but is very questionable.

We included in Table 1 non-CK receptor histidine kinases (CKI1, ETR1) as they may play a role in CK signaling. When one such kinase (AtCKII) was spontaneously overexpressed, the mutated phenotype mimicked the effect of massive CK treatment (Kakimoto, 1996). The receiver domain of another histidine kinase, ethylene receptor ETR1, also can interact with MSP signaling intermediates acting downstream from CK receptors (Zdarska et al., 2019). The background TCS activity of these proteins seems to be sufficient to rescue the basic phenotype of Arabidopsis triple mutants lacking all three CK receptors and no longer responding to CKs (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Romanov, 2009). All this indicates the possible role of CKI1 and ETR1 in MSP signaling. In total, the estimated number of genes directly involved in CK signaling (MSP) in DM potatoes is 31.

For CK degradation, potatoes possess seven CKX orthologs (Table 1). Genes for putative CK conjugation are included only in Supplementary Table 1 because potato StUGTs have
no direct homology to Arabidopsis UGT genes responsible for CK O- and N-glucosylation (Supplementary Figure 3). Also, genes encoding CK transporters (Liu et al., 2019)—StPUP (10), StENT (4), and StABC14 (2) [possibly also StABCI19-21 and StAZG1,2 (Kim et al., 2020; Tessi et al., 2020), see Supplementary Table 1]—have active orthologs in Arabidopsis and are obviously of particular importance. The exceptions are gene-orthologs of PUP14 transporters of Arabidopsis that are missing in potatoes (Supplementary Figure 4). Overall, the central part of the CK regulatory system in the core potato genome comprises 70 genes, the number close to that in Arabidopsis (Supplementary Figure 1). Among the potato genes, 28 (40%) are TCS homologs, whereas the remaining genes are not TCS-related.

**THE EXPRESSION PATTERN OF CK-RELATED GENES**

To elucidate the molecular events underlying potato growth, productivity and stress tolerance, the list of families of paralog genes is a useful but insufficient characteristic. Knowledge of absolute and relative spatiotemporal gene expression is necessary to address this issue. The expression pattern of genes involved in the CK action in the diploid potato line RH89-039-16 is shown in Figure 1. Clearly, most of these genes are expressed differently depending on the organ and stage of development. Among CHK receptors, the expression of StHK2/3 predominates in leaves and flowers, while StHK4 is mostly expressed in roots. In the latter organ, StHK2/4 genes are quite active. Among phosphotransmitters, StHP1a expression predominates in every organ, following by StHP1b, which is also expressed quasi-constitutively but to a much lesser extent. Interestingly, all three genes for the phosphotransfer-like proteins StPHP4b, 4c, 6, which are supposed to suppress CK signaling, are hardly expressed in RH89-039-16 line. As for RR-B transcription factors, StRR1b is expressed most uniformly in all organs, StRR1a acts similarly but much more weakly, and StRR1c is almost not expressed. Among all B-type RR genes, StRR1a, StRR1b, StRR11, and StRR18a are the most strongly expressed. Roots represent the site where almost all RR-B genes are expressed, dominated by StRR11.

By means of cluster analysis of the organ-specific expression of genes encoding receptors and StRRs type B (Supplementary Figure 5), we identified four types of prevailing signaling chains: (1) in leaves and flowers, StHK2/3→StHP1a→StRR1b+/+; (2) in shoot apical meristems, stolons, and mature tubers, StHK2/4→StHP1a→StRR1b+/+; (3) in stems and young tubers, StHK2/4→StHP1a→StRR1b/11/18a; and (4) in roots and tuber sprouts, StHK4→StHP1a→StRR11/18a.

Cytokinins synthesis genes StIPT3/5 and StCYP735A are expressed mainly in roots (similarly to Arabidopsis). CK-perception and synthesis genes (StHKs, StIPTs, and StCYP735A) are also actively expressed in tuber sprouts, where StHK4 transcripts prevail over transcripts of other CK receptor genes. A special group of CK-activation genes termed StLOGs are active in stolons, and StLOG3b expression is mainly restricted to this organ (Figure 1). It is noteworthy that organs (leaves, stolons) in which StLOG genes are strongly expressed, are mostly devoid of transcripts of other CK-synthesizing genes (StIPT, StCYP735A) (Figure 1). The above observations, based on the gene expression data for the diploid potato RH89-039-16, are generally

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1 http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml
consistent with organ-specific analysis of the transcriptome in the commercial tetraploid potatoes “Désirée” (Lomin et al., 2018b, and data not shown). However, the organ/tissue patterns of the expressed genes in different potato lines/cultivars (DM, RH89-039-16, “Désirée”) were not strictly identical, indicating some cultivar-specificity of gene functioning in potatoes. For example, RH89-039-16 and “Désirée” share similar organ-dependent expression patterns for StHP1a, StPHP4b, StPHP6, StRR1b, StRR14, StRR18a, StRR9a, StCYP735A, and StABCGL14a, although marked differences in these patterns were observed for StRR11, StRR9c, StPT75, and StCKX3. The expression patterns of remaining CK-related genes coincide moderately.

**DISCUSSION**

Here we present a global view on the CK regulatory system in potatoes. Generally, each hormonal regulatory system, in particular the cytokinin one (Romanov, 2009), includes complex units ensuring hormone biosynthesis, translocation, perception, and inactivation, as well as the primary response structures to targeted signal transduction. These units consist of corresponding proteins encoded by cognate genes. Here, the CK-related gene sequences were retrieved from the annotated core genome of the doubled monoploid DM1-3 S16 R44 potato Phureja (the genes whose role in the CK system is proven or highly probable, Table 1) and thus represent the minimal gene set of the potato CK system. There is no doubt that commercial varieties of potatoes, mostly tetraploids, possess much many genes related to CK system. This is corroborated by data on the tetraploid “Désirée” variety, in which at least six authentic CK receptors have been identified (Lomin et al., 2018b), twice as many (3) CK receptors encoded by the DM genome. However, additional genes are close paralogs of the “core” genes, so the number of gene clades remains unchanged. Other genes that could theoretically acquire the status of CK “core” genes are listed in Supplementary Table 1, but their involvement requires detailed research and seems unlikely at this time.

In terms of nomenclature, we propose to follow the tradition of naming gene/protein according to the best homology to its Arabidopsis counterpart. Thereby, the potato genes were named according to their closest orthologs in Arabidopsis (see Supplementary Figures 2–4) in Supplementary Data. When many potato genes turned out to be orthogonal to the same Arabidopsis gene, the former are marked with an additional letter at the end (a, b, c, etc.). Such nomenclature has already been used to designate potato CK-receptors (Lomin et al., 2012; Steklov et al., 2013) and other potato genes/proteins (Lomin et al., 2018b). The exceptions are StPUP genes since their homology to any of Arabidopsis PUPs is not obvious. In any case, this compiled gene set (Table 1) can serve as a convenient basis for studying genes, constituting the basic part of the CK system in potato.

As expected, most of CK-related enzymatic activities are encoded in the core potato genome by small (mostly 2–10 members) gene families. At the moment, 16 such families can be counted, of which eight families correspond to intracellular signal transduction (MSP), totally 31 genes encoding 41 proteins (Table 1). Among these proteins, only three (StHKs) have contact directly with hormonal ligands, while others obviously do not, though this has not been definitely proven. In fact, this part of the global CK system includes 28 authentic TCS homologs, which generate or affect signal transmission through His-Asp phosphorelay. The exceptions are proteins with degenerated TCS homology, which cannot directly participate in MSP because of structural deficiency. These non-functioning in MSP genes and proteins are evidently not bona fide members of the central part of the CK system. Some of them are displayed in Supplementary Table 1 containing yet not excluded but possible candidates for the CK system in potatoes. In fact, CKs almost monopolized the MSP system, using it as a signaling part of their global regulatory circuit. This statement is supported by the fact that every functional phosphotransmitter is promiscuous, i.e., is able to transmit “hot” phosphate from any CK receptor to any RR-B in the nucleus (Hutchison et al., 2006; Dortay et al., 2008; Lomin et al., 2018a; Arkhipov et al., 2019). Other hormones—in particular, ethylene, whose receptors are also TCS homologs—had to switch to a signal transduction pathway other than MSP (Pekárová et al., 2018).

Data on the relative expression of selected genes allowed us to outline the most plausible protein chains transmitting the CK signal from receptors to primary response genes. This corresponds to the activity of the prevailing potato MSP. We suggested four types of main signaling chains, which are delineated above. These chains are partially redundant, especially in relation to the transfer stage from the cytosol to the nucleus, where phosphotransmitter StHP1a predominates in all organs of the diploid potato. However, predominant receptors and B-type response regulators may differ depending on the organ, and this CK-signaling specificity should be taken into account when researchers manipulate the potato genome. To date, we consider the stolon as the most promising organ for engineering potato productivity and early maturation. Apparently, this is where the most important events leading to tuber initiation should take place. The most noticeable feature of the transcriptome of the CK system in stolons is the increased activity of the LOG-genes (StLOG1/3a/3b/7c/8a) without visible changes in the expression of other CK-related gene families. Moreover, the expression of StLOG3b is stolon-specific. In contrast, the activity of other CK synthesis genes, IPTs and CYP735A, was much weaker here than in other organs. Thus, stolons are likely sites where CK had to switch to a signal transduction pathway other than MSP.

| Table 1 |
Collectively, since CKs were known to be implicated in many aspects of potato growth and productivity, the presented genome-wide data characterizing the CK-regulatory system of potatoes can be useful for the exploration and breeding of this important crop.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml.

AUTHOR CONTRIBUTIONS

SL, YM, OK, IG, ES, SD, and DA presented and analyzed their experimental and/or bioinformatic results. SL prepared main illustrative materials. GR wrote the version of the manuscript.

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