Plant Omics Journal POJ

POJ 5(6):553-558 (2012) ISSN:1836-3644

Regulatory TGACG-motif may elicit the secondary metabolite production through inhibition of active Cyclin-dependent kinase/Cyclin complex

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Abstract

Cell division and expansion have a pivotal importance for enhancement of efficient metabolite production through cell cycle regulation in transcriptional levels. The Gap1/Synthesis (G1/S) transition of the cell cycle could predict the products for synthesis as well as the distribution of the secondary metabolite between the cell and the medium with cessation of cell cycle in this transition. In this study, 1000 bp upstream sequences of G1/S transition genes as promoter regions from *Arabidopsis thaliana* were analyzed to introduce new generation of pharmaceutical metabolite stimuli. Identification and analysis of potential cis-regulatory motifs with using Botany Array Resource database (BAR) detected ten cis-regulatory elements that had significant differences than other cis-regulatory elements. Then, we evaluated the function of ten Cis-regulatory elements with PlantCARE database. TGACG-motif as Methyl jasmonate responsive element was found among ten putative cis-regulatory elements. The presence of this element in promoter region of cell cycle inhibitors such as Kip-related proteins (KRPs) suggests inhibiting active Cyclin-dependent kinase/Cyclin complex in G1/S checkpoint. Cell cycle arrest in G1/S checkpoint will also increase in the production of plant secondary metabolites in non-cycling cells.

Keywords: Plant cell cycle, cis-regulatory element, core genes, secondary metabolites, methyl jasmonate.
Abbreviations: ABA- abscisic acid; CDC- cell division cycle; CDK- cyclin-dependent kinase; CYC- cyclin; G1/S-gap/synthesis; IC-interolog confidence; ICV- interolog confidence value; Interolog- interaction ortholog; KRP- kip-related proteins; MJ- methyl jasmonate; PCC- pearson correlation coefficient.

Introduction

Plants produce a wide range of natural products with valuable activities as pharmaceuticals, nurtaceuticals, crop protection agents, food ingredients and personal care products (Balandrin and Klocke, 1988). With a growing public interest in 'naturals' and policy drivers to increase sustainability of chemical production, there is now a major opportunity to increase the supply of phytochemicals available for industrial applications. Elicitation of plant cells, tissues and organs represent a useful approach to improve the production of these valuable metabolites (Karuppusamy, 2009). It seems that elicitation processes regulate complex relationships among cell cycle, cell expansion and genes involved in plant secondary metabolism (Hall and Yeoman, 1987; Yanpaisan et al., 1998). Some of elicitors exert their effects through the cell cycle progression machinery. Investigation of plant cell cycle progression and control with a molecular point of view made it able to identify some specific agents and subsequently their proper application for increasing in target metabolites (Hall and Yeoman, 1987; Fett-Neto et al., 1994; Srinivasan et al., 1995). Analysis of cell cycle activity and population dynamics indicated that the population of non-cycling cells may be specialized for secondary metabolite production (Mak and Doran, 1993; Yanpaisan et al., 1998). The cell cycle, by a series of checkpoints, coordinates a variety of cellular events such as DNA replication and cytokinesis through G1/S and G2/M phase transitions (De Veylder et al., 2003). It has been shown that the activity of the regulatory factors including cyclin-dependent kinases (e.g. CDKA;1), cyclin partners (e.g. CYCsA, B, and D), and CDK inhibitory proteins (e.g. Kip-related proteins (KRPs) and WEE1) (Inze and De Veylder, 2006; Sorrell et al., 2002) is omitted or arrested during the different phases of plant cell cycle (De Veylder et al., 2001). It is generally believed that the control of checkpoints through cell cycle genes will be an effective way of pharmaceutical metabolite accumulation. The G1/S phase transition constitutes an important regulatory point (De Veylder et al., 2003) among the different checkpoints in cell cycle. In this transition, plant cells decide to divide (Stals and Inze, 2001), differentiate or be inactive (Francis, 2007). The importance of this phase is considered by essential gene expression for DNA replication in S phase of cell cycle. Therefore, G1/S checkpoint is sensitive in response to various exogenous stimuli. Despite the undeniable importance of post-transcriptional and post-translational regulation, the transcriptional regulation makes a considerable control on core cell cycle regulators (Menges et al., 2005). In this way, identification and characterization of DNA motifs which are probably involved in transcriptional

Table 1. The interolog method utilizes ICV and PCC to validate genes relationship.

Protein 1	Protein 2	ICV	IC	PCC	Annotation 1	Annotation 2
At3g48750	At2g27970	780	High	0.616	CDKA;1	CKS2
At1g18040	At5g27620	504	High	0.829	CDKD1;3	CYCH;1
At3g48750	At2g27960	315	High	0.877	CDKA;1	CKS1
At3g48750	At5g11300	132	High	0.793	CDKA;1	CYCA2;2
At3g48750	At1g02970	84	High	0.662	CDKA;1	WEE1
At3g48750	At1g18040	20	High	0.557	CDKA;1	CDKD1;3
At3g48750	At1g47230	18	High	0.734	CDKA;1	CYCA3;4
At3g48750	At1g80370	18	High	0.597	CDKA;1	CYCA2;4
At3g48750	At5g27620	16	High	0.647	CDKA;1	CYCH;1
At1g66750	At5g27620	8	Medium	0.721	CDKD1;2	CYCH;1
At3g48750	At3g50630	6	Medium	0.64	CDKA;1	KRP2
At3g48750	At5g25380	6	Medium	0.368	CDKA;1	CYCA2;1
At3g48750	At2g32710	4	Medium	0.496	CDKA;1	KRP4
At2g23430	At4g34160	4	Medium	0.232	KRP1	CYCD3;1
At3g48750	At2g23430	4	Medium	0.059	CDKA;1	KRP1
At3g48750	At4g34160	2	Medium	0.622	CDKA;1	CYCD3;1
At2g27970	At4g34160	1	Low	0.891	CKS2	CYCD3;1
At1g66750	At4g28980	1	Low	0.833	CDKD1;2	CDKF;1
At1g47230	At2g27960	1	Low	0.797	CYCA3;4	CKS1
At3g48750	At1g47210	1	Low	0.703	CDKA;1	CYCA3;2
At3g48750	At1g70210	1	Low	0.636	CDKA;1	CYCD1;1
At3g48750	At1g15570	1	Low	0.608	CDKA;1	CYCA2;3
At2g22490	At2g27960	1	Low	0.585	CYCD2;1	CKS1
At3g48750	At2g22490	1	Low	0.578	CDKA;1	CYCD2;1
At3g24810	At3g48750	1	Low	0.556	KRP5	CDKA;1

Protein1 and 2= Accession number of interacted proteins, ICV= interolog confidence value, IC= interolog confidence, PCC= Pearson correlation coefficient, Annotation 1 and 2= the name of accession numbers.

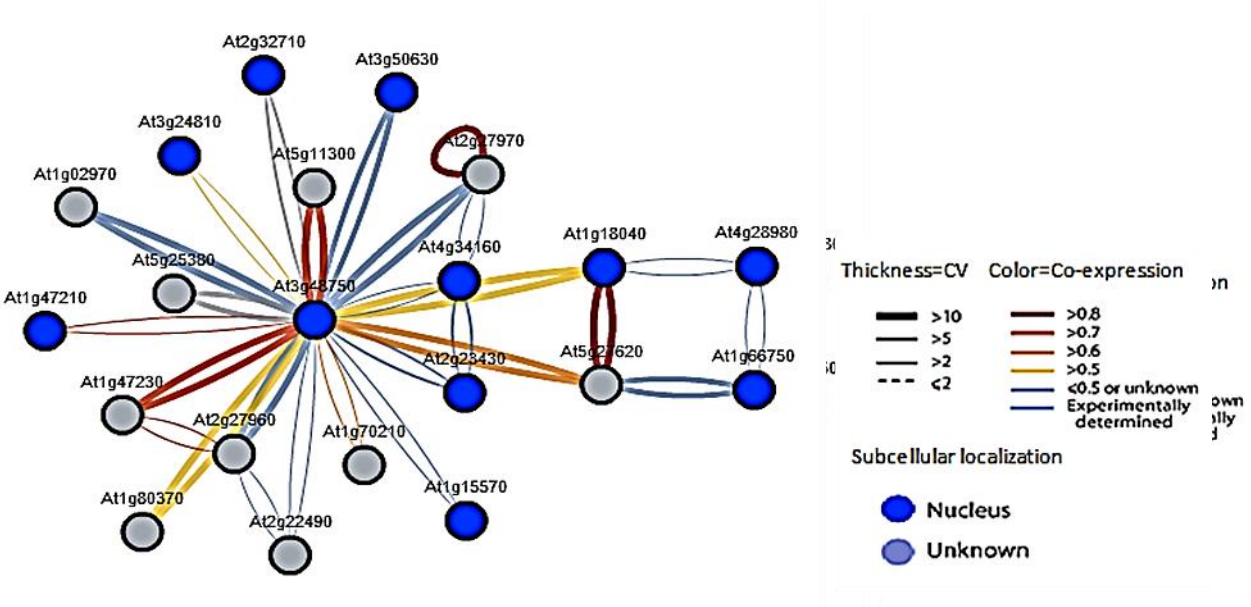

Fig 1. Interaction view of G1/S genes. All predicted interactions are evaluated by CV (line thickness) and co-expression (line color). Nodes are color coded with predicted subcellular localizations. The Botany Array Resource database (BAR) contained an interactome tool that reveals protein-protein interaction based on interolog method. The interactome tool predicts interacting proteins by computing a confidence value (CV) that CV>10, 2<CV<10 and CV=1 defines as high confidence, medium confidence and low confidence between proteins or co-regulated genes respectively.

regulation of the genes can help us to find and apply proper elicitor(s) for a higher efficient metabolite production. A wide variety of methods such as MEME (Bailey et al., 2006), Gibs Sampler (Newberg et al., 2007), PLACE (Higo et al., 1999) and PlantCARE (Lescot et al., 2002) web servers have been developed to find and analyze the motifs. Elicitation mechanism of methyl jasmonate was significantly determined in the plant cell cycle with increase in the percentage of cells in G0/G1 (Naill and Roberts, 2005). Here, we report that there are some potential cis-regulatory elements in promoters of co-regulated genes of G1/S checkpoint which allow methyl jasmonate to arrest cells in G1/S transition. In addition, these cis-regulatory elements will let the cells to increase in capacity of secondary metabolite accumulation.

Results and Discussion

Identification of interaction and Co-expressed connections of G1/S genes

To demonstrate that the accumulation of secondary metabolites are dependent on regulation of G1/S genes at the

transcriptional level, we selected 42 core cell cycle genes involved in G1/S checkpoint according Vandepoele et al., (2002) studies. These genes related to the G1/S checkpoint were introduced to the Botany Array Resource database (BAR) to determine the co-expression level among the interacting proteins. BAR is a web server to gain access to *Arabidopsis thaliana* microarray data associated with the corresponding experimental details. The database contained an interactome tool that reveals predicted interactions or interaction ortholog (interolog) based protein-protein interaction. Pearson correlation coefficient (PCC) is also used in order to validate the co-expression level among the interacting proteins (Geisler-Lee et al., 2007). Out of the 42 introduced genes, 21 genes with different confidence values were recognized and confirmed to be interactive and/or co-regulated at the G1/S checkpoint as Arabidopsis interacting proteins. As shown in Table. 1, nine genes had high interolog confidence level with the average of PCC equal to 0.7 which was the highest correlation value among the three classes of interolog confidence. Gene pairs with a low interolog confidence level relatively gained high Pearson correlations as an index for co-expression of a gene pair. Among three groups of interolog confidence, CDKA;1 had the highest ICV with CKS1, CKS2, CDKD1;3, CYCH;1, CYCA2;2, WEE1, CYCA3;4, and CYCA2;4. CDKD1;3 also revealed this relationship with CYCH;1. Cyclin formed complex with CDK and CKSs as docking factor in collaboration with this binding are caused cell cycle progression during cell division (Francis, 2007), although, WEE1 as an inhibitor suppresses the active CDKA;1/CYC complexes in G1/S checkpoint (De Schutter et al., 2007). In medium confidence, CDKA;1 was related to KRP1, KRP2, KRP4, CYCA2;1, and CYCD3;1. The CYCD3;1 and CDKD1;2 interacted with KRP1 and CYCH;1, in this group, respectively. In addition, Kip-related proteins (KRPs) are switching the cell cycle that induce cell cycle arrest or to delay cell cycle progression in response to intracellular or extracellular signals (Verkest et al., 2005). The result of this inhibition is production and accumulation of secondary metabolites in plant cells. Last group which presents the ortholog interaction with the lowest ICV, showed nine protein interactions, which CDKA;1 took part in most of them. Interaction view of the G1/S genes (Fig. 1) demonstrated that CDKA;1 occupied a central position within the predicted interactions and co-expression patterns. It also directly interacted with four Kip-related proteins (KRPs) as inhibitory agents for cell cycle progression. Some CDK/cyclin complexes conduct the major pathway regulating at the G1/S transition. Growth factors such as sucrose and some plant growth regulators (Richard et al., 2002) induce D-type and A-type cyclins (CYCA/D) to interact with the A-type CDK (CDKA;1), and consequently form an inactive CDKA;1/CYCDs and CDKA;1/CYCAs complexes (Breyne and Zabeau, 2001; Potuschak and Doerner, 2001) (Fig. 2). CDK subunit (CKS) proteins act as docking factor in interaction between cyclins and CDKA;1 and are expressed from late G1 until the end of mitosis (De Veylder et al., 1997). CDK-activating kinase pathway, which involves CDKF and CDKD associated with an H-type cyclin (CYCH) may be able to active the CDKA;1/CYCDs and CDKA;1/CYCAs complexes through phosphorylation (Shimotohno et al., 2004; Yamaguchi et al., 2003) (Fig. 2). Our data showed that the high interolog confidence for CYCA2;2, CYCA3;4, and CYCA2;4 in interaction with CDKA;1 (Table. 1) may correspond to their crucial importance in regulating G1/S checkpoint. On the other hand, some antimitogenic stimuli including abscisic acid (ABA) (Wang et al., 1998), cold temperature, sucrose starvation

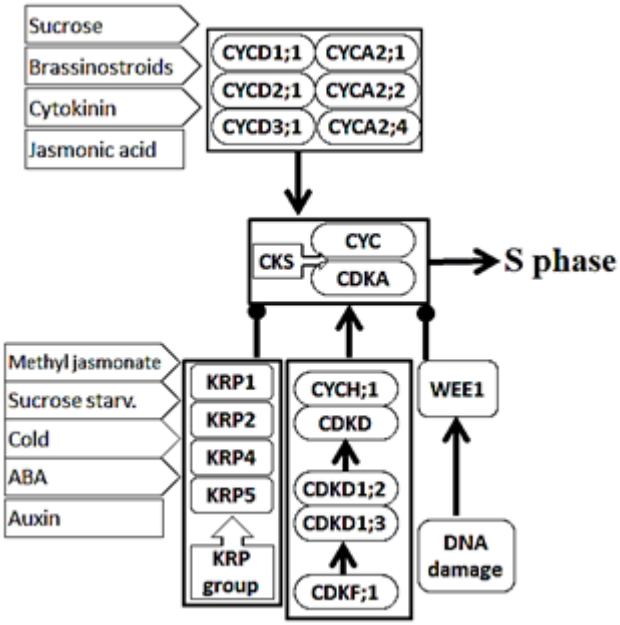

Fig 2. A representative model for G1/S transition of plant cell cycle. The intrinsic and extrinsic signals involve in regulation of the G1/S checkpoint genes. The rectangles and the pentagon's show signals to stimulate and inhibit the gene complexes, respectively. The functional effect of gene sets was also designed with lines including round and arrow end which have inhibitory and stimulatory effects on the other gene complexes, respectively.

(Planchais et al., 2004) can inhibit the activated CDKA;1/CYCDs and CDKA;1/CYCAs complexes (Fig. 2). In addition, there is a negative regulation on CDK activity by phosphorylation activity of WEE1 kinase (Sorrell et al., 2002) that DNA damage as a stress factor has an effective role in the activation of WEE1 kinase. But, the CDC25-related kinase, if existing, removes the phosphate groups and activate CDK complex (Landrieu et al., 2004). These results, in accordance with our data, showed that CDKA;1 is a central core of G1/S checkpoint and it is co-expressed with many genes specially Kip-related proteins (KRPs). Fig. 1 is the results of the BAR database that it included all predicted interactions involved in G1/S transition. It depicted and confirmed that more than 90 percent of G1/S core genes communicate with CDKA;1. In total, Cell growth seems to be suppressed or reduced in response to abiotic and biotic stimuli and external elicitors through proteins such as Kip-related proteins (KRPs) which expression level of them plays a crucial role in progression of cell cycle and consequent secondary metabolite production (Lui et al., 2000; Zhou et al., 2002).

Predicting Cis-regulatory elements of G1/S genes

The symphony of cell cycle genes expression may be regulated by putative and potential cis-regulatory elements. Cis-regulatory elements are one of the most important factors of gene expression in transcriptional level in response to internal and external cues (Li et al., 2008). To identify the elements, we analyzed promoter sequences of G1/S cell cycle genes of *A. thaliana*. The "Promomer" tool of BAR database was used to determine possible cis-regulatory elements. We inserted 21 accession numbers of G1/S cell cycle genes to this tool and were statistically discovered significant

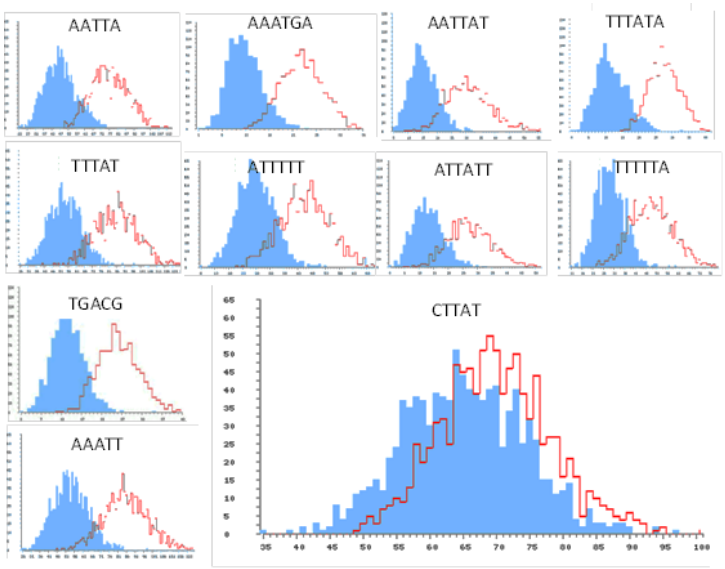

Fig 3. Identification of ten putative cis-regulatory elements with Statistical significance in promoter region of G1/S cell cycle gene. Two distributions are shown from 1000 bootstrapped sets of equal number. The first is obtained by sampling for the frequency of occurrence of motifs found from the given gene cluster promoter set bootstrapped 1000 times, and the second is from 1000 whole genome promoter data sets of equal number to the cluster set. The distribution of occurrence of a given element in both data sets is then obtained and plotted, and significant differences are highlighted as shown in above Figure. In comparison, the distribution for variation of the CTTAT is also shown. These 5-mers is not significantly over-represented in the cluster in question.

degenerate motifs in promoter sequences of individual or co-regulated genes (Fig. 3). The best features of Promomer that make it distinct from other motif finders are using both alignment and enumerative methods (Toufighi et al., 2005). Our cis-element analysis uncovered ten motifs in different lengths with a P-value of 0.001 which may cooperate in co-expression of the genes (Fig. 3). Putative function of these elements was identified using PlantCARE software (Lescot et al., 2002). The data indicated all putative motifs to be TA rich with an exception of TGACG motif. There was evidence that the TA rich sequences in the core promoter region may act as potential building transcription factor site with a rule in promoting or repressing of the gene transcription level (Hobson et al., 1988). Functional analysis of the motifs was carried out using PlantCARE and the Homology Test of PLACE database. The data demonstrated that TGACG-motif was a responsive element to methyl jasmonate (Fig. 3). PLACE Homology Test showed that TGACG motif has 100 percent similarity to the known methyl jasmonate responsive motif. According to this result, existence of TGACG-element on cell cycle G1/S checkpoint genes may be involved in arresting or delaying cell cycle progression in response to methyl jasmonate signal.

TGACG motif may regulate KRPs in response to methyl jasmonate

Kip-related proteins (KRPs) are the most important cell cycle inhibitors (De Veylder et al., 2001). These proteins interact with CDKA;1 (Lui et al., 2000; De Veylder et al., 2001; Zhou et al., 2002) and potentially arrest CDKA;1/CYC complexes. Our data showed that KRP2 and KRP3 contain 3 and 1 copy, respectively, of TGACG motif at their promoter regions. In spite of KRPs, CDKA;1 as a highly connected hub gene at the G1/S gene network did not contain TGACG motif that known cis-acting regulatory elements involved in methyl jasmonate responsive. The presented results suggested that MJ elicits KRPs genes through TGACG motif and the activation of KRPs consequently arrest CDKA;1/CYC complex towards inhibition of cell cycle progression and increase in secondary metabolites production.

Materials and methods

Selection of candidate genes

Candidate G1/S genes from *Arabidopisis thaliana* which were introduced by Vandepoele et al, (2002), were selected and used to survey potentially cis regulatory elements involved in G1/S cell cycle arrest

Interaction analysis

The interaction among all G1/S genes was evaluated by using "Interactome viewer" tools of BAR database as described by Geisler-Lee et al., (2007) (http://bar.utoronto.ca/interactions/cgi-bin/arabidopsis_interactions_viewer.cgi). AGI IDs of the genes were used as input data for Arabidopsis Interactions Viewer.

Regulatory motifs finding

To identify putative and potential regulatory motifs, AGI IDs of the candidate genes were analyzed by "Promomer" tool from Botany Array Resource (BAR) database (http://bbc.botany.utoronto.ca.). The part of "Identify a Statistically Over-represented Element" in a "Group of Genes" were selected for the motif analysis. As a default, the range of five to ten nucleotides with 75 percent occurrence was considered.

Identification of motif function

In order to find cis-regulatory function, the potential regulatory motifs were submitted in "Search for Care" tool of PlantCARE database. "Homology Test of PLACE" tool of

PLACE database was used to verify PlantCARE analysis results (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999). With exception of "Search reverse strand" which turned on the other parameters were left as they were.

Identification of motif number

In order to find number of the regulatory motifs, 1000 bp upstream of all candidate genes as promoter region were acquired using TAIR database. The sequences were submitted in "Search for Care" tool of PlantCARE database.

Conclusion

Our main assumption was that the accumulation of secondary metabolites might be dependent on regulation of G1/S genes at the transcriptional level. Analysis of potential cis-regulatory motifs suggests that methyl jasmonate through the regulatory TGACG-motif may inhibit active CDK/CYC complex in G1/S checkpoint. Consequently, cessation of cell cycle through the MJ responsive element may enhance synthesis of secondary metabolites in non-cycling cells. We hope that expression control in transcriptional levels of cell cycle genes can be a powerful tool in production of secondary metabolites in near future and environmental different signals direct plant cells to the accumulation of secondary metabolites through cell division control.

Acknowledgements

I would like to thank the Shahid Beheshti University of Iran for allowing me to embark on this project. My deepest appreciation also goes to God that makes everything possible.

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