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Environmental stresses and transcriptional responses for safe chloroplast operation

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Higher plants are exposed to various environmental stresses which affect their survival and growth. Organellar perturbation which is caused by environmental stresses results in modulation of nuclear gene expression as a feedback regulation to relieve these stresses. To understand mechanisms of environmental adaptation that is achieved in part through the chloroplast signaling, or retrograde signaling, we are analyzing the transcriptional network that orchestrates stress responses in higher plants. Determination of *cis*-regulatory elements in the promoter region is an old subject in plant science but still provides essential information on the transcriptional network, and, it is now much more easily and more comprehensively conducted with the aid of our recently developed methodologies of bioinformatics. Functional analysis of synthetic promoters that contain only one or two *cis*-regulatory elements provides precise information on the upstream signals driving the *cis*-elements, which is also necessary to understand the network. In addition, biochemical analysis of DNA-protein interaction that is scalable with the aid of oligo DNA and a cDNA resource of Arabidopsis transcription factors (TFs) enables empirical determination of a middle-scale transcriptional network, which is under trial in our laboratory.

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Abbreviations: TF: transcription factor; ETC: electron transport chain; ROS: reactive oxygen species; LDSS: local distribution of short sequences; REG: Regulatory Element Group; RAR: Relative Appearance Ratio; MEME: Multiple Expectation Maximization for Motif Elucidation

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Introduction

Higher plants are facing environmental and biological stresses every day, such as excess light, UV-B, unsuitable temperatures which is higher or lower than optimal growth temperature, lack of nutrients, toxic metals in soil, attack by insects and infection of pathogens, and many of them are critical issues for plants to survive and grow. Environmental stress cause perturbation of mitochondria and chloroplasts (Huner et al. 1998; Taylor et al. 2002). This organellar perturbation can trigger nuclear gene expression and relieve these stresses by so called stress acclimation (Fernández and Strand 2008; Rhoads and Subbaiah 2007).

Mitochondrial perturbation under stress conditions causes several symptoms including inhibition of the mitochondrial electron transport chain (ETC) and changing the redox status, changing balance of mitochondrial metabolites, and increase of mitochondrial ROS (reactive oxygen species) (Schwarzländer and Finkemeier 2013). Recent studies have revealed that a ROS species, H_2O_2, plays a role in the mitochondrial signaling in Arabidopsis (De Clercq et al. 2013).

Chloroplasts are the place for photosynthesis, and nuclear transcription for supplying chloroplast proteins should be coordinated for safe and effective operation of the organelle (Chan et al. 2016; Chi et al. 2015; Fernandez and Strand 2008; Oelmüller 1989). As in the case of the mitochondrial signaling, H_2O_2 is involved in the chloroplast signaling for regulation of nuclear gene expression (Karpinski et al. 1999). Chloroplast signals that are independent of H_2O_2 are also suggested (Chi et al. 2015; Kimura et al. 2003; Triantaphylides et al. 2008), but these signals are not clearly shown at the molecular level yet.

Our laboratory is interested in environmental adaptation of higher plants and regulation of chloroplasts, where chloroplast signaling, or retrograde signaling, plays an important role. Our current activity focuses on synthetic promoter projects and empirical identification of the transcriptional network that orchestrates stress responses for environmental adaptation in higher plants.

Development of methods for promoter prediction

Expression profile of a gene is primarily determined by a set of transcriptional regulatory elements in its promoter

region. Because currently identified *cis*-regulatory elements are limited and far from covering all the elements in a plant genome, recognition of promoter structure demands more detailed analysis instead of using known *cis*-regulatory elements in the region.

In order to complement our limited knowledge of regulatory elements, we have developed two prediction methods of *cis*-regulatory elements in a promoter region (Figure 1). One is called local distribution of short sequences (LDSS) analysis (Yamamoto et al. 2007). In this method, a distribution profile in relative distance from the TSS was calculated for each short sequence, i.e., octamer, and, sequences showing localized distribution profiles are judged as LDSS positive and extracted as promoter elements. Examples of LDSS positive and negative profiles are illustrated in Figure 1A.

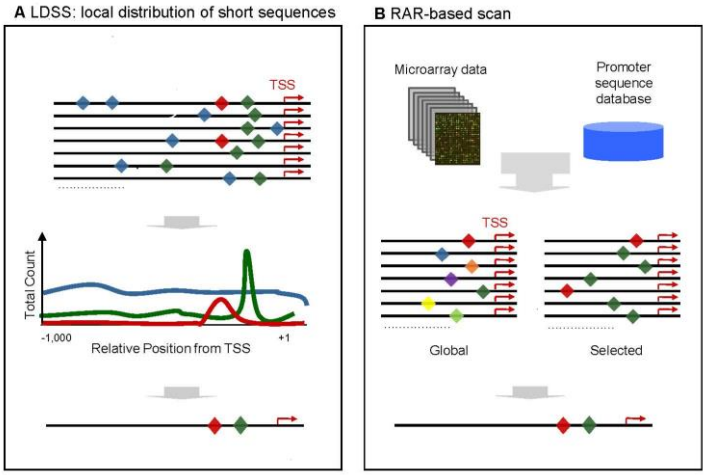

Figure 1. Two approaches for prediction of transcriptional regulatory elements. (A) The top panel illustrates promoter sequences aligned at the most major transcription start site (TSS) for each gene. Position of TSS and direction of transcription is shown as an arrow. Using the aligned sequence set which covers all the genes in a genome, appearance of short sequences (e.g., octamers or hexamers, shown as rhombuses) is counted. The counts are then summed up according to the promoter position, giving a distribution profile for each octamer (or hexamer) as shown in the middle panel. All the profiles are subjected to screening for ones showing local distribution. In the panel, a sequence showing the green profile is selected as LDSS-positive sequence. One with the red profile is also selected though the sequence is not a major one considering its total appearance ratio in the promoter region. A sequence with the blue profile, which is a major one in the promoter region, is not selected because the profile does not show local distribution, and thus it is LDSS-negative. These analyses provide a list of LDSS-positive elements. When the list is applied to a promoter sequence, a structure as shown in the bottom panel can be obtained. Using this method, we have identified common core elements shared with animals (TATA Box), plant-specific core elements (Y Patch, GA and CA Elements), and a group containing diverse sequences named as REG, for Regulatory Element Group. One unique feature of REG elements is that their biological roles are not considered at all for the extraction. (B) Prediction of transcriptional regulatory elements based on large-scale gene expression data. In this method, a gene set whose members share some transcriptional response is extracted using microarray or RNAseq data. Referring to a promoter sequence database (blue barrel in the top panel), two promoter sequence sets, a global set and the extracted set, is prepared (middle panel). For each short sequence (e.g., octamers, shown as rhombuses), degree of over-representation in the selected promoter set over the global set is calculated as Relative Appearance Ratio (RAR). In the analysis of octamers, RAR values are provided to all the possible octamers, that are 4^8 sequences. When the prepared RAR table is applied to a promoter, loci with high RAR values are detected if the promoter contains octamers with high RAR (bottom panel, red and green rhombuses). Such loci are the sites predicted as the putative *cis*-regulatory elements related to the transcriptional response which is used to select the promoter set.

In our analysis several groups have been identified including the TATA box, and a group called as REG (Regulatory Element Group) included known and novel transcriptional regulatory elements (Yamamoto et al. 2007).

Another prediction method is detection of regulatory elements using a parameter called Relative Appearance Ratio (RAR) (Yamamoto et al. 2011) (Fig 1B). RAR is a degree of over-representation of a short sequence, i.e., octamer, in a promoter set sharing the same expression profile over a control set of all the promoters in the genome of object. The focused promoter set is identified using microarray or RNAseq data. Conventional prediction methods including Gibbs Sampler and MEME (Multiple Expectation Maximization for Motif Elucidation) primarily determine consensus sequences in the focused promoter set, but our prediction analyzes frequency of appearance and over-represented elements in the focused set over the global set. Our method is good at detection of elements with specific function, and has much higher accuracy and sensitivity than conventional prediction methods (Table 1) (Yamamoto et al. 2011).

Prediction-oriented functional analysis of plant promoters

Using our methods of promoter prediction, we have established prediction-oriented promoter analysis in order to identify novel transcriptional regulatory elements and characterize their functions including cross talks with environmental or phytohormone-related signals.

Recently we have analyzed two Arabidopsis promoters that strongly respond to multiple environmental stresses. One is a promoter of a malate transporter gene, *ALUMINUM*

ACTIVATED MALATE TRANSPORTER 1 (*AtALMT1*). This transporter secretes malate into soil from roots, which is critical in acquisition of resistance to acid soil (Tokizawa et al. 2015). Consistent with its role, gene expression of *AtALMT1* is strongly activated by low pH and Al ion, which elutes from popular feldspar under acidic conditions. We used microarray data of Al-treatments and mutants of *STOP1*, a transcription factor of the zinc finger group whose mutation is known to reduce expression of *AtALMT1* for promoter prediction of *AtALMT1*. According to our analysis, 8 sites in the promoter was predicted as Al-related elements. Their functional analysis by introducing mutations in the promoter has revealed that 7 out of the 8 sites have function in transcription (Table 2).

Table1. Comparison of promoter prediction methods. Well-established prediction methods, Gibbs Sampler and MEME, were compared with our RAR-based prediction method. Promoters that were subjected to the prediction test have been intensively studied and thus most of functional elements in their promoters are considered to be reported in literature. In this analysis, we assessed each prediction with the aid of information in literature.

Method	Accuracy rate	Sensitivity rate
Gibbs Sampler	0%	0%
MEME	0%	0%
RAR-based prediction	**58-67%**	**88-89%**

Table 2. Summary of RAR-based prediction of novel elements. The acid soil-responsive AtALMT1 promoter and the light stress-responsive ELIP2 promoter were subjected to RAR-based promoter prediction, and the predictions were assessed by *in planta* functional analysis. In the "Function" row, type of response (alminum ion response (Al), high light (HL), UV-B, cold, and circadian oscillation) or signal-independent activation or suppression is shown for each element. An arrow of the row indicates signal-independent activation or suppression. Totally 11 predicted elements were examined and 10 of them turned out to be functional promoter elements.

	ALMT1								ELIP2		
Position from TSS (bp)	-526	-360	-320	-297	-282	-270	-257	-191	-783	-583	-149
Name	A	B	C	D	E	F	G	F	C	B	A
Function	Al	Suppr-ession	Al	Activ-ation	Activ-ation	Activ-ation	-	Al	Suppr-ession	HL, UV-B, Cold Circadian	
TF			CAMTA2	STOP1						HY5	

Accuracy rate=91% (10/11)

The other promoter is of *ELIP2*, which is thought to be involved in light protection as a pigment-binding thylakoid protein, and its expression is also strongly activated by high light (HL), UV-B, and cold stresses (Hayami et al. 2015). With the aid of public and private microarray data of HL, UV-B, and cold treatments, we predicted three sites in the promoter. For their functional analysis, we directly prepared synthetic promoters containing one or more predicted elements, and introduced them into plants. After the analysis, we found that all the three predicted elements are functional (Table 2). Further analysis has revealed that among the three elements, A, B, and C, combination of A and B is necessary for stress responses, but promoters with either one element are not enough for the response (Figure 2). We also knew that the same combination of A and B gives response to HL, UV-B, and also cold stresses.

Table 2 summarizes results of the two promoters, and as shown, 10 out of 11 predicted elements have been shown to have any function in transcription. These data demonstrate that our prediction can be successfully applied for detection of novel transcriptional regulatory elements in plant promoters.

Identification of promoter-transcription factor interactions

Once a transcriptional regulatory element is revealed, identification of the corresponding transcription factors (TFs) is possible. It helps understanding the signaling pathway to the promoter of interest. In our laboratory, protein-DNA interaction is detected by luminescence-based *in vitro* binding assay called AlphaScreen™ because of its high throughput characteristics. In this assay, a TF protein, prepared *in vitro*, and a synthetic oligonucleotide probe labeled with biotin are mixed together with donor beads that

produce singlet oxygen ($^1O_2$) by light excitation and acceptor beads that emit light by $^1O_2$. Binding activity is detected as a light-induced luminescence signal.

In the case of identification of a corresponding TF for a specific promoter element, it is easy if a nucleotide sequence of the regulatory element suggests a specific TF family. One example is identification of a TF binding to a CGCG box in the *AtALMT1* promoter (Tokizawa et al. 2015). In this work, we examined three candidate TFs in the CAMTA family, and one of them, CAMTA2, bound *in vitro* to the CGCG box, and showed involvement in the Al tolerance, revealed by mutant analysis. If there is no candidate TFs for a specific promoter element, screening of two thousand TFs by binding assay (AlphaScreen or Y1H) is necessary, and the required amount of labor considerably increases.

The binding assay is also applicable for identification of corresponding promoter elements for a specific TF in focus. This approach was also recently tried by us. Candidate DNA probes were prepared according to our promoter prediction using microarray data of knockout mutants and/or overexpressors of the focused TF. Using this strategy, we have identified target elements of STOP1, a zinc finger protein (Tokizawa et al. 2015), HY5, a bZIP family protein (Hayami et al. 2015) and RRTF1, an AP2 family protein (Matsuo et al. 2015).

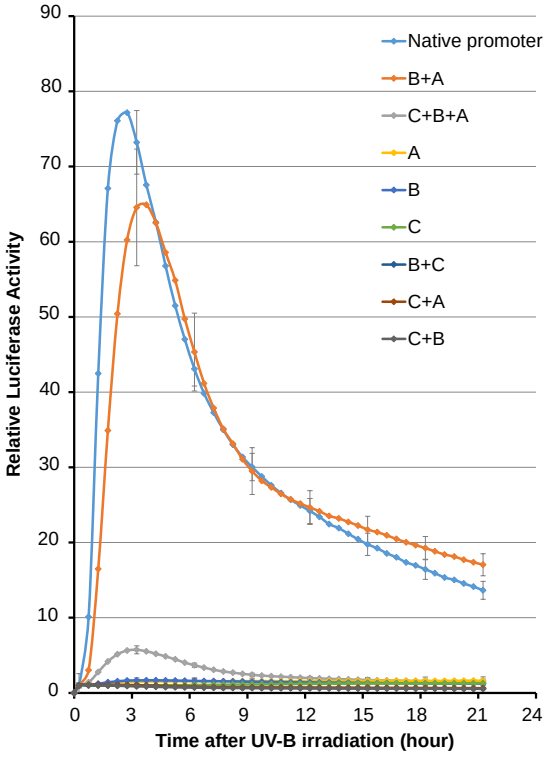

Figure 2. Response of synthetic promoters to an UV-B pulse. Response to an UV-B pulse of Arabidopsis seedling containing synthetic promoters fused to a luciferase reporter is shown. An native ELIP2 promoter which is the source of the synthetic elements is also analyzed. *In vivo* luciferase activity was measured every 30 minutes for 24 h following a pulse of UV-B irradiation (52.6 μmol m^{-2}). For each constructs, average of four to five independent transgenic lines is shown, with SD values provided once for every 3-h interval. The absolute level of luciferase activity at 0 h is designated as 1.0 for each construct.

Empirical determination of transcriptional network in a middle scale

Our knowledge about transcriptional responses to the environmental stress in higher plants is still fragmented, and the whole picture of these responses, from sensors to final responses through a transcriptional cascade, has not been revealed. We are trying for such an empirical identification of a transcriptional network in a limited situation.

H_2O_2 is a signal transducer for a very wide range of stress responses of higher plants, including both biotic and abiotic stress responses (Neill et al. 2002). As mentioned above, it is also known as a mediator oft he mitochondrion and chloroplast signals. Our microarray analysis has revealed that ~60 TFs are involved in the transcriptional cascade of H_2O_2 response in Arabidopsis. Using these data, we tried identification of the transcriptional network composed of these TFs (Figure 3). We utilized three types of information. 1) Time points of induction for these TFs after H_2O_2 treatments. They suggest a rough idea about order of gene activation among the TFs. 2) Transcriptome data of knockout mutants and overexpressors of each TFs. These data, sometimes available in public databases, indicates downstream TFs of the TF, whose expression data is analyzed, revealing *in vivo* relationship between regulating and regulated TFs. 3) *In vitro* binding analysis of an upstream TF protein to an downstream TF promoter. These data together provides enough information about a potential transcriptional network that functions in H_2O_2 responses, in stressed plants.

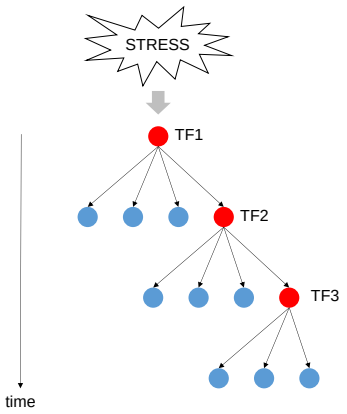

Figure 3. Stress-induced transcriptional network. When plants are exposed to some stress, a cascade of gene activation by transcription factors (TFs, red circle) occurs. The cascade, or transcriptional network, has a role in generation of the transcriptional response of a variety of genes (red and blue circles). One of our goals is identification of direct regulation among involved transcription factors.

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