ATTED-II: a database of co-expressed genes and *cis* elements for identifying co-regulated gene groups in *Arabidopsis*

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ABSTRACT

Publicly available database of co-expressed gene sets would be a valuable tool for a wide variety of experimental designs, including targeting of genes for functional identification or for regulatory investigation. Here, we report the construction of an *Arabidopsis thaliana trans*-factor and *cis*-element prediction database (ATTED-II) that provides co-regulated gene relationships based on co-expressed genes deduced from microarray data and the predicted *cis* elements. ATTED-II (http://www.atted.bio.titech.ac.jp) includes the following features: (i) lists and networks of co-expressed genes calculated from 58 publicly available experimental series, which are composed of 1388 GeneChip data in *A.thaliana*; (ii) prediction of *cis*-regulatory elements in the 200 bp region upstream of the transcription start site to predict co-regulated genes amongst the co-expressed genes; and (iii) visual representation of expression patterns for individual genes. ATTED-II can thus help researchers to clarify the function and regulation of particular genes and gene networks.

INTRODUCTION

High-throughput techniques have produced vast amounts of sequence, expression and structure data. One of these techniques, DNA microarray analysis, produces information on relative expression levels for thousands of genes simultaneously. In addition, large collections of microarray data contain information about concerted changes in transcript levels in these datasets beyond the original purpose of each dataset. Microarray data have been collected in several general repositories, including ArrayExpress (1), Gene Expression Omnibus (GEO) (2) and the Center for Information Biology Gene Expression Database (CIBEX) (3). However, it is generally difficult for experimental researchers who lack bioinformatics expertise to retrieve the information they seek.

For the model plant *Arabidopsis thaliana*, The *Arabidopsis* Information Resource (TAIR) (4) and the Nottingham *Arabidopsis* Stock Centre Arrays (NASCArrays) (5) are species-specific repositories for microarray data. Although it is easy to search the data for individual genes or samples, it is still difficult to retrieve gene-to-gene relationships or simply browse gene expression patterns. Several databases have become available as secondary database for microarray data. The comprehensive systems-biology database (CSB. DB) (6), Botany Array Resource (BAR) (7), *Arabidopsis*

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Co-expression Tool (ACT) (8) and Genevestigator (9) provide co-expressed gene relationships calculated from the array data stored in TAIR and/or the NASCArrays. Such gene relationship information is valuable for predicting gene function, because co-expressed genes are often involved in the same or related pathways. Approaches for specifying experimental targets using such co-expressed relationships have already been reported (10–13). Each database has its own means of displaying co-expression data. One of the tools provided in ACT, 'clique finder' returns a group of co-expressed genes from the co-expressed gene network, although it does not show the network itself (8). Visualized expression patterns are provided in BAR (7), because a gene expression pattern is difficult to comprehend from lists of expression values. Computational prediction of *cis* elements is another important feature for microarray analysis. Interactive *cis*-element prediction from co-expressed genes is also provided in BAR (7). It could enforce the estimation of regulatory scheme of the target genes deduced from the limited amount of experimentally determined *cis* elements, which are stored in *cis*-element databases (14,15).

Although these databases are well equipped with such user-friendly tools, additional interfaces are still required. The following three points need to be addressed: (i) Co-expressed gene lists are too long to allow visualization of complete co-expression patterns. (ii) Although the color-coded 'heat maps' provided by BAR (7) are suitable for comparison of a set of genes, they do not represent quantitative expression changes well. (iii) The interactive *cis*-element prediction tool provided by BAR (7) can extract *cis* elements from the user-selected genes, but the results are hard to exhaustively compare between different queries.

To support the needs of non-bioinformatics experts, we constructed a database named *A.thaliana trans*-factor and *cis*-element prediction database (ATTED-II) for retrieving gene-to-gene relationships similar to the other databases for co-expressed genes. ATTED-II contains the following three original aspects. (i) Network representation of co-expressed gene relationships, which, in addition to the original lists of co-expressed genes, facilitates understanding of the structural basis of gene co-expression (16). (ii) Stored pre-calculated results for *cis*-element prediction are linked to every gene and every functional category, and are presented along with several characteristics of the *cis* elements. (iii) The gene expression patterns are graphically represented—the display of bar graphs for individual genes makes it easy to see quantitative expression changes for many experimental conditions.

ATTED-II is based on the framework of the *A.thaliana* tissue-specific expression database (ATTED), which was opened in 2003 as the repository for our original data for tissue-specific gene expression (17). With the subsequent availability of public microarray data, we imported these data into the original ATTED and stored the calculated co-expression information and the predicted *cis* elements. ATTED-II now contains co-expressed gene networks for 22 263 loci and for 1102 functional categories as well as predicted *cis* elements represented by 304 heptamers. All expression data for these contents are GeneChip data (25k) released by TAIR (4), update of which will be incorporated into ATTED-II. In the following sections, the contents of ATTED-II and its usefulness are described with examples

for obtaining information on co-expressed genes (Figure 1) and predicted *cis* elements (Figure 2).

DATABASE CONTENTS

ATTED-II contains four types of pages; locus page, co-expressed genes page, *cis* element page and functional category page.

(i) The *locus page* contains information for each of 31 128 loci, which covers all *Arabidopsis* protein-coding loci defined by TAIR (4). Of these loci, 22 263 have gene expression patterns measured by GeneChip. For each locus, co-expressed genes are calculated and the 12 most strongly correlated genes are represented in the *locus page* as a network and a list. These co-expressed genes are supported by the following information provided in the same *locus page*: organized functional gene annotation from other databases, subcellular localization predicted by TargetP (18) and WoLF PSORT (19), and graphs of the gene expression patterns. Predicted *cis* elements in the 200 bp region upstream from the transcription start site (TSS) are also shown to support co-expressed gene relationships (Figure 2C). The upstream sequence used in ATTED-II is obtained from TAIR (4), where for the loci without TSS annotation the sequences from translational start site are provided. Details about the predicted *cis* elements can be obtained in the *cis element page*.

(ii) A *co-expressed genes page* is prepared for each of the 22 263 loci for which there are gene expression data. This page contains a longer list of co-expressed genes (300 genes) than that found in *locus page* and presents statistical test results of the nearest 50 genes for functional categories and for *cis*-element appearances (Figure 2D). For extended analysis, whole lists—including more than 300 co-expressed genes—are downloadable from ATTED-II as tab-delimited text files.

(iii) The *cis element page* provides information for each predicted *cis* element. In this prediction, *cis* elements are represented by heptamers. The extracted heptamers are matched with reported *cis* elements stored in the Database of Plant *Cis*-acting Regulatory DNA Elements (PLACE) (14) (Figure 2E). The *cis*-element profiles for position and for experimental conditions are provided to reduce false positives and to consider biological functions (Figure 2E). Also provided are results of a statistical test for functions of the genes having a predicted *cis* element and a list of transcription factors predicted to bind the *cis* element. List of transcription factors in ATTED-II were downloaded from AGRIS (20).

(iv) A *functional category page* is constructed for each gene function category using the 3481 terms defined by GO (21), KEGG (22), AraCyc (23) and KaPPA-View (24). The 15 most significant co-expressed genes are selected from the genes annotated by each functional term, and co-expressed gene networks are drawn (Figure 1C). Because two-thirds of the categories have
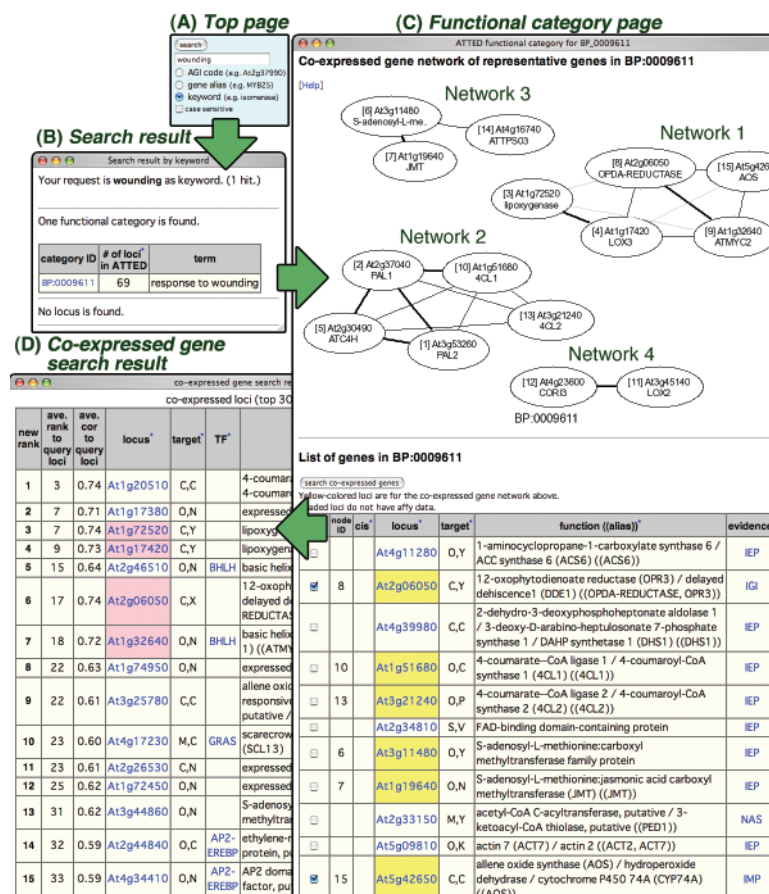
Figure 1. Screenshots of ATTED-II pages relevant to the search for wounding-responsive gene groups. (**A**) Top page. (**B**) Search results. (**C**) Functional category page. Four co-expressed gene groups were found for wounding response. (**D**) Results of the co-expressed gene search. 'Target' in tables in (C) or (D) indicates predicted subcellular localization. For example, 'C,C' indicates both TargetP and WoLF PSORT predict that the gene product is localized in chloroplasts. Yellow-colored AGI codes in the table in (C) indicate genes in the co-expressed gene network. Pink-colored AGI codes in the table in (D) indicate query genes used to search for co-expressed genes.

no significantly co-expressed genes, the networks are drawn for 1102 categories in total. Lists of all genes in the categories are described under the network (Figure 1C). The method for selecting the genes used to draw the networks is described in the help page in ATTED-II.

EXAMPLE 1: OBTAINING CO-EXPRESSED GENES FOR A PARTICULAR FUNCTION

As an example, consider the study of plant defense systems for wounding and injury. As a non-motile organism, a plant cannot move to a more favorable place but must bear and adapt to its environments using complex defence mechanisms. Identification of the genes in a system is the first step for deeper understandings of the system. For gene identification, it is efficient to list and classify genes of co-functional candidates using co-expressed genes (10–13). For this purpose, ATTED-II could be used as follows (Figure 1). (Note that larger figures for this example are shown in the help page in ATTED-II.).

(i) Search 'wounding' as a keyword using the search form to the right of the title logo in ATTED-II (Figure 1A).

(ii) As a result, a GO category 'response to wounding' is found. Click the 'Category ID' (Figure 1B).

(iii) The *functional category page* for 'response to wounding' provides co-expressed gene networks of 15 representative genes in this category (Figure 1C). Each circle represents a co-expressed gene. The AGI code, which is locus ID in *Arabidopsis* (25), a short description and the node ID are enclosed by the circle. The thickness of the lines connecting the circles corresponds to the relative strength of co-expression. The table under the network contains longer descriptions, where the genes in the networks are numbered by node IDs and highlighted by yellow-colored AGI codes. Four networks of co-expressed genes are drawn for this category. To consult each network, the information of the external links in each *locus page* is useful in addition to the information presented in ATTED-II. In this example, publications for each locus provided by TAIR (4) were referred for the interpretation of the results obtained. Each gene network clearly corresponds to a biological function in response to wounding as follows:

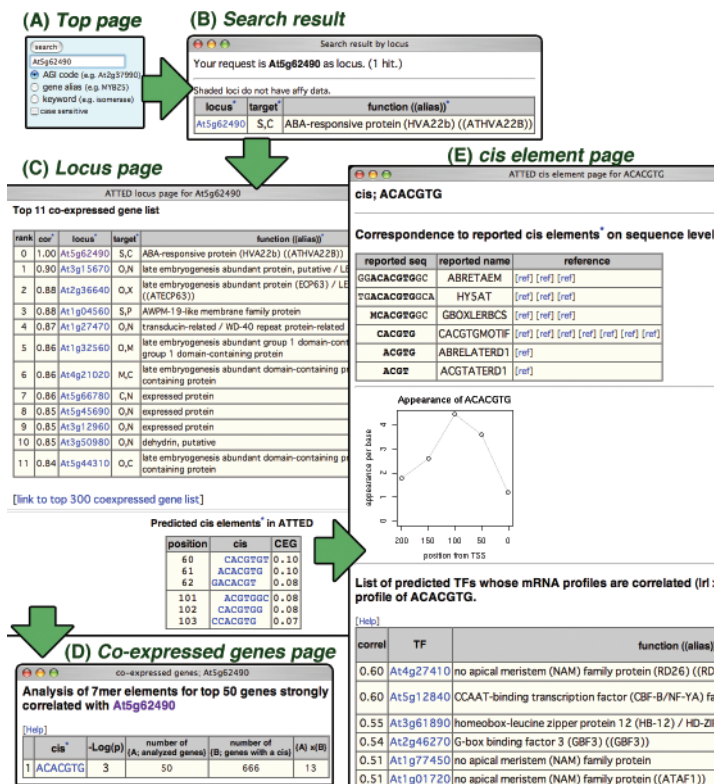(1) Network 1 contains five genes, four of which are enzymes for biosynthesis of jasmonic acid (JA),

Figure 2. Screenshots of ATTED-II pages relevant to the search for a predicted regulatory scheme of At5g62490. (**A**) Top page. (**B**) Search results. (**C**) Locus page. The co-expressed gene list and predicted *cis* elements for At5g62490. (**D**) Co-expressed genes page. Results of a statistical test for the presence of a *cis* element in the 50 co-expressed genes. (**E**) *Cis* elements page and detailed information for ACACGTG. For simplicity, some graphs are omitted in (C) and (E).

which is the phytohormone that has a central signaling role in the wounding response. The other gene, AtMyc2, encodes a transcription factor that regulates JA-responsive genes (26). Gene expression profiles prepared in each *locus page* show that all of these genes are upregulated at an early stage of wounding (data not shown in Figure 1).

(2) Network 2 contains five genes that are enzymes for biosynthesis of flavonoid, one of the major secondary metabolites functioning as a phytoalexin or protectant against photodamage.

(3) Network 3 contains three genes that are enzymes for volatile signaling compounds for defence response. JMT and At3g11480 are enzymes used to produce methylated forms of JA and salicylic acid, respectively (27,28). ATTPS03 is an enzyme for terpene biosynthesis (29).

(4) Network 4 contains two JA-responsive genes. CORI3 is an enzyme, cystine CS lyase (30), which is regulated by JA signaling. This enzyme is involved in the sulfur metabolism participating in defence mechanism. Although LOX2 had been thought to act in JA biosynthesis (31), this gene is known to respond to wounding and JA stimulus at a late stage (32,33) and thus may be involved in JA synthesis at a late stage or function for other purpose in the wounding response.

In this example, four groups of wounding-responsive genes are found. To make a complete list of genes related to the wounding response, next search for co-expressed genes from each group. To select genes to query, click on the checkboxes to the left of each appropriate entry—for example, the group of genes for the JA biosynthetic pathway above—and the click the button 'search co-expressed genes' just above the table.

(iv) The result of the co-expressed gene search is a list of the 300 most strongly co-expressed genes from the five query genes for JA biosynthesis (Figure 1D). This list contains putative functional and unknown genes in addition to many known JA biosynthesis genes. In the same way, we can obtain co-expressed genes for other gene groups. Finally, gene lists containing putative wounding-responsive genes are obtained. These lists of genes are supported by other biological information or *cis*-element information and can be used to design experiments to identify gene functions.

EXAMPLE 2: OBTAINING PREDICTED CIS ELEMENTS

Information of predicted *cis* elements can provide support for proposed co-expressed gene relationships. When co-expressed genes share particular *cis* elements in their promoters, we can expect that these genes are co-regulated through these *cis* elements and that they function together. As an

example in obtaining information on predicted *cis* elements, consider the regulation of an abscisic acid (ABA)-responsive protein At5g62490. ABA is a phytohormone for stress response signaling. ATTED-II indicates that this gene can be up-regulated at embryogenesis through an ABA-responsive element (ABRE) (Figure 2). (Note that larger figures for this example are shown in the help page in ATTED-II.)

 (i) Search 'At5g62490' as an AGI code using the search form to the right of the title logo (Figure 2A).
 (ii) Click the AGI code in the resulting table to go to the *locus page* (Figure 2B).
(iii) In the *locus page* for At5g62490, *cis* elements are predicted at 60–62 bp and 101–103 bp upstream from the TSS (Figure 2C). Although six predicted heptamers are provided, they are grouped into two *cis* elements for position, GA<u>CACGTGT</u> and <u>CCACGTGGC</u>, sharing CACGTG core. The co-expressed gene list indicates that this gene is co-expressed with genes for late embryogenesis. Graphs of At5g62490s expression pattern are also provided, showing that this gene is upregulated by osmotic and salt stresses as well as ABA treatment (data not shown in Figure 2C). Dramatic expression during embryogenesis is also shown, suggesting that this gene may function in embryogenesis. Click 'link to top 300 co-expressed gene list'.
(iv) In the *co-expressed genes page* for At5g62490, appearance of ACACGTG in the co-expressed genes is statistically abundant, suggesting that these genes, including At5g62490 itself, are commonly regulated through this *cis* element (Figure 2D). To obtain detailed information on this *cis* element, return to the *locus page* and click the link 'ACACGTG' to go to the *cis element page*.
 (v) In the *cis element page* for ACACGTG (Figure 2E), users can see that this predicted heptamer corresponds to the reported *cis* elements named ABRE and G-box. The ABRE is a central *cis* element of ABA signal transduction in response to stress treatment (34). This element prefers the position from 50 to 100 bp upstream of the TSS, indicating that the ABRE-like element found in At5g62490 appears in an appropriate position (60–62 bp upstream). The graph for *cis*-element specificity for various experimental conditions shows that genes containing this *cis* element in their promoters are usually up-regulated responding to ABA as well as osmotic or salt stress (data not shown in Figure 2). This pattern is consistent with the expression pattern of At5g62490, supporting the possibility that this gene is regulated through this *cis* element. A list of transcription factor candidates that bind this heptamer is also provided on this page. This list includes known transcription factors to bind ABA-responsive *cis* elements or G-box. In fact, ABF3, a well-known transcription factor to bind ABRE (35), is found in the list, although its correlation value is not high for this sequence itself. When we focus on the regulation during embryogenesis, RD26 (At4g27410) is a good candidate because this gene is highly expressed during embryogenesis and up-regulated by ABA as well as osmotic or salt stress, as indicated by their expression patterns on their respective *locus page*. Furthermore,

RD26 functions in ABA-dependent stress signaling (36), and its homologous transcription factors recognize a CACG core (37). G-box binding factor 3 (GBF3, At2g46270) is another candidate, since it binds G-box sequence for ABA signaling (38). The *cis element pages* for the second group of heptamers found in the *locus page* for At5g62490 contain almost identical information except for the position preference, which is at 100 bp upstream of the TSS, indicating that the second *cis* element also appears at its most appropriate position (101–103 bp).

In summary, information in ATTED-II suggested the following regulatory scheme for the test case of At5g62490. Osmotic or salt stress stimulates ABA signaling. The ABA signal up-regulates At5g62490 together with co-expressed genes for late embryogenesis, potentially through two ABREs and the transcription factors RD26 and GBF3.

OTHER METHODS TO ACCESS PAGES IN ATTED-II

ATTED-II also provides lists of functional categories and *cis* elements to browse. Direct access by entering the URLs is also possible. For example, using http://www.atted.bio.titech.ac.jp/locus/(AGI code of the gene of interest).html will bring up the *locus page* of the gene of interest.

DATA SOURCES AND CALCULATION

Definition of co-expressed genes in ATTED-II

To define co-expressed genes, gene expression profiles were compared. The profiles were constructed from 1388 Gene-Chip data downloaded from TAIR (4), which were manually divided into 53 experimental groups and normalized by RMA method (39). To quantify the similarity of the gene expression profiles, pairwise Pearson's correlation coefficients were used (40). The 12 most strongly correlated genes for each gene (including the gene itself) were used to generate the co-expressed gene networks. The 300 most strongly correlated genes for each gene were listed in the *co-expressed genes page*. The lists of all co-expressed genes are downloadable from ATTED-II as tab-delimited text files. Co-expressed gene networks were pictured by simply drawing a line between co-expressed genes. Details of the method and description of the experiments used in ATTED-II are available in the help pages of ATTED-II.

Prediction of *cis* elements

Previous reports on comprehensive *cis* element prediction for *Arabidopsis* have used reported *cis* elements to search the genome (20,41) or have extracted conserved motifs from the genome (42,43). Our approach using microarray data complements their approaches. To estimate gene regulatory schemes, *cis* elements in the region 200 bp upstream of the TSS, which is downloaded from TAIR (4), were predicted based on the method described previously (44). Briefly, oligomers were used as *cis*-element candidates, and correlations between gene expression changes and presence of the *cis*-element candidates in the gene promoters were estimated

by calculation of Pearson's correlation coefficients. This value is named CEG (correlation between gene expression and a defined gene group) in ATTED-II. We modified this method to distinguish the position of the heptamers from the TSS to reduce false positives.

The region used to extract *cis* elements was also examined. The peak of position specificity was concentrated within 200 bp of the TSS. Moreover, estimation of expression changes using the extracted *cis* elements by a linear model (44) was best for *cis* elements within 200 bp upstream of the TSS, indicating that most of the *cis* elements predicted out of this region are false positives (data not shown). These results agreed with a previous report about ABA-responsive elements (34). Additionally, the shorter region can save the calculation cost. Thus we decided to use the region 200 bp upstream from the TSS.

As a result, 304 heptamers were extracted from all 16 384 ($=4^7$) possible heptamers and registered in ATTED-II. The properties of position specificities are presented in the *cis element pages*. Details of our methods are described in the help pages in ATTED-II.

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