

RiceFOX: A Database of Arabidopsis Mutant Lines Overexpressing Rice Full-Length cDNA that Contains a Wide Range of Trait Information to Facilitate Analysis of Gene Function

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(Received October 26, 2010; Accepted December 3, 2010)

Identification of gene function is important not only for basic research but also for applied science, especially with regard to improvements in crop production. For rapid and efficient elucidation of useful traits, we developed a system named FOX hunting (Full-length cDNA Over-eXpressor gene hunting) using full-length cDNAs (fl-cDNAs). A heterologous expression approach provides a solution for the high-throughput characterization of gene functions in agricultural plant species. Since fl-cDNAs contain all the information of functional mRNAs and proteins, we introduced rice fl-cDNAs into Arabidopsis plants for systematic gain-of-function mutation. We generated >30,000 independent Arabidopsis transgenic lines expressing rice fl-cDNAs (rice FOX Arabidopsis mutant lines). These rice FOX Arabidopsis lines were screened systematically for various criteria such as morphology, photosynthesis, UV resistance, element composition, plant hormone profile, metabolite profile/fingerprinting, bacterial resistance, and heat and salt tolerance. The information obtained from these screenings was compiled into a

database named 'RiceFOX'. This database contains around 18,000 records of rice FOX Arabidopsis lines and allows users to search against all the observed results, ranging from morphological to invisible traits. The number of searchable items is approximately 100; moreover, the rice FOX Arabidopsis lines can be searched by rice and Arabidopsis gene/protein identifiers, sequence similarity to the introduced rice fl-cDNA and traits. The RiceFOX database is available at <http://ricefox.psc.riken.jp/>.

Keywords: *Arabidopsis thaliana* • Full-length cDNA • Gene function • *Oryza sativa* • Trait • Transgenic plant.

Abbreviations: CaMV, cauliflower mosaic virus; CDS, coding sequence; fl-cDNA, full-length cDNA; FOX hunting, full-length cDNA overexpressor gene hunting; FT-NIR, Fourier transform near-infrared; GC-TOF/MS, gas chromatography-time-of-flight mass spectrometry; GO, gene ontology; HCA, hierarchical cluster analysis; MS medium, Murashige and Skoog medium; SA, salicylic acid.

Plant Cell Physiol. 52(2): 265–273 (2011) doi:10.1093/pcp/pcq190, available online at www.pcp.oxfordjournals.org

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Introduction

The generation of loss-of-function and gain-of-function mutant resources is one of the effective approaches for the identification of plant gene functions (Kuromori et al. 2009). For loss-of-function mutant analysis, many loss-of-function mutants have been generated by T-DNA and Ds-transposon insertions in *Arabidopsis thaliana* (Springer et al. 1995, Martienssen 1998, Ito et al. 2002, Alonso et al. 2003, Kuromori et al. 2004). Similarly, in rice, knockout mutants have been prepared by Tos17 retrotransposon and Ds-transposon insertions (Hirochika et al. 2004, Kolesnik et al. 2004, van Enckevort et al. 2005). These mutant resources are widely used for the analysis of gene functions disrupted by the insertion elements. With regard to gain-of-function approaches, several procedures have also been applied to investigate gene function in plants. For example, the activation tagging method is based on the random insertion of cauliflower mosaic virus 35S (CaMV 35S) transcriptional enhancers into the plant genome. This method was first applied to *Arabidopsis*, and allowed us to isolate mutants with severe morphological traits (Weigel et al. 2000, Li et al. 2001, Nakazawa et al. 2003, Yoshizumi et al. 2006). Likewise, the method was applied to rice (Hsing et al. 2007). However, the CaMV 35S enhancer can affect gene expression up to several kilobases from the insertion site (Hsing et al. 2007), sometimes resulting in difficulties in the identification of genes responsible for the observed mutant traits.

Therefore, in order to improve the identification of genes responsible for the observed mutant traits, we have developed transgenic *Arabidopsis* lines overexpressing full-length cDNAs (fl-cDNAs). These transgenic lines, named FOX (full-length cDNA Over-expressor gene) *Arabidopsis* lines, express 1–2 *Arabidopsis* fl-cDNAs under the control of the CaMV 35S promoter (Ichikawa et al. 2006). The FOX hunting system is unique in that only fl-cDNAs are required for the functional analysis of genes, and has an advantage for plants that have large genomes or a long life cycle. Advances in methods for transforming *Arabidopsis* without the need for tissue culture have shortened the time required for gene function analysis (Clough and Bent 1998). We obtained various morphological and physiological mutants from >30,000 rice FOX *Arabidopsis* mutant lines using rice fl-length cDNAs (Kondou et al. 2009).

A large number of mutants have been generated and analyzed using loss- or gain-of-function approaches. Informational resources from these studies have been organized into databases and published on the Internet. These databases contain a large volume of information on mutants (Li et al. 2003, Samson et al. 2004, Sakurai et al. 2005, Zhang et al. 2006, Dalmais et al. 2008, Larmande et al. 2008). However, most of the records in these databases include few paired descriptions of genomic information (e.g. tag insertion position on the genome and introduced gene) and traits corresponding to each mutant (Kuromori et al. 2006). Here, we describe our novel database, RiceFOX, which aims to identify effectively the function of each

gene. Moreover, this database has useful search functions, including the ability to refer to the introduced rice fl-cDNA in addition to the broad range of morphological and invisible traits. RiceFOX access is available via the web interface at <http://ricefox.psc.riken.jp/>.

Database contents

Rice FOX *Arabidopsis* mutant phenotypes were categorized into 19 primary aspects that consist of eight morphological and 11 invisible aspects (Table 1). The RiceFOX database houses wide-ranging information on 17,985 rice FOX *Arabidopsis* lines. Most of the mutant line entries provide information on the introduced rice fl-cDNA and pictures related to each morphological trait. The overview of the records in RiceFOX is summarized by screening categories in Table 1. The briefs of those screening categories are described below. Contents on the RiceFOX database are licensed under a Creative Commons Attribution 2.1 License, which permits non-commercial use, distribution and reproduction in any medium, provided that the original work is cited properly.

Morphological traits

Visible traits in the T_1 generation can be observed in parallel with producing lines, because traits of the rice FOX *Arabidopsis* lines are basically dominant (Ichikawa et al. 2006). With regard to these morphological traits, we focused on the whole plant, root, rosette and cauline leaf, stem, flower, silique and seed, and described their features such as speed of growth, shape, color, number, and so on via our own description. For example, the items for rosette leaf were 'large', 'small', 'long', 'short', 'wide', 'narrow', 'epinastic', 'hyponastic' and 'spiral' for shape; 'dark', 'pale' and 'variegated' for color; and 'many' and 'few' for number. The traits of lines showing abnormal morphology in the T_1 generation were checked in the T_2 generation and described in the database. Additionally, for the seed trait, several numerical data such as diameter and seed color code were included in the records of many rice FOX *Arabidopsis* lines in the T_2 generation.

Plant hormone profiles

Plant hormones play important roles as signaling molecules in the regulation of almost all phases of plant development, from seed germination to senescence. For instance, cytokinins are involved in the regulation of leaf senescence, apical dominance, shoot development and root–shoot balance; auxins in apical dominance, phyllotaxis and root development; ABA in seed dormancy and stress response; and gibberellins in seed germination and leaf development (Davies 2004). The contents of these plant hormones were analyzed by liquid chromatography–tandem mass spectrometry (Kojima et al. 2009) and included in the RiceFOX database.

Table 1 Overview of the entries in the RiceFOX database summarized by screening categories

Screening categories			No. of lines	
			Screened	Objective
Morphological trait	Adult plant	Growth	256	33,623
		Plant height	655	
		Flowering	122	
	Root	Number	75	
		Others	6	
	Rosette leaf	Shape	1,603	
		Color	651	
		Number	537	
		Others	883	
	Cauline leaf	Shape	406	
		Color	102	
		Number	113	
		Others	239	
	Stem	Shape	679	
		Color	66	
		Number	83	
		Others	766	
	Flower	Shape	68	
		Others	87	
	Silique	Shape	280	
Others		127		
Seed	Shape	3,014		
	Color	480		
	Number	330		
Invisible trait	Plant hormones		73	175
	Elements		283	9,869
	Pigments		35	4,302
	Photosynthesis	Chl fluorescence	129	9,947
		High light stress	66	4,570
	UV signaling		51	7,352
	Metabolite	GC-TOF/MS profile	13	350
		FT-NIR fingerprint	23	3,003
	Salicylic acid sensitivity		53	21,200
	Resistance to bacterial pathogen		70	20,000
	High-salinity tolerance		46	21,048
Thermotolerance		3	20,184	
UV stress tolerance		43	7,199	

Element accumulation

Elements are essential not just for plant growth, but also for human nutrition and health. We tried to isolate rice genes that cause higher accumulation of elements in plants to support crop breeding for bioremediation (Guerinot and Salt 2001) and to improve human health in developing countries (Daar et al. 2002). On this basis, a system was developed to measure ratios of any number of elements in plants. We simultaneously measured the ratios of potassium, calcium, iron, zinc, manganese, magnesium, sulfur and silicon in rice FOX Arabidopsis lines in the T₁ generation.

Pigment accumulation

It is known that pigments function as protectors against high light (Steyn et al. 2002) and UV irradiation in plants (Jin et al. 2000). Additionally, they contribute to the taste and color of fruits, vegetables, grains and flowers. Thus, biotechnologies for the control of these compounds offer potential benefits. In this study, we measured the optical density of a solution extracted from the rosette leaves of rice FOX Arabidopsis lines at wavelengths of 305 and 530 nm in order to detect accumulation of UV-absorbing compounds (Mazza et al. 2000) and anthocyanins (Hodges et al. 1999), respectively.

Photosynthesis

Photosynthesis is one of the most important determinants of plant productivity. Chl fluorescence has been widely used to identify photosynthesis mutants because it reflects the state of photosynthetic electron transport (Baker 2008). We used Chl fluorescence imaging to isolate photosynthesis-related mutants from rice FOX Arabidopsis lines. The time course of changes in Chl fluorescence intensity and photosynthetic parameters of rice FOX Arabidopsis lines were compared with those of the wild type.

Light is critical for the growth and development of plants. However, too much light is known to result in photo-oxidative damage (Asada 1999). Although the response of the photosynthetic apparatus to high irradiation must have an underlying regulatory mechanism, little is known about this mechanism. To isolate high light-tolerant rice FOX Arabidopsis lines, Chl fluorescence was measured after high light treatment. One of the photosynthetic parameters, maximal quantum yield (F_v/F_m), was used as an indicator of photo-oxidative damage.

UV signaling

In plants, UV light damages DNA and the photosynthetic machinery and generates reactive oxygen species that damage macromolecules. However, low UV-B fluence rates can stimulate the transduction of signals that regulate the plant's protective response. Some of these UV-B responses are activated not by DNA damage but specifically by radiation in the UV-B range (280–320 nm), and regulate the expression of a wide range of genes (Jenkins 2009). Therefore, it is important that we understand how signal transduction leads to the regulation of the expression of genes that mediate protection against UV-B light. We tried to isolate rice genes that cause hypersensitivity to low UV-B irradiation by looking for the inhibition of hypocotyl elongation by UV-B doses below the level that triggers damage responses in plants (Suesslin and Frohnmeier 2003).

Metabolite phenotyping using two different metabolomics approaches

Metabolomics allows comprehensive phenotyping, filling a niche between systems biology and functional genomics. It thus contributes greatly to integrated functional genomics. We applied two different metabolomics approaches—metabolite profiling and metabolite fingerprinting—to screen changes in the metabolite composition of FOX Arabidopsis mutants. Metabolite profiling is one of the strategies used to study the metabolome. This approach can be used for in-depth investigation of metabolite responses. Gas chromatography–time-of-flight mass spectrometry (GC-TOF/MS) is one of the most widely used techniques and a key technology in metabolite profiling. We performed GC-TOF/MS analysis using aerial parts of rice FOX Arabidopsis mutants (Albinsky et al. 2010).

Metabolite fingerprinting is used in metabolomics because it enables rapid, high-throughput analysis and provides

information from the spectra of total metabolite compositions. Fourier transform near-infrared (FT-NIR) spectroscopy has great potential for metabolite fingerprinting because it is simple to operate, and various types of samples (liquid, solid and powder) can be analyzed non-destructively. In addition, the spectral traits can be systematically extracted by multivariate statistical analysis. Therefore, we used FT-NIR for metabolic screening of rice FOX Arabidopsis mutant seeds (Suzuki et al. 2010).

Salicylic acid sensitivity

The signaling pathway mediated by salicylic acid (SA) plays a crucial role in the defense responses of plants (Durrant and Dong 2004). Rice also has the SA signaling pathway, but only a small number of regulatory components in this pathway have been identified (Takatsuji et al. 2010). To identify new components of the pathway or regulatory components that modulate the pathway in rice, we screened for SA hypersensitivity in rice FOX Arabidopsis lines. Ten T_2 seeds of each FOX line were sown on Murashige and Skoog (MS) medium plates with 50 μ M SA and grown for 12–14 d at 22°C under 16 h light and 8 h dark photoperiods. Lines that showed dwarf and white or pale green phenotypes similar to the *npr1* mutant (Zhang et al. 2003) were selected as 'SA-hypersensitive'.

Resistance to bacterial pathogen

The interaction between Arabidopsis and the bacterial pathogen *Pseudomonas syringae* has been used as a model for investigating the various mechanisms and processes involved in host resistance and bacterial virulence (Kim et al. 2008). The main objective of this research is to look for rice genes that confer resistance to bacterial pathogens, genes that will confer resistance to host plants in a wide variety of genera to various pathogenic organisms. The rice FOX Arabidopsis lines were screened for resistance to compatible *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) for this purpose. Three-week-old T_2 plants were inoculated with $0.5\text{--}2 \times 10^8$ cfu ml⁻¹ of *Pst* DC3000 and disease symptoms were evaluated 6 d after inoculation. Wild-type plants were apparently killed by the screening condition. In contrast, the plants from some rice FOX Arabidopsis lines survived 6 d after inoculation, similar to the resistant control plants, *cpr5-2* (Boch et al. 1998). These lines were estimated to be resistant. The number of lines that survived after three rounds of screening is listed in **Table 1**. Detailed methods and results were recently described (Dubouzet et al. 2011).

High-salinity tolerance

High salinity is one of the major extrinsic factors affecting plant growth and crop productivity. To sustain agricultural productivity and improve agricultural practices, an increase in salinity tolerance of plants is needed by either traditional breeding or genetic manipulation. We used the rice FOX Arabidopsis mutants to collect rice genes with the potential to increase

salinity tolerance by molecular breeding. About 15 T₂ seeds of each line were sown on 1/2 MS medium containing 1% sucrose, 0.8% agar and 150 mM NaCl, and were incubated at 22°C under continuous light for 1 week. Based on visual assessment, lines with a higher germination rate and greener cotyledons were selected (Yokotani et al. 2009).

Thermotolerance

High temperature poses a substantial constraint on the productivity and geographic range of crops, even with the highly sophisticated management systems of today's agriculture. There is a risk that increasing global temperatures will change the optimum sites and conditions for crop production and harm agriculture. We used the rice FOX Arabidopsis mutants to collect rice candidate genes related to a thermotolerant trait. About 15 T₂ seedlings of each line were grown on agar medium at 22°C. A Petri dish containing 4-day-old seedlings was sealed with vinyl tape and dipped into water warmed to 42°C for 90 min. Subsequently, the Petri dish was incubated at 22°C for 10 d. Tolerance was estimated based on whether seedlings green (Yokotani et al. 2008).

UV stress tolerance and root elongation

Plants are continuously exposed to environmental stresses such as UV-B light. UV-B radiation causes growth retardation of plants by inhibiting cell proliferation. This leads to decreased productivity. Genetic manipulation could improve the UV-B tolerance of plants. Roots are essential for resource capture, plant stability and anchorage, so the enhancement of root growth should increase yield. To identify rice genes that confer resistance to UV-B or promote root growth in rice FOX Arabidopsis lines, we screened several long-root mutants from the rice FOX Arabidopsis lines using a root-bending assay (Sakamoto et al. 2003, Takahashi et al. 2005) with modifications. Seedlings were exposed to 7–8 kJ m⁻² UV-B and then incubated under continuous white light for another 3 d. After incubation, lines that had roots longer than those of the wild type were selected as candidate mutant lines. Screenings were performed three times to confirm the identification of mutant lines.

Introduced rice fl-cDNAs

We generated a rice fl-cDNA expression library by using approximately 13,000 rice fl-cDNAs under the control of the CaMV 35S promoter. The rice fl-cDNAs we used are a part of the rice fl-cDNA collection of the National Institute of Agrobiological Sciences (NIAS) (Kikuchi et al. 2003). So far, we have sequenced 6,522 rice fl-cDNAs amplified from 14,401 rice FOX Arabidopsis lines. For the purpose of aiding the understanding of traits in Arabidopsis gene function, Arabidopsis coding sequence (CDS) information was attached to the rice cDNA sequence records, as many rice CDS are homologous to those in Arabidopsis.

Information organizing and database implementation

We have performed comprehensive screening of the rice FOX Arabidopsis mutant lines as described above, and a large number of the comprehensive screening records have been collected. In addition, the introduced rice fl-cDNAs in each rice FOX Arabidopsis line have been sequenced and identified. It is important to integrate the results of the various screening categories in order to analyze gene function effectively. **Fig. 1** shows an overview of the RiceFOX implementation. We classified the screening categories by data type. Next, in order to import the results into a relational database, we organized and formatted the screened results of the rice FOX Arabidopsis mutant lines. With regard to specific results categories such as seed morphological screening and hormone profile, we then performed statistical analyses. Rice fl-cDNA information such as sequence, similarity-based annotation and ontology were also imported into the database. Finally, these information entries were integrated to form the searchable RiceFOX database.

Database access and interface

Search interface of RiceFOX

To browse housed rice FOX Arabidopsis mutant entries, RiceFOX provides a web-based search interface enabling keyword, traits, several identifiers (IDs) related to introduced cDNA function, and sequence similarity searches. RiceFOX has 19 primary and 103 secondary categories for selectable traits. Searches with keyword strings are possible with the National Center for Biotechnology Information (NCBI) BLAST (Altschul et al. 1997) definitions, as well as with IDs from databases such as original rice fl-cDNA (Yazaki et al. 2004), GenBank (Benson et al. 2010), MSU rice genome annotation release 5.0 which is previously known as the TIGR rice genome annotation (Ouyang et al. 2007), RAP-DB annotation release 2 (Tanaka et al. 2008), TAIR8 Arabidopsis locus (Swarbreck et al. 2008), InterPro release 16.2 (Hunter et al. 2009) and gene ontology (GO) (Ashburner et al. 2000) assigned in the InterProScan results. NCBI BLAST has also been implemented on RiceFOX. These search interfaces provide users with effective access to rice FOX mutant entries by using various types of queries.

Screening result and introduced fl-cDNA annotation

Detailed information pages of each rice FOX Arabidopsis line consist of a summary, the screening result and annotation of the introduced rice fl-cDNA. First, the summary contains a text-based outline with hyperlinks to corresponding information on the upper part of the page (**Fig. 2A**). This summary portion allows researchers to determine the annotation status of the searched entries and the annotation most likely to be of

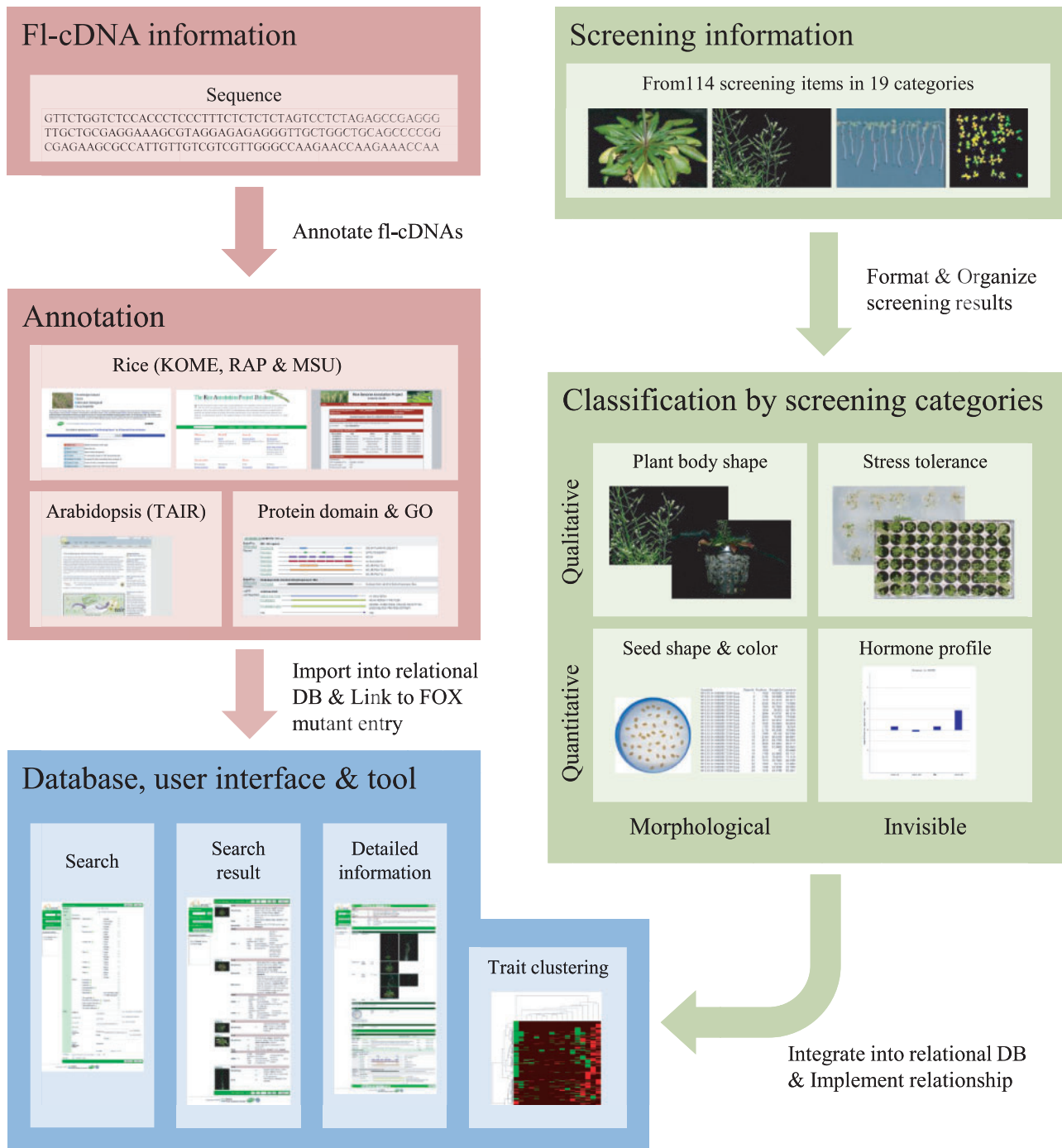


Fig. 1 Overview of workflow to organize the results of FOX mutant screening and database implementation. RiceFOX provides several ways to search the results from screening by IDs, keywords or sequence similarity.

interest, and facilitates users seeking relationship between traits and genes. Subsequently, the screening result provides pictures corresponding to each trait as well as trait information (Fig. 2B). The annotation of the introduced rice fl-cDNA is shown on the lower part of the page, and, whenever possible, links to the original data for each similar hit against RAP-DB, MSU rice genome, protein domain and GO annotation are provided to enable browsing of additional related information

(Fig. 2C). For domain-based functional annotation, the fl-cDNA sequence data were submitted to a domain search using InterProScan. Users can browse each of the results of the protein domain search, along with the predicted GO classification.

Trait hierarchical cluster analysis

The trait hierarchical cluster analysis (HCA) tool is a web-based application and facilitates identification of the relationships of

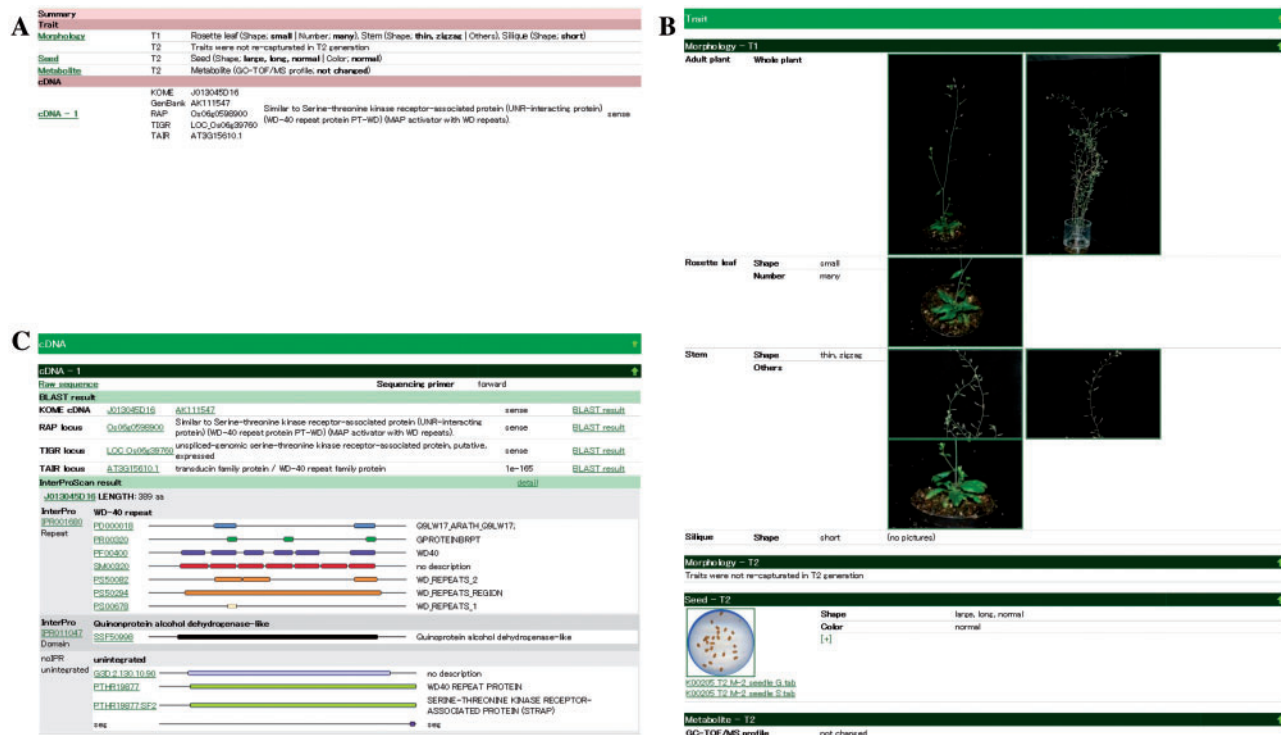


Fig. 2 Example of the detailed information for a rice FOX Arabidopsis line (K00205). (A) Summary table for a rice FOX Arabidopsis line containing the observation results and introduced rice fl-cDNA. (B) Detailed information of the observation results. (C) Annotation of the introduced rice fl-cDNA with links to the original data for each similar hit against RAP-DB and MSU rice genome annotation, and protein domains by InterPro.

mutant traits. In order to enable HCA by mutant trait, all screening results that the RiceFOX database contains were converted from trait vocabularies to numerical values (Supplementary Table S1). The numerically converted trait information can be applied to the HCA by optional distance and clustering methods, screening items and related genes. Therefore, this clustering tool aids the understanding of relationships among the traits of interest. The procedure is divided into two main steps. In the first step, in order to retrieve the observation records of the rice FOX Arabidopsis mutants, users select the generation (T₁/T₂), search condition (and/or) and screening items that are of interest. Next, in order to cluster the retrieved mutant line records in the first step, users choose the clustering method (average, single, complete, ward, mcquitty, median and centroid) and distance method (manhattan, euclidean, maximum, binary and minkowski), and then select the screening items that are to be calculated by HCA execution. Finally, users are able to obtain the trait HCA result and supplementary files for confirmation.

Conclusion

There are some databases similar to the RiceFOX database in that they contain a lot of information on mutant lines. However, RiceFOX has useful search functions, and it is possible

to refer to the introduced rice fl-cDNA in addition to the morphological features of a broad range of traits and invisible traits. This is the first time such a large and comprehensive amount of data pertaining to the traits of plant mutants and their related candidate genes has been collected for reference from a single system. The database should accelerate progress in plant genomics and gene functional annotation, as well as facilitate rapid characterization of useful crop traits. For further enrichment of the information concerning gene function, we will update this database and develop new tools for the advancement of plant genomics.

The rice FOX Arabidopsis line screening is ongoing and RiceFOX will be updated as new data become available.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Japan Science and Technology Agency [a Special Coordination Fund for Promoting Science and Technology].

Acknowledgments

We thank Drs. Satoshi Tabata (Kazusa DNA Research Institute), Yuichiro Watanabe (University of Tokyo), Kenzo Nakamura (Nagoya University) and Naoyuki Umemoto (Kirin Co. Ltd.) for their helpful discussions.

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