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Juan Ling  
*Huazhong Agricultural University*

Renjie Li  
*Huazhong Agricultural University*

Chinedu Charles Nwafor  
*Benson Idahosa University*

Junluo Cheng  
*Huazhong Agricultural University*

Maoteng Li  
*Huazhong University of Science and Technology*

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Authors
Juan Ling, Renjie Li, Chinedu Charles Nwafor, Junluo Cheng, Maoteng Li, Qing Xi, Jian Wu, Lu Gan, Qingyong Yang, Chao Liu, Ming Chen, Yongming Zhou, Edgar B. Cahoon, and Chunyu Zhang
Development of iFOX-hunting as a functional genomic tool and demonstration of its use to identify early senescence-related genes in the polyploid *Brassica napus*

Juan Ling1,†, Renjie Li1,†, Chinedu Charles Nwafor1,2, Junluo Cheng3, Mao teng Li3, Qing Xu1, Jian Wu4, Lu Gan1, Qingyong Yang1, Chao Liu1, Ming Chen5, Yongming Zhou1, Edgar B. Cahoon1,5 and Chunyu Zhang1,∗

1National Research Centre of Rapeseed Engineering and Technology, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China
2Department of Crop Science, Benson Idahosa University, Benin City, Nigeria
3Department of Biotechnology, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China
4Jiangsu Provincial Key Laboratory of Crop Genetics and Physiology, Yangzhou University, Yangzhou, China
5Center for Plant Science Innovation and Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, NE, USA

Summary

Functional genomic studies of many polyploid crops, including rapeseed (*Brassica napus*), are constrained by limited tool sets. Here we report development of a gain-of-function platform, termed ‘iFOX (inducible Full-length cDNA OverexPrexors gene)-Hunting’, for inducible expression of *B. napus* seed cDNAs in Arabidopsis. A Gateway-compatible plant gene expression vector containing a methoxyfenozide-inducible constitutive promoter for transgene expression was developed. This vector was used for cloning of random cDNAs from developing *B. napus* seeds and subsequent Agrobacterium-mediated transformation of Arabidopsis. The inducible promoter of this vector enabled identification of genes upon induction that are otherwise lethal when constitutively overexpressed and to control developmental timing of transgene expression. Evaluation of a subset of the resulting ~6000 Arabidopsis transformants revealed a high percentage of lines with full-length *B. napus* transgene insertions. Upon induction, numerous iFOX lines with visible phenotypes were identified, including one that displayed early leaf senescence. Phenotypic analysis of this line (rsl-1327) after methoxyfenozide induction indicated higher degree of leaf chlorosis. The integrated *B. napus* cDNA was identified as a homolog of an Arabidopsis acyl-CoA binding protein (ACBP) gene designated *BnACBP1*-like. The early senescence phenotype conferred by *BnACBP1*-like was confirmed by constitutive expression of this gene in *Arabidopsis* and *B. napus*. Use of the inducible promoter in the iFOX line coupled with RNA-Seq analyses allowed mechanistic clues and a working model for the phenotype associated with *BnACBP1*-like expression. Our results demonstrate the utility of iFOX-Hunting as a tool for gene discovery and functional characterization of *Brassica napus* genome.

Keywords: iFOX-Hunting system, functional genomic platform, *Brassica napus*, Arabidopsis thaliana, early leaf senescence, *BnACBP1*-like, acyl-CoA binding protein.

Introduction

Reverse genetic studies based on loss-of-function and gain-of-function mutations are widely used to identify novel genes for understanding of basic processes in plants and for introduction of valuable variations in crop genomes (Kuromori et al., 2009). Usually, in the loss-of-function mutant system, RNAi (Wang et al., 2013), Ds-transposon (G van Enckevort et al., 2005), T-DNA insertional (Hirochika et al., 2004) or more recently CRISPR/Cas9 mutagenesis (Ma et al., 2015) are used to either knock out or disrupt gene activity. Indeed, many loss-of-function mutations have been generated for functional genomic studies in Arabidopsis thaliana (Alonso et al., 2003; Kuromori et al., 2004) and rice (G van Enkevort et al., 2005; Kolesnik et al., 2004). In contrast, the gain-of-function mutant systems have typically used, gene activation tags and FOX (full-length cDNA overexPrexors gene)-Hunting techniques to investigate gene function in plants. The activation tag method is based on the random insertion of transcriptional enhancers into the plant genome to induce ectopic, constitutive expression of genes adjacent to the insertion sites (Nakazawa et al., 2003; Weigel et al., 2000). A chemical-inducible activation tagging system has been used to identify important genes controlling the vegetative-to-embryonic transition in Arabidopsis (Zuo et al., 2002). FOX-Hunting is based on overexpression of a single or limited numbers of full-length random cDNAs from a target species or organ in individual transgenic plants (Ichikawa et al., 2006; Sakurai et al., 2011). The FOX-Hunting approach has been effective for the identification of genes associated with a variety of traits including heat and salt tolerance and nitrogen metabolism (Albinsky et al., 2010; Yokotani et al., 2008, 2009a,b).

Reverse genetic approaches for functional genomic studies have been largely used for species such as Arabidopsis and rice...
that are more amenable to high-throughput transformation. Unfortunately, functional genomic tools for identifying useful genes from many crop species, especially polyploids, are lacking. Our particular crop of interest is *B. napus* or rapeseed. Globally, rapeseed ranks third behind palm and soya bean as a source of vegetable oils and is the most important oilseed crop in the cooler climates of China, Canada and northern Europe (Hu et al., 2016). Rapeseed production faces significant challenges including significant losses globally to diseases such as clubroot and *Sclerotinia* induced stem rot (Rondanini et al., 2012). Yield enhancement and improvement of its seed oil content and composition are also major rapeseed breeding and biotechnological targets.

Currently, no loss-of-function or gain-of-function mutant collections are available for systematic analyses of rapeseed gene functions to support genetic improvement efforts. The lack of these tools for rapeseed is due in part to its large genome size (~1200 Mb), multiple copy number of homologous genes and the low efficiency of existing transformation methods. In addition, the high frequency of gene redundancy resulting from its polyploid nature reduces the likelihood of obtaining phenotypes from loss-of-function mutations (e.g. T-DNA insertions) in single genes. In addition, activation tagging for generation of gain-of-function mutants often results in activation of multiple genes distal from the T-DNA or transposon insertion site, which complicates efforts to link specific genes to observed phenotypes (An et al., 2003; Sallaud et al., 2004). These difficulties associated with development and use of mutant populations in rapeseed are also encountered with many other polyploid crops including wheat.

FOX-Hunting is one gain-of-function system that has potential utility for crops such as rapeseed that are less tractable for production of mutants for functional genomic studies. This method involves construction of a full-length cDNA (fl-cDNA) library from the species or organ of interest and cloning of the cDNAs under control of a constitutive promoter (e.g. CaMV35S) into a plant expression binary vector. The resulting library is then transformed into *Arabidopsis* for screening of ectopic expression phenotypes (Ichikawa et al., 2006; Nakamura et al., 2007). A limitation of the FOX-hunting method is that genes that result in lethality or strongly reduced growth upon ectopic expression are missed in the FOX-Hunting mutant screens (Papdi et al., 2008). In addition, strong constitutive promoters such as CaMV35S can cause transgene silencing (Du et al., 2008; Kei-ichiro et al., 2005). To facilitate *B. napus* functional genomic studies and gene discovery, we have developed an inducible FOX-Hunting system termed ‘iFOX-Hunting’ involving the use of an inducible promoter for the expression of rapeseed seed cDNAs in *Arabidopsis*. In addition to the ability to identify genes that induce lethality or impaired growth, the use of an inducible promoter allows one to finely tune expression to specific developmental stages to gain more insights into gene function. This report describes the development of the iFOX-Hunting system using a methoxyfenozide-inducible promoter and its application especially to rapeseed functional genomic studies. As demonstrated, the close genetic relation of rapeseed and *Arabidopsis* allows for predictable translation of findings from the Arabidopsis screening system to rapeseed. We also show for the first time how the iFOX-Hunting-inducible promoter can be coupled with RNA-Seq studies in rapeseed to gain basic insights into the functions of genes identified in mutant screens.

### Results

#### Generation, evaluation and screening of Rapeseed iFOX mutant library

We took advantage of the Gateway® cloning technology to establish a rapeseed full-length cDNA (fl-cDNA) entry vector. To eliminate deleterious effects associated with cDNA overexpression by a strong constitutive promoter, such as the CaMV35S promoter, we developed a conditional gene regulation vector, based on the interaction of a chemical inducer methoxyfenozide, that is compatible with the Gateway® technology. This destination vector harbours methoxyfenozide-responsive gene switch and a Basta resistance gene for selection of transformants. To evaluate the cDNA entry library, 10 000-fold dilution of cDNA library was cultured overnight on LB solid medium and an average of 45 clones was counted, which could be interpreted as \(3.5 \times 10^7\) clones of the total library, sufficient to represent most of the genes expressed in *B. napus* seed. PCR insert fragment of randomly picked 347 clones ranged 0.5–2.0 kb with an average size at 1.26 kb (Figure 1a). Furthermore, a blast search of sequenced fragments revealed that 2% noncoding RNA, 27% non-full-length cDNA and 69% full-length cDNA, which include 72% low abundant and 28% high abundant cDNAs, were contained in the entry library (Figure S1a and b). We used the Gateway LR Clonase II to catalyse the reaction of the entry vector with the destination vector containing the methoxyfenozide-inducible expression system to generate the plant expression vector. The vector structure with restriction enzymatic sites is shown in Figure S1b. The plant expression library was introduced into Agrobacterium by electroporation and subsequently used for transforming Arabidopsis ecotype Columbia-0 (Col-0) by floral dip transformation (Clough and Bent, 1998).

Following floral dip transformation, we generated a total of >6000 T1 seed lines. Seed from transformed plants was germinated, and 4298 positive T1 transgenic plants were selected after screening with Basta. The size of fl-cDNA inserts based on PCR analysis ranged from 1 to 2 kb (Figure 1b). Most of the fl-cDNA (77%) amplified single cDNA fragments and had sequence size above 1.5 kb (Table S1). This inserted cDNA sequence size was comparable to that found in the library used for plant transformation (Figure S1a and b). Although copy number of transgenes was not surveyed in the recovered lines, it is assumed that one or more transgenes is likely present in the selected lines, as is typical for Agrobacterium-based floral dip method in Arabidopsis. This ultimately necessitates additional functional confirmation of genes identified in the iFOX screen, as described in the example below.

#### Observing visible phenotype of T2 generation in induced condition

We subsequently established an efficient screening system to facilitate discovery of visible phenotypes resulting from inducible overexpression of rapeseed fl-cDNAs in a selected portion of the T1 lines (1000 lines). First, we determined the optimal inducer concentration (61.3 𝜇M) required to achieve maximal level of transgene transcript accumulation (Koo et al., 2004) (Figure S1e). Next, 200 positive T1 transgenic plants were self-pollinated to generate T2 seed stock. The resulting T2 progenies were screened with methoxyfenozide, and visible phenotypic changes were identified in these transgenic lines under induced condition (Figure 1d). To determine whether the observed phenotype
resulted from gain or loss of function, we planted the same lines from T2 seed stock under induced and noninduced conditions. Interestingly, no visible phenotypes were observed in most lines without induction. Meanwhile, phenotypic changes were observed in a number of the induced lines. Such mutations were considered to be gain-of-function mutations. However, a few transgenic plants displayed similar visible phenotypes under noninduced and induced conditions. Such lines were considered to be loss-of-function mutants due to insertional mutagenesis, although it cannot be excluded that the inducible promoter was ‘leaky’ in these lines. This process was repeated for over 3000 positive T1 plants advanced to the T2 generation. From this population, 37 transgenic candidates with visible morphological changes compared to noninduced lines and wild-type plants were obtained. These included 31 gain-of-function mutants and 6 loss-of-function mutants (Figure S1d). The gain-of-function lines included 22 early leaf senescence mutants and two mutants displaying lethality (Table S2, Figure 2a and Data S1). These mutants maintained stable, inducible phenotypic expression through subsequent generations.

To determine whether transcript induction correlates with observed phenotypes following induction, we analysed the transgene expression pattern in different tissues of a randomly selected iFOX line (rsl-1375) 36 h after induction. Enhanced transcript abundance was detected in all organs tested, which confirmed ectopic gene expression was achieved by inducible promoter (Figure 1c). We then selected 23 gain-of-function transgenic lines, rescued and sequenced their individual fl-cDNAs. Sequence analysis revealed they had single fragment of rapeseed fl-cDNA, and these fl-cDNAs had Arabidopsis homologs coding for various protein with diverse functions (See Table 1 for more detail). Briefly, that is, Line rsl-1947 had dwarf phenotype and contained rapeseed fl-cDNA with protein sequence predicted as At3G49910 homolog, a translation protein SH3-like family involved in cold response. Induction of rapeseed fl-cDNA integrated in Line rsl-1181 conferred narrow leave phenotype and its predicted sequence had no known function in Arabidopsis. Interestingly, Line rsl-805 showed a very strong lethal phenotype under induced condition (Figure 2b), and the recovered cDNA sequence had high protein similarity with Arabidopsis.
AT5G45890 annotated as Senescence-associated gene 12 (Table 1). This lethal phenotype of Line rsl-805 following induction confirmed earlier reports of deleterious effect of CAMV35S as a constitutive promoter for ectopic expression fl-cDNA(s) (Papdi et al., 2008). This result also underscored the usefulness of the inducible promoter to identify genes deleterious genes and to controllably obtain phenotypes at desired developmental stages.

Characterization of early leaf senescence in rapeseed iFOX Arabidopsis line (rsl-1327)

Among the 22 lines that displayed early leaf senescence phenotype in the T2 generation, line rsl-1327 showed dramatic leaf senescence just after bolting under induced condition (Figure 3a) and was selected for further analysis. Morphological characters such as plant height, leaf shape, floral organ formation, flowering time and fruit set were not significantly different between induced and noninduced plants (Control) (Table S3). However, leaf ageing rate and the number of senesced leaf (Figure 3b) varied significantly. Consistent with programmed cell death (PCD) (Lim et al., 2007), the chlorophyll content of rsl-1327 was significantly lower than that of the control (Figure S2a), and the relative expression of two senescence-related marker genes (SAG12, SEN1) was significantly up-regulated in rls-1327 plants compared to wild-type plants (Figure 3c).

To identify the transgene integrated in the rsl-1327 line, we amplified the fl-cDNA and sequenced it with insert specific primers. Phylogenetic analysis revealed Rsl-1327 homolog gene in Arabidopsis is ACBP1 (AT5G53470) and acyl-CoA binding protein (Xiao and Chye, 2011), while in B. napus it is similar to BnACBP1 (BnaA02g10270D) and NCBI BLAST® indicated that the Rsl-1327 protein sequence is BnACBP1-like (Figure 3d). Results from subsequent studies using GFP-tagged protein expressed in protoplasts were consistent with plasma membrane and ER-localization of the BnACBP1-like protein (Figure 3e).

To confirm that induced-overexpression of the BnACBP1-like triggered the accelerated early leaf senescence phenotype, we performed independent retransformation of Arabidopsis wild-type Col-0 lines with the recovered fl-cDNA fused to a constitutive promoter (CaMV35S). After retransformation, the early senescence phenotype was observed in all regenerated plants (Figure S2b and c). The chlorophyll content of these plants was also significantly reduced compared to wild-type plants (Figure S2d). Similar phenotypes were also observed with transformation of the BnACBP1-like cDNA under control of the CaMV35S promoter in B. napus (Figure 4a). In the two confirmed transformation events (Figure 4b). When premature leaf senescence was assessed in detached leaves, leaves from two different transgene lines showed accelerated senescence marked by yellowing 8 d after detachment, while those of the wild type remained green (Figure 4a). In particular, lines Bnrs1-1327-29-OE and Bnrs1-1327-44-OE plants had very severe leaf etiolation and significantly low chlorophyll content after 2 months (Figure 4c). Consistent with these phenotypes, the BnACBP1-like transcript level in Bnrs1-1327-OE lines was approximately 80-fold higher than in the control lines (Figure 4d). Together, these results demonstrate that the BnACBP1-like overexpression phenotype in B. napus is essentially the same as observed in the Arabidopsis rsl-1327 line.

Transcriptome analysis of rsl-1327 lines

To gain insights into the function of BnACBP1-like, RNA-Seq studies were conducted using four-week-old rsl-1327 plants. Samples were collected from noninduced and induced plants at 2 h and 4 h after application of water (noninduced) or methoxyfenozide (induced) (Figure S3a). The results of RNA-Seq data preprocessing and read alignment are reported in Table S4, Data S1 (including Figures S3b-d, S4d and S5). At 2 h, significant changes ($P < 0.01$, FDR = log2FC > 1) in gene expression were detected in 608 genes between the induced and noninduced plants (Table S5). Approximately 87% of these genes were up-regulated and 13% were down-regulated in the induced versus noninduced plants (Table S6). Functional annotation revealed that 575 genes were assigned to cellular component, 535 genes were assigned to molecular functions, and 558 genes were assigned to biological process (Figure S4a–c). The result of gene enrichment test revealed significantly ($P < 0.05$, $P < 0.01$) enriched genes mostly related to defence responses, which included genes related to jasmonic acid (JA) biosynthesis (AT1G76680, AT2G06050, AT3G25760, AT3G25780, AT1G72520); signalling processes (AT1G19180, AT5G47220, AT3G11820); and JA stimulus (AT1G18570, AT2G34810, AT5G13220); and oxylipin biosynthetic and metabolic process (AT2G06050, AT3G25760, AT1G17420, AT2G26560, AT1G72520). Others up-regulated genes encoded SNARE superfamily proteins (AT3G11820, AT3G52400, AT3G52400).
Sequencing the fragments in different lines and blasting them in TAIR to obtain the information of homologous genes in Arabidopsis.

| Line   | Homologous gene in Arabidopsis | Annotation in database | Query cover | Phenotype               |
|--------|--------------------------------|------------------------|-------------|-------------------------|
| rsl-70 | AT2G41280                      | Late embryogenesis abundant protein (M10) | 73%         | Early leaf senescence   |
| rsl-116| AT2G32930                      | Zinc finger nuclease 2 | 87%         | Narrow leaves           |
| rsl-395| AT1G06760                      | Winged-helix DNA-binding transcription factor family protein | 66%         | Early leaf senescence   |
| rsl-486| AT3G43810                      | Calmodulin 7           | 100%        | Early leaf senescence   |
| rsl-805| AT3G45890                      | Senescence-associated gene 12 | 78%         | Early leaf senescence/lethal |
| rsl-935| AT3G29550                      | LOB domain-containing protein 41 | 82%         | Early leaf senescence   |
| rsl-1181| AT1G62870                      | Unknown protein        | 96%         | Narrow leaves           |
| rsl-1300| AT1G51980                      | Insulinase (Peptidase family M16) protein | 85%         | Dwarf                   |
| rsl-1327| AT5G3470                       | Acyl-CoA binding protein 1 | 85%         | Early leaf senescence   |
| rsl-1346| AT1G01720                      | NAC, ATAF              | 86%         | Early leaf senescence   |
| rsl-1375| AT1G20693                      | High mobility group B2 | 87%         | Early leaf senescence   |
| rsl-1436| AT5G62260                      | AT hook motif DNA-binding family protein | 77%         | Early leaf senescence   |
| rsl-1472| AT1G04270                      | Cytosolic ribosomal protein S15 | 93%         | Pale silhouette         |
| rsl-1479| AT1G36380                      | KH domain-containing protein | 70%         | Early leaf senescence   |
| rsl-1512| AT5G65500                      | Cpn60 chaperonin family protein | 84%         | Yellow silique          |
| rsl-1752| AT4G25570                      | Ferric reductase transmembrane protein family | 86%         | Early leaf senescence   |
| rsl-1767| AT2G33880                      | Homeobox-3             | 72%         | Slow growth             |
| rsl-1810| AT5G19990                      | Regulatory particle triple-A ATPase 6A | 89%         | Early leaf senescence/lethal |
| rsl-1947| AT3G49910                      | Translation protein 533-like family protein | 89%         | Dwarf                   |
| rsl-1988| AT1G75990                      | PAM domain (PCVPINT associated module) protein | 80%         | Early leaf senescence   |
| rsl-2208| AT3G39970                      | Proteasome inhibitor-related | 74%         | Early leaf senescence   |
| rsl-2295| AT5G47120                      | BAX inhibitor 1        | 88%         | Botting early           |
| rsl-2301| AT1G65720                      | Unknown protein        | 66%         | Early leaf senescence   |

Sequencing the fragments in different lines and blasting them in TAIR to obtain the information of homologous genes in Arabidopsis.

AT4G23210) and transmembrane receptor genes (AT5G41750, AT5G44910, AT2G39200, AT1G66090, AT5G41740, AT1G51270, AT1G29690) that function in signal transduction, immune system response and PCD (Table S7).

After 4 h of induction, fewer responsive genes (123) based on $P < 0.01$, $FDR = \log_{2} FC > 1$ were observed (Table S8). About 89% of these differentially expressed genes (DEG) showed significant up-regulation, while 11% were down-regulated (Table S6). Functional annotation and enrichment (FDR < 0.05, $P < 0.01$) uncovered significant biological processes similar to what has been observed in the 2 h treatment, including enrichment of genes related to JA stimulus and oxylipin metabolic process, oxidative stress and defence responses, and PCD (Table S9). Furthermore, evaluation of expression profile revealed significant co-expression relationship between genes involved JA biosynthetic and signalling pathways (Figure 5a). In this regard, JA was of interest and prompted further analysis.

Inducible expression of BnACBP1-like conferred senescence phenotype of rsl-1327 by promoting high accumulation of JA and oxylipins

To confirm whether the significant up-regulation of JA synthesis and signalling genes resulted from inducible expression of BnACBP1-like, we measured JA content in both induced and control plants 2 h after inducer treatment. Consistent with the RNA-Seq results, which showed significant up-regulation of JA genes in early response to BnACBP1-like inducible expression, JA content was significantly high in induced lines compared to water-treated control plants (Figure 5b). This finding suggests that JA may play a significant role in promoting accelerated leaf senescence phenotype of the iFOX line rsl-1327 (He et al., 2002).

Given the overlap in the enrichment of genes related to JA, oxylipin and PCD and reports that JA enhances expression of several oxylipin genes through a feedback loop that amplifies signal transduction and cell death (Savchenko et al., 2014; Sun et al., 2014), we measured the oxylipin levels in Arabidopsis lines constitutively expressing BnACBP1-like in comparison with wild-type Col-0. The results shown in Figure 5c revealed very high accumulation of oxylipins: 9-HOD (9-hydroxy linoleic acid), 9-HOT (9-hydroxy linolenic acid), 13-HOD (13-hydroxy linoleic acid), 13-HOT (13-hydroxy linolenic acid) and 9-KOD (9-keto linoleic acid), 13-KOD (13-keto linoleic acid) in the transgenic lines, confirming that ectopic expression of BnACBP1-like induced metabolism of oxylipins. Together, these results suggest that BnACBP1-like could mediate early leaf senescence through induction of JA and oxylipin signal transduction. Moreover, as JA is a signalling molecule, any significant changes in its level of biological activity will constitute an indirect regulation of metabolic, developmental and defensive processes in rsl-1327 (Devoto and Turner, 2003). Additionally, because the sublocation of BnACBP-1like protein is in the endoplasmic reticulum and plasma membrane (Figure 3e), it is tempting to speculate that BnACBP-1like might indirectly induce senescence-associated PCD through unsaturated fatty acid metabolism via a pathway that involves lipoxygenase genes.

**Discussion**

The identification of BnACBP-1like in the studies reported here highlights advantages of the iFOX-Hunting as a functional tool for polyploid Brassica napus...
genomic tool, particularly compared to the previously described FOX-Hunting system. Because its expression induces an early senescence phenotype, BnACBP1-like, for example, may not have been identified without the use of an inducible promoter. In addition, the ability to translate findings from ectopic expression in Arabidopsis to rapeseed was shown by the observation of a similar early senescence phenotype from constitutive expression of BnACBP1-like in transgenic B. napus lines. Furthermore, the inducible promoter in the iFOX-Hunting system was particularly advantageous for gene function characterization by unravelling the network of genes responsive to BnACBP1-like expression through the combined use of transcriptomic analyses and metabolomics. With a noninducible promoter, it is likely that this network of genes and the temporal aspects of the network’s response to BnACBP1-like expression would not be so clearly defined.

We produced about 6000 transgenic lines using the iFOX-Hunting system. From this population, 4298 positive T1 lines were obtained that harboured the inducible plant expression vector and the rapeseed fl-cDNAs. Size distribution pattern and sequence analysis of fl-cDNAs in randomly selected iFOX mutants were not significantly different from fl-cDNA contained in the entry vector (Figure S1a and b). Although the rapeseed seed-specific fl-cDNAs used in the study were not

Figure 3  Characterization of early leaf senescence in iFOX Arabidopsis line (rsl-1327). (a) The phenotype of rsl-1327 in induced condition. Rsl-1327 shows significantly early leaf senescence compared with WT and noninduced. (b) The relative leaf senescence ratio in different growth stage. Values are means ± (n = 96) of three independent experiments. (c) The transcript levels of senescence mark genes SAG12, SEN1 in 4 weeks 35SBNACBP1-like seedling leaves. The values are the means ± SD for three biological replicates (**P < 0.01). (d) Evolutionary relationships of ACBP1 in Arabidopsis and Brassica napus. The analysis involved 13 amino acid sequences. Evolutionary analyses were conducted in MEGA7. (e) Subcellular localization of BnACBP1-like in protoplast transformation. pBip-RFP is an endoplasmic reticulum-localized marker; ARPIP1, RFP is plasma membrane marker. At least three independent transformation experiments were performed using the two constructs.
normalized, it is interesting that the diversity of fl-cDNAs was largely maintained in the iFOX lines (Figure S1a and b). Future application of the iFOX strategy would likely benefit from normalization to increase the diversity of the genes screened.

From the 4298 confirmed transgenic lines, 37 T2 lines with altered visible phenotypes were identified using the methoxyfenozide inducer. Line rsl-1327 was chosen for gene characterization as an example of the application of iFOX-Hunting in functional genomic research. This line was found to contain a cDNA corresponding to the BnACBP1-like gene (BnaA02g10270D), which shares the highest identity to the Arabidopsis ACBP1 (AT5G53470) gene (Figure 3d). In contrast to our findings with the BnACBP1-like gene, it was previously reported that overexpression of ACBP1 in Arabidopsis does not induce an early senescence phenotype (Lung and Chye, 2016). Instead, a phenotype similar to that observed with overexpression of the BnACBP1-like gene in Arabidopsis and rapeseed was reported for overexpression of the more distantly related Arabidopsis ACBP3 gene (Xiao et al., 2010). These findings indicate that although our results with BnACBP1-like gene expression are similar in Arabidopsis and rapeseed, it is not possible to predict the functions of rapeseed genes based solely on high levels of identity with Arabidopsis genes.

BnACBP1-like mediates early leaf senescence in rsl-1327 through apparent induction of senescence-associated PCD

Our findings with BnACBP1-like are consistent with a role of this gene in PCD induction. These findings include the severe leaf chlorosis of rsl-1327 plants after induction, up-regulation of senescence marker genes, and increased JA and oxylipin levels (9-HOD/T, 13-HOD/T and 9/13-KOD) (Sun et al., 2014). Notably, it is well documented that jasmonates initiate adaptive defence processes that lead to senescence, and oxylipins mediate ROS (reactive oxygen species) production and cell death in plants (Fan et al., 2013; Montillet et al., 2005). Specifically, in our RNA-Seq data, we observed significant increase in the expression levels of lipoxygenase 3 (AT1G17420 = log2FC: 1.61711) and lipoxygenase 4 (ATIG72520 = log2FC: 2.09071), genes known to be involved in fatty acid hydroperoxide formation. Lipoxygenases catalyse the oxygenation of fatty acids by addition of molecular oxygen to unsaturated fatty acids to yield an unsaturated fatty acid hydroperoxide (Schneider et al., 2007). Additionally, these LOX-hydroperoxy fatty acids serve as precursor of diverse oxygenated fatty acids including jasmonates in plant (Caldelari et al., 2011; He et al., 2002), and studies have reported that LOX-dependent hydroperoxy fatty acid formation is required for

Figure 4 The characteristic of two different BnACBP1-like overexpression transformation lines in B. napus. (a) The phenotype of BnACBP1-like overexpression transformation lines. After 8 days dark treatment, the leaves of two lines show different senescence degree, the T-DNA insert plants all show senescence, no insert and wild type did not show significant senescence. All the leaves come from 3 months seedling at the same position. (b) PCR analysis of the T-DNA insert in two Brassica lines. Insert specific primers were used. (c) The BnACBP1-like relative expression in different lines by qRT-PCR. RNA was extracted from 3-month leaves. Error bars indicate s.d. from three technical replicates. The transcript levels of each gene were normalized to Actin7. (d) Relative chlorophyll contents of two different BnACBP1-like overexpression transformation lines. After 8 days of dark treatment, total chlorophyll content was measured and normalized per gram fresh weight of sample. Asterisks indicate significant difference from the wild type at the same treatment (*P < 0.05 or **P < 0.01). Values are means ± SD (n = 3) of three independent experiments.

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hypersensitive cell death development in plant, such as cotton (Jalloul et al., 2002; Marmey et al., 2007), pepper (Hwang and Hwang, 2010) and tobacco (Garcia-Marcos et al., 2013). For these reasons, we hypothesize that BnACBP1-like may interact with linoleic acid and linolenic acid in PC/PA of the ER. Recombinant AtACBP1 has been previously shown to bind PA and PC (Du et al., 2013). Possibly, BnACBP1-like enhances PC/PA exchange and enhances the linolenic acid content in plastid. The substrate increase might lead to oxylipins accumulation by LOX 3/4 catalytic peroxidation, and this may directly cause PCD or indirectly cause JA levels to increase and then lead to senescence-associated PCD. Based on the above hypothesis, we proposed a potential working model for role of BnACBP1-like in leaves early senescence upon inducible overexpression (Figure 6). This model shows major cellular and physiological interaction in response to inducible overexpression of BnACBP1-like in the iFOX line rsl-1327. Given that the fl-cDNA library screened by iFOX-Hunting was prepared from developing seeds, it is likely that BnACBP1-like has functions related to seed metabolism. Although this gene may also mediate fatty acid oxygenation via lipoxygenases in seeds, additional studies are needed to confirm this.

Although the iFOX-Hunting system was used for visual screening of Arabidopsis phenotypes associated with the expression of a seed library prepared from rapeseed, this system can be applied to a wide range of screens for identification of gene function especially for polyploids crops species. For example, we have conducted additional screens, including gas chromatography (GC) of fatty acid methyl esters of seed oils from induced plants, to find new gene functions in rapeseed, including those related to oil metabolism.

The iFOX-Hunting methodology can be easily adapted to the high-throughput characterization of genes from other plant species as well as genes from specific organs, developmental stages or conditions (e.g. water stress). The example reported here also highlights the value of the inducible promoter of the iFOX-Hunting system to identify genes whose ectopic expression

Figure 5 RNA-seq results and JA and oxylipin content in response to induction. (a) The heat map of JA synthetic and signalling pathway after induced 2 h and 4 h. (b) The JA levels were enhanced after 2-h induction in three-week-old seedlings. The values are the means ± SD for six biological replicates. The asterisks indicate statistically significant differences between the transgenic and WT plants (*P < 0.05, **P < 0.01). (c) Major oxylipin compositions for 9 of 13 hydroxy-FAs and keto-FAs in the WT and 35sBnACBP1-like transgenic line leaves. 9- / 13-HOT, 9- / 13-hydroxy octadecatrienoic acid; 9- / 13-HOD, 9- / 13-hydroxy octadecadienoic acid; 9- / 13-KOD, 9- / 13-keto octadecadienoic acid. The values are the means ± s.d. for three biological replicates. The asterisks indicate statistically significant differences between the transgenic and WT plants (*P < 0.05, **P < 0.01).
is deleterious to growth and for use in transcriptomic studies to further dissect the function of genes identified from screens of the mutant populations. Overall, the iFOX-Hunting method described here is a tool to expand plant functional genomic studies that is especially useful for crops such as rapeseed that currently lack high-throughput transformation protocols and have considerable gene redundancy arising from polyploidy.

Experimental procedures

Plant materials and growth conditions

Arabidopsis plants (Wide-type Col-0) and the transformed lines were grown at 22 °C under a photoperiod conditions (16-h light and 8-h dark).

Construction of B. napus fl-cDNA expression library, plant transformation and selection

A rapeseed fl-cDNA iFOX expression vector was produced according to the method described by Weiste et al. (2007) with slight modification. Briefly, seeds at different development stages were collected. Total RNA was isolated by CTAB, the mRNA was purified from total RNA using illistra™ mRNA Purification Kit, and equal amount of mRNA were pooled to obtain composite sample for cDNA library construction. Using the Gateway® cloning technology, first we established an enriched rapeseed full-length cDNA entry vector (Weiste et al., 2007). The M13 primers were used to test and evaluate the cloned insert. Next, a Gateway® compatible destination 24101-1 vector was constructed to include methoxyfenozide-chemical-inducible promoter (Koo et al., 2004) and the resistant gene for Basta. Following site-specific recombination, the entry vector containing the rapeseed fl-cDNA was transferred to the destination vector. The resulting binary vector was subsequently transformed into Escherichia coli. Afterwards, plasmid mixture extracted from transformed Escherichia coli containing the destination vectors was used to transform Agrobacterium tumefaciens GV3101. The resultant transformants harbouring the destination vector were selected by spectinomycin. A pool of these selected Agrobacteria was grown in liquid medium for plant transformation.

Arabidopsis plants were transformed by the floral dipping method using Agrobacterium (GV3101). Subsequently, leaves randomly selected from T1 plants were used for DNA extraction and PCR analysis to confirm fl-cDNA diversity. Transformed T1 seeds were selected with 120 mg/L of Basta solution, and T2 seeds were screened for visible phenotype with 100 μL/L methoxyfenozide (Dow AgroSciences LLC 22.6%w/v). Phenotypes were scored based on morphological changes such as germination, leaf size, shape and colour. Other parameters include flowering time and senescence. All plants showing visible phenotypes were transferred to a new growing tray. Rosette leaves were collected from T2 plants showing visible phenotype for further analysis.

Genomic DNA isolation, PCR and sequencing

To identify integrated cDNAs, genomic DNA prepared from leaves of randomly selected 1064 T1 transgenic plants was the template for PCR amplification with primers complementary to vector sequences flanking the attB1 and attB2 sites (RS-LBRY-L985: GAGGACACCGTGAACTGAGGACACCGTGAACGAT and Plinex-r: CTGGTGATTTTGTGGGACTCTTGTTGATTTTGGG GACT). The PCR condition was 95 °C for 30 s for denaturation, 55 °C for 30 s for annealing and 72 °C for 60 s for elongation. The PCR products were gel purified and sequenced with the same primers. The identity of the transcript was revealed by sequence homology search using the TAIR BLAST tool. To validate the phenotype conferred by the inducible expression of fl-cDNA, the cDNA was isolated and inserted into pGly35sRed3 expression vector driven by 35S promoter for Agrobacterium-mediated transformation of Arabidopsis wild-type Col-0.

RNA extraction and RT-PCR

To evaluate the expression pattern of transgenes in different plant tissues after induction, semi-quantitative RT-PCR was performed on randomly selected mutant rsl-1375. The TRizol Reagent Kit (Ambion™) was used following manufacturer’s instructions to extract total RNA from leaf, stem, flower bud and pod, respectively, of 4-week-old plant. First-strand cDNAs were synthesized from each RNA preparation using the Thermo Scientific RevertAid Kit following manufacturer’s instructions. The specific sequences of each of the primer pairs used in semi-quantitative reverse transcription (RT-PCR) are listed in: 1375-f TGCTAAAG CAGCAGTGCAGA; 1375-r ACACAGACTTTGTCAGATCC.

Subcellular localization

PCR-generated open reading frame of BrnACBP1-like without stop codon was subcloned in-frame upstream of the GFP gene in the 35S-GFP vector (Bottanelli et al., 2012). The construct was validated by sequencing (Forward primer: GACCCGGTCGCCGGG GATCCATGGTGTTGATTTTGGTT, reverse primer: CCTTGCTCAC CATGGATCACAGAATCTCTTCTCTC). Next tobacco (Nicotiana benthamiana) leaf protoplasts were isolated according to Aggarwal et al. (2014). The resulting constructs were transiently expressed in tobacco protoplast according to the method described by Batoko et al. (2000). Tobacco leaf protoplasts were isolated according to Aggarwal et al. (2014), subsequently, GFP signal was detected at room temperature after 24 h of expression with confocal fluorescence microscopy (Zeiss, LSM510 Meta, Carl Zeiss Germany).

Figure 6 Proposed model defining the role of BrnACBP1-like in leaf early senescence. BrnACBP1-like may interact with PA/PC of linoleic acid and linolenic acid in ER. Recombinant AtACBP1 has been previously shown to bind PA and PC (Du et al., 2013). Possibly, BrnACBP1-like enhances PC/PA exchange and increases the linolenic acid content in plastids. The resulting substrate increase, in turn, leads to oxylipin accumulation by LOX 3/4 catalytic oxidation and/or JA accumulation, leading to PCD induction.
RNA sequencing

For RNA-Seq, total RNA was extracted according to The TRIzol Reagent Kit RNA quality and quantity were determined using a Nanodrop 8000 (Thermo Scientific, Wilmington, DE) and a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Before RNA extraction, rsl-1327 transgenic line was sprayed with the inducer ‘methoxyfenozide’, for control, and rsl-1327 transgenic lines were water sprayed. Leaves were collected after 2, 3, 4 and 5 h of inducer and control treatment. All samples were collected in three biological replicates. Afterwards, samples from each biological replicate at each time point were pooled. For RNA sequencing, only sample from 2 h and 4 h were used. In total, 12 samples were used to construct cDNA library with Illumina® TruSeq® RNA Sample Preparation Kit following the manufacturer’s instructions. All samples were sequenced using an Illumina HiSeq 2000 sequencer at the National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University.

Analysis of sequence data

After image analysis, estimation of error and base calling with Illumina Pipeline, a hundred-bp paired-end sequences data were generated (paired-end reads that were 100 bp in length). Next, reads from different samples were identified in the sequence data using indexed primers and low-quality reads were removed using NGS QC tool kit as described by Wu et al. (2016). Reads that passed the QC were considered suitable for further analysis after passing quality control.

Afterwards, short-read alignment and mapping of reads were carried on Arabidopsis genome annotation (TAIR 10) with the software TopHat v2.0.11 using the default parameters (Trapnell et al., 2009). Only, uniquely mapped reads were considered for gene expression analysis. Cufflinks v2.2.1 programme (Trapnell et al., 2010) was used to estimate transcript abundance and differential gene expression. Reads mapping to annotated transcripts were summed for each gene model and normalized by FPKM. Differentially expressed genes (DEGs) were identified with Cuffdiff implemented in Cufflinks software, and FDR was set at P value < 0.01, log2FC = >1.

For functional annotation, enrichment analysis, cluster and pathway analysis differentially expressed genes showing significant enrichment in response to control and induced treatments and comparisons were analysed using TAIR (http://www.arabidopsis.org/tools/bulk/go/index.jsp), Agrigo (Du et al., 2010), Genesis (Sturn et al., 2002) and KEGG databases.

Validation of RNA-Seq data

Quantitative real-time PCR (qRT-PCR) was performed on cDNA obtained from one of the biological replicates belonging to control and induced treatment, which was used for RNA sequencing. First-strand cDNA was synthesized using Thermo Scientific RevertAid Kit following manufacturer’s instructions. qRT-PCRs were performed with SYBR Green Premix system (Newbio Industry) and specific primers (Data S1 and Table S10) using the CFX ConnectTM Real-Time PCR Detection System (BIO-RAD, Hercules, CA). The expression profiles of 22 genes were analysed, with Actin7 (AT5G09810) used as constitutive gene for normalization. PCR conditions were 95 °C for 1 min, followed by 44 cycles at 95 °C, 12 s, 60 °C, 30 s and 72 °C, 30 s. After cycling, melting curves of the reaction were run from 55 °C to 95 °C. Relative expression was calculated with the software LINREG, as described by Ramakers et al. (2003).

Measurement of jasmonic acid and oxylipin content

Quantification of JA and oxylipins was performed as described by Sun et al. (2014). Samples for JA and oxylipins analysis were prepared in parallel, six replicates for each according to Liu et al. (2012). For JA, 10 ng (±) – 9, 10 – dihydro – JA (Sigma) was added to each sample as internal standard. The samples were stored at –80 °C before the quantification. The JA levels were quantified using an HPLC-MS/MS system (AB SCIEX Triple Quad 5500 LC/MS/MS) with JA (Sigma) as the external standards. To quantify oxylipin levels, we used 9-/13-HOD, 9-/13-HPOT, 9-/13-HOD, 9- or 13-HOT or 9-/13-KOD (Cayman Chemical Co) as the external standard.

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**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1** Evaluation of the mutant library and Arabidopsis iFOX line (rsl-1327).

**Figure S2** Characterization of 35SBrACBP1-like and the chlorophyll content of rsl-1327 in different growth stage.

**Figure S3** Experimental design of RNA-Seq and data quality assessment.

**Figure S4** GO annotation and cluster analysis of RNA-Seq data.

**Figure S5** qRT-PCR validation of RNA-Seq data.

**Table S1** Summary of PCR analysis of transgenes.

**Table S2** Number of gain-of-function and loss-of-function mutants.

**Table S3** Growth stages for induced and non-induced (control) mutants.

**Table S4** Summary of RNA-Seq read alignment results.

**Table S5** List of all differentially expressed genes in the pairwise comparison of non-induced and induced at 2 h time point.

**Table S6** Evaluation of significant up- and down-regulated genes in each pairwise comparison between time points.

**Table S7** Functional enrichment analysis of DEGs at 2 h timepoint.

**Table S8** List of all differentially expressed genes in the pairwise comparison of non-induced and induced at 4 h time point.

**Table S9** Functional enrichment analysis of DEGs at 4 h timepoint.

**Table S10** Summary of quantitative real-time PCR (qRT-PCR) primer.

**Data S1** Loss of function phenotype and RNA-Seq data analysis.