Molecular and biochemical studies of fragrance biosynthesis in rose
Sun, Pulu

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Chapter 4

Transcriptional regulation of
*Nudix hydrolase 1* in roses
Chapter 4 Transcriptional regulation of Nudix hydrolase 1 genes in roses

General outline

In this chapter, which is presented in the form of an article, we describe: 1) Testing of NUDX1 promoter activities, and 2) Testing of transcription factors potentially acting on different NUDX1 promoters. These experiments were performed to investigate how NUDX1 genes are transcriptionally regulated. Although the same NUDX1 genes were found in both scented and unscented rose, the difference in floral scent phenotypes (scented or not) correlated with the NUDX1 expression levels. Therefore, knowledge of the regulation of NUDX1 genes, such as promoter structure or specialized transcription factors (TFs), could help explaining, at least partially, the difference in rose floral volatile profiles. The promoter of RcNUDX1-1a, RcNUDX1-1b and RwNUDX1-2 were obtained and used for qualitative and quantitative activity studies via transient expression assays in rose petals and leaves of Nicotiana benthamiana. The qualitative experiment showed that all three tested promoters could drive the expression of the β-glucuronidase (GUS) reporter gene in rose petals. However, a quantitative study could not be performed due to the difficulties in isolating rose protoplasts, which was an essential step for quantifying the GUS activity. In addition to the promoter study, two TFs, which showed similar expression patterns as NUDX1, were selected using RNA-Seq on rose petals (details are presented in Chapter 5) bearing differences in their scent profiles. Transient transactivation assays in N. benthamiana were used to evaluate the capacity of these TF to activate the different NUDX1 promoters. However, no significant transactivation was observed in any of the promoter-TF combinations, and different explanations for this phenomenon will be presented.
Transcriptional regulation of Nudix hydrolase 1 genes in roses

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Introduction

Transcriptional regulation is an essential process in all the living organisms. It influences the level of expression of a certain gene, and acts like a “switch” to decide whether or when a gene is expressed. The main role players in transcriptional regulation are promoters and transcription factors (TF). A promoter is a short DNA sequence where the general TFs and the RNA polymerase assemble to initiate the transcription of a gene. TFs bind to the regulatory sequences, which can locate near or far upstream of the promoter or even within the introns or downstream of the gene, and regulate the rate of transcriptional initiation (Alberts et al., 2007).

According to the TF binding motif, they can be classified into many different groups: helix-turn-helix, zinc finger, leucine zipper, basic helix-loop-helix (bHLH), and others. Among these groups, bHLH family members play important roles in metabolic and developmental processes of all organisms. For example, in plant, a bHLH TF named BIS1 was shown to control the biosynthesis pathway of indole alkaloid in Catharanthus roseus (Van Moerkercke et al., 2015). In Arabidopsis thaliana, a bHLH TF named SPATULA promoted the development of the carpel margins and pollen tract tissues (Heisler et al., 2001) and it also regulated the expression of gene GA3ox – gibberellic acid 3-oxidase to mediate the seed germination upon the light and temperature (Penfield et al., 2005). The core DNA sequence generally targeted by bHLH proteins is called E-box (CANNTG). The canonical E-box is CACGTG but other non-canonical E-boxes were also found in animals to serve as a binding site for bHLH proteins (Yoo et al., 2004, Yoshitane et al., 2014).

Our recent results showed that geraniol and E,E-farnesol production in rose petals was correlated with the expression of RcNUDX1-1a and RwNUDX1-2 genes, respectively. Both genes were found in the genomic DNA of both rose cultivars: Rosa chinensis cv. ‘Old Blush’ (OB), which produces geraniol but not E,E-farnesol, and R. x wichurana (Rw), which produces E,E-farnesol but not geraniol. However, only one of the two genes was expressed in each cultivar, suggesting that the production of geraniol and E,E-farnesol was due to a transcriptional regulation. In this study, we investigated the promoters of genes RcNUDX1-1a and RwNUDX1-2 and examined the transcription factor candidates to determine their influence on the expression of both rose NUDX1 genes.

Material and methods

Testing of NUDX1 promoter activities

1. Genomic DNA extraction and Genome-walking

Rose genomic DNA was extracted according the modified protocol described by (Xu et al., 2004a). Briefly, 400 mg of fresh leaves were ground in liquid nitrogen and the ground powder was washed with thoroughly vortexing in 2 ml of washing buffer [consisted of 100 mM Tris-HCl pH8.0, 5mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.35 M glucose, 2% polyvinylpyrrolidone (PVP) and 4% β-mercaptoethanol] and incubated on ice for 30 min. The supernatant was discarded after centrifuging the mixture at 2800g for 10 min to remove contaminants, organic molecules and excessive water. The pellet was resuspended in 2 ml DNA extraction buffer and incubated at 65°C for
30 min, followed by the addition of 2 ml chloroform-isooamylalcohol (24:1) and 200 μl of 5M potassium acetate, which was gently shaken until the tissue, buffer and chloroform were homogenized. The suspension was centrifuged at 5000g for 15 min and the extract (supernatant) was then subjected to a purification process with 1.5 ml chloroform-isooamylalcohol (24:1), followed by a precipitation process with 1.5 ml of isopropanol, 150 μl of 3M sodium acetate and incubation at – 20 °C for 30 min. Resulting pellet was washed with 1.5 ml 70% (v/v) ethanol, air-dried and redissolved in 1 ml autoclaved TE pH 8.0 buffer, which was subjected to RNase treatment followed by precipitation and washing steps to obtain clean genomic DNA. The integrity of genomic DNA was evaluated using electrophoresis and the quantity was determined using NanoDrop.

In order to study the promoter activities of three NUDX1 genes: RcNUDX1-1a, RcNUDX1-1b and RwNUDX1-2, their 5’ regions before ATG were obtained. As the rose genomic sequence is not available yet, genome-walking technique was applied to obtain the sequence before the start codon of the genes. Genome-walking experiment was performed according to the manufacturer’s protocol Universal GenomeWalker™ 2.0, Clontech Laboratories, California, USA. Primary and nested PCR primers used in these experiments are listed below (Table 1). When 5’ and 3’ sequences of each NUDX1 gene were determined, another pair of primers was used to obtain around 2000 bp of NUDX1 upstream sequences: 1) primers OB1a Promo F2 (GGGGACAAGTTTGTACAAAAAAGCAGGCT GCTTCTTGTGAATGCATTTG) and OB1a Promo R2 (GGGGACCACCTTTGACAAGAAAGCTGGGT GCTTTTGATATGGCTTTTTC) to obtain 2258 bp 5’ region sequence of RcNUDX1-1a; 2) OB1b Promo F2 (GGGGACAAGTTTGTACAAAAAAGCAGGCT TTTGCCCACTCGATTAACCA) and OB1b Promo R2 (GGGGACCACTTTGACAAGAAAGCTGGGT GCTTTTGATATGGCTTTTTC) to obtain 2258 bp 5’ region sequence of RwNUDX1-2. Resulting amplicons were cloned into Gateway® pDONR™ 221 entry vectors (Thermo Fisher Scientific, Massachusetts, USA) via BP reaction. The verified entry vectors were subjected to LR reaction to transfer the target fragment into a destination vector – pGWB3, which contains a GUS (β-glucuronidase) reporter gene (Nakagawa et al., 2007). These destination vectors were transformed into the A. tumefaciens strain GV3101 (pMP90) that contained a firefly luciferase (LUC) driven by the cauliflower mosaic virus (CaMV) 35S promoter (35S-LUC) (and/or 35S-YFP and/or 35S-GFP) were used as negative controls and those that contained 35S-GUS (and/or 35S-YFP-GUS) were used as positive controls. The bacterial suspensions were kept in the dark at room temperature for at least 1 hour prior to infiltration. Stage four petals of R. x hybrida cv. ‘Akito’ and R. x hybrida cv. ‘The McCartney rose’, and according to the gene origin, they were named pOB1a-GUS, pOB1b-GUS and pRw-GUS.

2. Transient trans-activation assay and GUS staining for the qualification of promoter activity

For transient trans-activation assay, A. tumefaciens strain GV3101 (pMP90) cultures harboring promoter-reporter constructs (pOB1a-GUS, pOB1b-GUS and pRw-GUS) were grown overnight and diluted in infiltration buffer [pH5.6, 2% glucose, 5g/l Murashige & Skoog medium basal salt mixture (MS salt), 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), 0.2 mM Acetosyringone] to obtain a final optimal density (OD₆₀₀) of 0.6. An A. tumefaciens strain GV3101 (pMP90) that contained a firefly luciferase (LUC) driven by the cauliflower mosaic virus (CaMV) 35S promoter (35S-LUC) (and/or 35S-YFP and/or 35S-GFP) were used as negative controls and those that contained 35S-GUS (and/or 35S-YFP-GUS) were used as positive controls. The bacterial suspensions were kept in the dark at room temperature for at least 1 hour prior to infiltration. Stage four petals of R. x hybrida cv. ‘Akito’ and R. x hybrida cv. ‘The McCartney rose’ (both purchased from Olijrozen B.V., De Kwakel, Netherlands) were detached and used for infiltration. They were put in closed Petri dishes with wet filter paper after the infiltration, and kept at 22 °C in dark for at least three days.

Three days later, a diameter of 0.6 cm of infiltrated rose petal was cut for GUS staining. The cut petals were submerged in GUS staining buffer [0.1 M pH7.2 NaHPO₄ buffer, 1 mM X-Gluc and 0.1% Triton X-100] and a vacuum was applied for 5-10 min. Subsequently, the tissue was incubated at 37 °C for 24 h. The next day, the staining buffer was removed and the tissue was incubated twice
with 70% ethanol. Transient transactivation experiments followed by GUS staining were performed three times.

### Table 1. PCR primers used for genome-walking

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<tr>
<th>Primary PCR primer</th>
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<th>Nested PCR primer</th>
<th>Sequence</th>
<th>Purpose</th>
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<td>CAACAGGCATACTA CACCGCCACCTTTT</td>
<td>GeW Rw R2</td>
<td>ACCCGAAGTTTCAGC CACTACTACTGT</td>
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</tr>
<tr>
<td>GeW Rw F1</td>
<td>CCGTCAGACGCCCA AGAATTTGAGCCAA</td>
<td>GeW Rw F2</td>
<td>ACTCTTTTGGCCTTTTG GAGAACGTTGTT</td>
<td>To obtain the downstream sequence of RcNUDX1-1a</td>
</tr>
<tr>
<td>GeW Rw R3</td>
<td>CCGTAAGCAGGTCATTTT GGCTCTCTCAAC</td>
<td>GeW Rw R4</td>
<td>GCCACTTTGATCAGG CTCGAGTTTCAG</td>
<td>To obtain the upstream sequence of RcNUDX1-1b</td>
</tr>
<tr>
<td>GeW Rw F3</td>
<td>CCGTCAAGAGCAGCAAGCAG</td>
<td>GeW Rw F4</td>
<td>ATTCCTGATGATGTTG GGATGGTATGAGTG</td>
<td>To obtain the downstream sequence of RcNUDX1-1b</td>
</tr>
<tr>
<td>GeW Rw R5</td>
<td>GGATCGACTGGTATGGTT CCGCTTTGTCCAA</td>
<td>GeW Rw R6</td>
<td>TCTCTGTGATGGTTG GGATGGTATGAGTG</td>
<td>To obtain the upstream sequence of RwNUDX1-2</td>
</tr>
<tr>
<td>GeW Rw F5</td>
<td>GGACAAAGCCGCAAC</td>
<td>GeW Rw F6</td>
<td>CAGGCGATGGAAC</td>
<td>To obtain the downstream sequence of RwNUDX1-2</td>
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**Testing of NUDX1 transcription factors**

1. Transcription factor activity measurement

This experiment was carried out to test the involvement of two newly identified putative transcription factors in the regulation of the RcNUDX1-1 and RwNUDX1-2 genes. Two putative basic Helix-Loop-Helix transcription factors (TFs), which we named RcbHLH79 (OB TF) and RwHLH79 (Rw TF), were found in the same expression sub-clusters as RcNUDX1-1 (sub-cluster 45) and RwNUDX1-2 (sub-cluster 58), respectively. These sub-clusters were generated from the bioinformatics analysis of RNA-sequencing (RNA-Seq) data of *R. chinensis* cv. ‘Old Blush’ (OB), *R. x wichurana* (Rw) and two of their progenies and the results are presented in detail in Chapter 5. These two TFs were amplified from the cDNAs of OB and Rw, respectively. Primers used for TF amplification are bHLH79 F (a forward primer, CACCATGGATCCGCCGGTGT) and bHLH79 R (a reverse primer, TTATGTTTGGGCTTTTCAAAGGCCCCAA). The amplified fragments were cloned into the vector pENTR, sequenced and then into the *in planta* expression vector pMDC32 (Curtis and Grossniklaus, 2003) using the Gateway cloning technique to generate 35S-RcbHLH79 or 35S-RwHLH79. These expression constructs were transformed into the *in planta* expression vector *Agrobacterium tumefaciens* strain GV3101::pMP90 for transient expression in *N. benthamiana* leaves. In addition, two promoter regions of OB, named “pOB1a” (promoter of RcNUDX1-1a) and “pOB1b” (promoter of RcNUDX1-1b), and the one from Rw named “pRw2” (promoter of RwNUDX1-2) each of them driving GUS in the pGWB3 vector (Nakagawa et al., 2007) were transformed to *A. tumefaciens* GV3101 as well.

For the transient trans-activation assay, *A. tumefaciens* cultures harboring the effector constructs expressing the two putative TFs (35S-RcbHLH79 or 35S-RwHLH79) and the reporter constructs (pOB1a-GUS, pOB1b-GUS or pRw-GUS) were grown overnight and then diluted in infiltration buffer (same composition as the one described in the Material and methods in Section 3) to obtain a final optical density (OD<sub>600</sub>) of 0.6. Co-expression of the GUS reporter-configuration driven by the tomato terpene synthase 5 (SiTPS5) promoter with the SiMYC1 transcription factor from tomato was used as positive control. Similarly, co-expression of 35S-RwNUDX1-2 (32Rw) in combination with the three promoter-GUS constructs were used as negative controls in order to identify their GUS background activities (Figure 1). As a negative control for transactivation, the construct *SiTPS5p-GUS* was co-infiltrated with 35S-RcbHLH79 and 35S-RwHLH79, respectively (Figure 1). *A. tumefaciens* cultures containing each of the effector constructs were mixed with the three reporter constructs respectively as well as with *A. tumefaciens* containing 35S-LUC construct in order to normalize the
difference in expression levels between leaves. The mixtures had a ratio effector:reporter:LUC of 5:5:2 and they were kept in the dark at room temperature for at least 1 hour prior to infiltration. Leaves of four-week old non-flowering N. benthamiana plants were used and the leaves were infiltrated with the effector-reporter-LUC mixture. Three days post the infiltration (3dpi), four leaf discs were collected and immediately frozen in liquid nitrogen and stored at −80°C. The transient trans-activation assay was only applied once to each transcription factor candidate (n=1).

Figure 1. The setup used for transient transformation-assay. Agroinfiltration was conducted to co-express each of the two putative transcription factor (TF) constructs (35S-RcbHLLH79 and 35S-RwbHLLH79) with any of the three promoter constructs (pOB1a-GUS, pOB1b-GUS and pRw-GUS) in four-weeks old N. benthamiana leaves. The promoter of terpene synthase SITPS5 (SITPS5-GUS) was co-expressed with the transcription factor – SIMYC1 from tomato (35S-SIMYC1) as a positive control or with the TF constructs 35S-RcbHLLH79 or 35S-RwbHLLH79 as negative controls. As a negative control to determine GUS background-activities, 35S-RwNUDX1-2 was co-expressed with one of the three promoter constructs that were tested: pOB1a-GUS, pOB1b-GUS or pRw-GUS. *GUS is β-glucuronidase.

GUS and LUC activity determination were conducted as described by Van Moerkercke et al. with slight modifications (Van Moerkercke et al., 2011). Frozen leaf discs were ground and proteins were extracted in 150 μl cell culture lysis reagent (CCLR) [25mM pH7.8 Tris-phosphate, 2mM dithiothreitol (DTT), 2 mM pH7.8 cyclohexane dinitrilo tetraacetic acid (CDTA), 10% Glycerol, 1% Triton® X-100, 1x proteinase inhibitor] by vortex. The suspension was then centrifuged at 13 000g, 4 °C for 20 min and 100 μl of cleared supernatant were transferred to a new tube, 10 μl of supernatant were added into 10 μl of CCLR and 20 μl MUG assay buffer [1 ml MUG assay buffer consisted of 1 ml CCLR, 1.4 μl β-mercaptoethanol, 10 μl 100 mM 4-methylumbelliferyl-β-D-galactopyranoside (MUG)] in a clear micro-titer plate, covered and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by adding 40 μl of freshly prepared 0.2 M Na2CO3 and the fluorescence was measured in a FluoroCount Microplate Fluorometer (Packard BioScience Company, Meriden, USA) at 360 nm excitation and a 460 nm emission. For the normalization based on transformation efficiency, LUC enzyme activity was also measured by mixing 20 μl of the cleared supernatant with 80 μl of LUC assay buffer [20 mM Tricine, 2.67 mM MgSO4, 0.1 mM EDTA, 3.33 mM DTT, 470 μM D-luciferine, 270 μM Coenzym A, 530 μM pH 7.8 adenosine 5′-triphosphate (ATP)] and measuring fluorescence in a FluoroCount Microplate Fluorometer at 560 nm emission.

2. Cloning and sequencing of bHLH79 transcription factors in eight progenies of OB and Rw

Cloning and sequencing of bHLH79 transcription factors in 8 progenies of OB and Rw (OW9011, OW9018, OW9035, OW9037, OW9047, OW9074, OW9082, and OW9204) (see Material and Methods in Chapter 3) were performed in order to study the correlation of their expression with NUDX1 gene expressions. A forward primer (bHLH79 F pEN, CACCATGGATCCGCGGTTG) and a reverse primer (Rw2 TFq R, CCCCTTCTGCTCGTACCTGATA) were used for amplifying a fragment of bHLH79 genes in selected 8 progenies. Resulting amplicons were cloned into vector Zero Blunt® (Invitrogen, Thermo Fisher Scientific, California, USA), followed by the transformation into
competent *E. coli* TOP10® (Thermo Fisher Scientific, California, USA) according to the manufacturer’s protocol. Resulting bacterial colonies were inoculated overnight and then subjected to plasmid extraction and purification using NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany). The purified plasmids were then sent for sequencing, which was carried by Eurofins Genomic (Ebersberg, Germany). The sequencing results were analyzed using Geneious® 8.1.7 software (Biomatters, Auckland, New Zealand) (Kearse et al., 2012).

**Results**

**Testing of NUDX1 promoter activities**

In order to investigate the activities of the promoters of each *NUDX1* gene, genomic fragments upstream of the ATGs of three *NUDX1* genes, *RcNUDX1-1a*, *RcNUDX1-1b* and *RwNUDX1-1*, were obtained using Genome-walking technique (Figure 2). The lengths of the promoter sequences were 2258 bp for pOB1a and pOB1b, and 1737 bp for pRW (Figure 2). As illustrated in Chapters 2 and 3, *RhNUDX1-1* (sequence from *R. x hybrida* cv. ‘Papa Meilland’ (PM) and *RcNUDX1-1a* (sequence from OB) had a similar function in roses; therefore, a small fragment of *RhNUDX1-1* upstream sequence (190 bp) was also used here for comparison (sequence was provided by Magnard J-L., unpublished data via genome-walking). Sequences of pOB1a and pPM are similar (90.5% similarity) while pOB1a is different from pOB1b (46.3%) and pRW (44.2%). Sequence of pOB1b is also different from the one of pRW (43.3%).

**Figure 2. (Part 1)**
Chapter 4 Transcriptional regulation of Nudix hydrolase 1 genes in roses

Figure 2. (Part 2)
Figure 2. (Part 3)
Figure 2. (Part 4) Nucleotide alignments of upstream regions of *R. chinensis* cv. ‘Old Blush’ *NUDX1* [pOB1a and pOB1b, both 2258 bp] and *R. x wichurana* *NUDX1* [pRwNUDX1-2 (pRw), 1737 bp]. The upstream sequence of *R. x hybrida* cv. ‘Papa Meilland’ [pPMNUDX1-1 (pPM)] was obtained by Magnard J.-L. The red boxes indicate sequence repeats. For pOB1a, the sequence of box 1, 2 and 4 have lengths of 38 bp, box 3 has 37 bp, while for pPM, the sequence of box 1, 3 and 4 have lengths of 38 bp, box 2 has 36 bp. The purple box indicates the canonical binding motif, E-box, for bHLH transcription factors in pPM and pOB1a. The yellow box indicates the start codon (ATG) of *NUDX1* genes.
When analyzing the promoter sequences in detail, we found four repeated sequences (Figure 2, Part 4, in red) that are present in pOB1a and pPM, but not in either pRW or pOB1b. Interestingly, the expression levels of NUDX1-1 is known to be high in PM ((Magnard et al., 2015)) and OB but not in Rw (see Chapter 3), while NUDX1-1b was not expressed in PM, OB or Rw. In addition, RwNUDX1-2 generally had a lower expression than RcNUDX1-1 (see Chapter 3). These findings suggest that the repetitive regions may play a role in the expression of NUDX1 genes. In addition, a bHLH-binding motif, named E-box (CACGTG), was found in the pPM and pOB1a, located close to the repeats (Figure 2, Part 4), suggesting there might be a bHLH TF involved in the regulation.

To test the hypothesis above, pOB1a, pOB1b and pRW were fused to a GUS reporter gene. These constructs were transiently transformed into detached rose petals (stage four) of R. x hybrida cv. ‘Akito’ (white rose, unscented) and R. x hybrida cv. ‘The McCartney rose’ (pink rose, scented). According to the results in Figure 3, most of the petals are not homogeneously transformed, even for the positive control. Two to three out of eight or nine (10%-30%) infiltrated petals show GUS staining (blue) and a similar pattern was also found in the two positive controls: 3SS-YFP-GUS and 3SS-GUS (Figure 3 I-III). Moreover, all negative control samples (35S-LUC and 35S-GFP) do not show any blue staining. Together, although the transformation efficiency needs to be improved, these results indicate that all three tested promoters, pOB1a, pOB1b and pRW, are active in rose petals.

In order to compare the level of activity of each promoter, quantification measurement by quantifying GUS activity in transformed rose would be required. Due to the low success rate of rose petal transformation as shown above, which could be influenced by the rose cultivar (Sylvie Baudino, personal communication), rose protoplasts were chosen for GUS activity quantification instead of rose petals, in order to obtain a more reproducible transformation. Several attempts were made but no viable rose protoplasts could be obtained, from either the petals of R. x hybrida cv. ‘Akito’ or R. x hybrida cv. ‘The McCartney rose’ and the quantification of promoter activity was therefore not continued.

According to the results above, we cannot conclude that the repetitive sequences in pOB1a and pPM act as a “switch” for the expression of NUDX1 genes, but they still may act as enhancers to increase the expression of NUDX1-1. One argument is that pRW does not possess any repetitive sequence and RwNUDX1-2 has generally lower expression levels than RcNUDX1-1a (Chapter 3 Figure 2).

In order to clarify the precise functions of these promoters, an experiment using rose protoplasts was attempted. However, the petal protoplast isolation wasn’t possible and therefore, the functions of these repeats in the promoter region remains unknown. This experiment should be repeated with an optimized protoplast isolation protocol or with different methods in order to specify the functions of these repeats.

The different expression levels of NUDX1-1 gene in different samples can be caused by different regulations due to differences in the promoter sequences or to the transcription factors that bind to the promoter. Therefore, another experiment using the two transcription factors candidates was conducted and the details of this experiment are illustrated below.
Figure 3. GUS staining of transiently transformed rose petal tissues. Each picture indicates one experimental replicate. The column at the left of each picture are the constructs that were infiltrated into rose petals: 35S-GFP (GFP), 35S-GUS (GUS), 35S-LUC (LUC), 35S-YFP (YFP), 35S-YFP-GUS (YFP-GUS), pOB1a-GUS (OB1a), pOB1b-GUS (OB1b), pRw-GUS (Rw). The row on the top of each picture indicates the replicates of each infiltrated constructs and the rose cultivars that was used for infiltration. AK indicates tissues from R. x hybrida cv. ‘Akito’ and MC indicates tissues from R. x hybrida cv. ‘The McCartney rose’. When there were more than 8 replicates, sample 9 and onward were placed in row F and the information (infiltrated construct and used rose cultivar) were stated below each sample (picture I-III) or above (picture II). In picture (II), only R. x hybrida cv. ‘Akito’ was used and therefore, no AK or MC was indicated in this picture.
Testing of NUDX1 transcription factors

1. Transcription factor activity measurement

In order reduce the number of putative rose RcnudX1-1a and RwnudX1-2-regulating TF candidates that will be tested in this preliminary screen, we selected those that had similar expression levels and patterns as the RcnudX1-1a (c34287_g1_i1, sub-cluster 45) and RwnudX1-2 (c92297_g1_i1, sub-cluster 58) genes (see chapter 5 for sub-cluster analysis in detail). The clustering results that were generated by edgeR (Robinson et al., 2010) are presented in Figure S2 (Supplemental data of Chapter 5). Two putative TF were found: one in the sub-cluster 45 and the other one in the sub-cluster 58. When BLAST their sequences against nucleotide database of National Center for Biotechnology Information (NCBI), the results showed that they both belong to the basic Helix-Loop-Helix (bHLH) family. Therefore, the one that was found in sub-cluster 45 (with RcnudX1-1a) was named RcbHLH79 (OB TF, transcript: c42493_g1_i2) and the one that was found in sub-cluster 58 (with RwnudX1-2) was named RwbhHL79 (Rw TF, transcript: c42493_g1_i4), respectively (Table 2).

In the RNAseq experiment (explained in details in Chapter 5), OB TF was expressed in OB and OW9047 (producing geraniol but not E,E-farnesol) and Rw TF was expressed in Rw and OW9035 (producing E,E-farnesol and not geraniol, see Chapter 3).

Table 2. List of annotated unisequences in sub-cluster 45 and sub-cluster 58. C34287_g1_i1 is corresponding to RcnudX1-1a and c92297_g1_i1 is corresponding to RwnudX1-2.

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<td></td>
<td>c46233_g1_i1</td>
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<td>c46331_g1_i2</td>
<td>Genome assembly common carp genome, scaffold LG24</td>
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<td>c46525_g2_i3</td>
<td>Leucine-rich repeat extensin-like protein 4</td>
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<td>MYC, Transcription factor bHLH79</td>
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<tr>
<td></td>
<td>c92297_g1_i1</td>
<td>NUDX hydrolase 1 (RwnudX1-2)</td>
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<td></td>
<td>c41558_g1_i2</td>
<td>Pectin acetyl esterase 8-like</td>
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The cDNAs of the \(bHLH\) genes were amplified and cloned into the destination vector pMDC32, in which two CaMV 35S promoters control the expression of inserted genes. Each of these TF-constructs was co-expressed with one of the three previously identified active rose promoter regions: \(pOB1a\)-\(GUS\), \(pOB1b\)-\(GUS\) and \(pRw\)-\(GUS\) (see Section 3). As positive control to evaluate the transactivation assay, we co-expressed the \textit{Solanum lycopersicum} \(TP55\)-gene promoter, \(SI\text{TP}55\), fused to the GUS reporter gene and the corresponding transcription factor \(\text{SIMY}C1\) (Spyropoulou et al., 2014b). The results in Figure 4 show that none of the \(bHLH\) candidates was able to transactivate any of the tested rose promoters (\(pOB1a\), \(pOB1b\) or \(pRw\)) in \(N.benthamiana\) leaves, even though a canonical E-box, which was a typical binding site for \(bHLH\) protein, was found in \(pOB1a\), located at \(-332\) bp upstream of the start codon of \(RcNUDX1\)-\(1a\) (Figure 2).

![Figure 4. GUS-reporter-based transactivation assay in \(N. benthamiana\) leaves to study promoter activities of two transcription factor candidates: (I) \(RcbHL79\) (OB TF) and (II) \(RwbHLH79\) (Rw TF). Promoter \(SI\text{TP}55\) and transcription factor \(\text{SIMY}C1\) were co-expressed to serve as a positive control for transactivation. The promoter-reporter constructs that were tested are: \(pOB1a\)-\(GUS\) (\(pOB1a\)), \(pOB1b\)-\(GUS\) (\(pOB1b\)) and \(pRw\)-\(GUS\) (\(pRw\)). Activity was determined by measuring the fluorescence generated from the reaction of GUS with MUG and the relative transactivation was relative to the expression of LUC. The construct pMDC32-Rw (32Rw) was used as a non-transactivating negative control to determine basal GUS activity in the promoter-reporter constructs. P-value (**: \(\leq 0.01\); ns: \(>0.05\)) was obtained by using independent student’s t-test comparing TF and 32Rw control.]

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Surprisingly, the TF candidate Rw TF could activate the *SITPS5* promoter, which resulted in doubling the GUS reporter signal compared to the effect of *SIMYC1* (independent student's t-test, p ≤ 0.001). This indicates that, at least, the TF candidate Rw TF was properly translated into a protein, which activated the *SITPS5*p promoter. However, a true negative control was missing in this case, a non-active construct should be co-infiltrated with the *SITPS5* promoter to verify this observation. Nevertheless, based on these results, the selected putative TF candidates do not seem to be the regulators of the promoters of the *RcNUDX1-1a* and *RwNUDX1-2* genes, unless there was an error during the experiment or the transactivation was not significant enough compared to the variation in plants. Therefore, a repetition of this experiment is necessary to obtain enough information to evaluate the cause of this outcome.

Interestingly, when comparing the transactivation level between pOB1a, pOB1b and pRw, pOB1b had higher activity compared to pOB1a and pRw, while pOB1a and pRw showed similarly low activity (Figure 4). This suggested that pOB1b may contain a promoter element that can be recognized in *N. benthamiana* leaves, while pOB1a and pRw do not possess this element, or pOB1a and pRw have rose petal-specific promoter elements, which could be inhibited or could not be recognized in *N. benthamiana* leaves while pOB1b does not have such elements.

2. Cloning and sequencing bHLH79 transcription factors in eight progenies of OB and Rw

In order to determine whether expression of the two bHLH candidates correlated with *NUDX1* expression in the F1 progeny described in Chapter 3, several primers were designed for qRT-PCR to quantify their expression levels among the progeny. However, OB TF and Rw TF are so similar that we were unable to find sequence specific primers to distinguish them (Figure 6). Therefore, sequencing of reverse-transcription polymerase chain reaction (RT-PCR) products was conducted on eight F1 individuals that have distinct expression of *NUDX1* genes (see Materials and methods), in order to distinguish OB TF and Rw TF. Two colonies of each amplification products were sent for sequencing and the sequencing results are shown in Figure 5. Two sequenced plasmids from OW9011, OW9047 and OW9074 contained the same fragment as the one obtained from OB TF, while the sequenced fragments from OW9018, OW9037 and OW9082 showed the same sequence as the one obtained from Rw TF. According to the results in Chapter 3, progenies OW9011, OW9047, OW9074 and OW9024 had high expression of *RcNUDX1-1a*, but almost no expression of *RwNUDX1-2*, while progeny OW9018, OW9035, OW9037 and OW9082 had the opposite expression pattern. In this case, the expression of *RcNUDX1-1a* is consistent with the expression of OB TF in OW9011, OW9047 and OW9074, and it is the same for *RwNUDX1-2* and Rw TF in OW9018, OW9037 and OW9082. However, for OW9035, both fragments of OB TF and Rw TF were found and only Rw TF fragment was found in OW9024, which is not consistent with the expression pattern of *RcNUDX1-1a* or *RwNUDX1-2* (Table 3), and in contradiction with the expression pattern obtained from RNA-Seq data (see Chapter 5).

Furthermore, RT-PCR were performed on another four rose F1 individuals: OW9021, OW9024, OW9049 and OW9069. OW9021 and OW9024 had high expression of both *RcNUDX1-1a* and *RwNUDX1-2* while OW9049 and OW9069 had low expression of both of *RcNUDX1-1a* and *RwNUDX1-2*. However, OB TF and Rw TF were similarly expressed in all four progenies (Table 3). These data seem to indicate that the two bHLH79 TF candidates are not involved in the regulation of either of the *NUDX1* genes. However, in the RNA-Seq assembly (details see Chapter 5), we identified five different transcripts of *RcbHLH79* (or *RwbHLH79*) (Figure 6). Transcript 1 (c42493_g1_i1), 3 (c42493_g1_i3) and 5 (c42493_g1_i5) were expressed in all four samples to different extents (Table 4). The PCR primers (bHLH79 F pEN and Rw2 TFq R) that I designed could very likely use the other transcripts (e.g. transcript 1 or 5) as templates, because the primer-binding sites are identical to that of OB TF and Rw TF. Therefore, unless transcript-specific primers or high affinity PCR are available to
discriminate each transcript variants, it is hard to conclude that these two TF candidates have no correlation with the expression of NUDXI genes.

Table 3. Expression of RcNUDX1-1a, RwNUDX1-2, RcbHLH79 (OB TF) and RwbHLH79 (Rw TF) in twelve rose F1 individuals. Symbol + indicates high expression, − indicates low expression. The samples that are highlighted in eunry are the samples, which showed inconsistency between expression of NUDXI genes and the corresponding TF genes.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Expression (qRT-PCR)</th>
<th>Expression (RT-PCR &amp; sequencing)</th>
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</thead>
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<tr>
<td></td>
<td>RcNUDX1-1a</td>
<td>RwNUDX1-2</td>
</tr>
<tr>
<td>OW9011</td>
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<td>−</td>
</tr>
<tr>
<td>OW9018</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>OW9035</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>OW9037</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>OW9047</td>
<td>+</td>
<td>−</td>
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<tr>
<td>OW9074</td>
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<tr>
<td>OW9204</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 4. Normalized expressions (FPKM values) of five sequences of bHLH79 genes in four sequenced rose progenies.

<table>
<thead>
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<th>FPKM value</th>
</tr>
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<td></td>
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<td>c42493_g1_i1</td>
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<tr>
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</tr>
<tr>
<td>c42493_g1_i3</td>
<td>60.1</td>
</tr>
<tr>
<td>c42493_g1_i4 Rw TF</td>
<td>368.3</td>
</tr>
<tr>
<td>c42493_g1_i5</td>
<td>47.2</td>
</tr>
</tbody>
</table>

*FPKM: Fragments Per Kilobase of transcript per Million of mapped reads. OB TF represents RcbHLH79 and RwTF represents RwbHLH79.
Figure 5. Nucleotide sequences and corresponding amino acid sequences (in colour boxes) of PCR fragments (from start codon to the middle of the coding region) of TF obtained from sequencing results of eight progenies of OB and Rw. The sequences highlighted in black are the differences between different samples. OB TF represents \( \text{RcbHLH79} \) and Rw TF represents \( \text{RwbHLH79} \). Green arrow boxes below the sequences indicate the primers (bHLH79 F pEN and Rw2 TF qR) that were used for amplification, which were again indicated in Figure 6 to show the location of the amplified DNA fragment.
Figure 6. (Part 1)
### Chapter 4 Transcriptional regulation of Nudix hydrolase 1 genes in roses

**Figure 6. (Part 2)**

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</tbody>
</table>

**Note:**

- Each line represents a sequence of nucleotides.
- The sequences are presented in a standard format for genetic analysis.
- The figure continues on the next page.
Chapter 4 Transcriptional regulation of Nudix hydrolase 1 genes in roses

Figure 6. (Part 3)
Figure 6. (Part 4) Nucleotide sequences and corresponding amino acid sequences (in colour boxes) of five isomers of bHLH79 genes found in roses assembled RNA-seq data. OB TF represents RcbHLH79 and Rw TF represents RwbHLH79. Green arrow boxes below the sequences indicate primers that were used for amplification of OB TF, Rw TF (forward primer bHLH79 F pEN and reverse primer bHLH79 R) as well as the ones to amplified the TF DNA fragment among the F1 progeny (forward primer bHLH79 F pEN and reverse primer Rw2 TF qR). Yellow box below the sequences indicates the start codon of the amino acid sequence, while the star in the black box within the amino acid sequences indicates the stop codon.

Discussion and conclusions

In this study, three rose NUDX1 promoters (pOB1a, pOB1b and pRw) and two bHLH79 TFs (OB TF and Rw TF) were isolated and tested. Qualitative experiments showed that all three promoters could be activated in rose petals, suggesting that theoretically, the genes that are located at the 3’ of these promoters should be all expressed in the petals, which was not the case in reality. Several sequence repeats were found in pOB1a as well as in pPM and they may play a role in the expression levels of NUDX1-1, since NUDX1-1 has relatively higher expression levels than RwNUDX1-2 (Chapter 3), whose promoter does not have the repeats. A similar situation was also observed in human cells in which tandem repeats in certain genes were found to be associated with cancer development by inducing increased levels of gene expression. A reporter assay that was conducted by Tsuge et al. demonstrated that plasmids containing three repeats of a CCGCC unit in the regulatory region of a gene showed higher luciferase activity than the ones containing two repeats, due to the enhancement of binding affinity to its transcription factor E2F-1 (Tsuge et al., 2005). In another study on human disease, a polymorphic (CCTTT)n repeat of nitric oxide synthase 2A (NOS2A) was found to have a negative correlation with the diabetic retinopathy (Warpeha et al., 1999). In addition, the ability to induce the expression of NOS2A gene varied depending on the numbers of CCTTT repeats. By using a luciferase reporter gene assay, it was shown that 14 repeats exhibited the highest luciferase activity among the tested constructs, while 9 repeats had the similar activity as the parent clone pNOS2-2000, which did not contain any CCTTT repeat (Warpeha et al., 1999). However, the function of the repeats in the rose promoters could not be confirmed due to the impossibility to obtain transformed rose petal protoplasts for quantitative measurement. Optimizing the protoplast extraction protocol would be useful to obtain viable protoplasts for further experiments. Besides, fragmenting the sequence repeats in the promoter would also facilitate the understanding of its role in the transcriptional regulation of rose NUDX1 genes.

For the bHLH79 TFs, even though their binding motif, E-box, was found in one of the tested promoters – pOB1a, no significant transactivation was detected, suggesting that the expression of NUDX1 genes did not depend on these two TFs. However, due to lack of a proper negative control, this experiment is not sufficient to exclude the regulatory functions of these two bHLH79 TFs on rose NUDX1 genes. Furthermore, five transcript variants of bHLH79 were found in the RNA-Seq data (details see Chapter 5). Hence, testing the correlation between the expression of bHLH79 and NUDX1 genes was not successful. Therefore, all together, current available data is not sufficient to conclude their actual functions. Another interesting phenomenon was observed during the promoter-TF transactivation experiments. pOB1b seemed to drive higher expression of GUS in N. benthamiana leaves than pOB1a and pRw, and its native gene, RcNUDX1-1b was not expressed in rose petals. This suggests that pOB1b may have leaf-specific motif that was missing in pOB1a and pRw, which may have petal-specific motifs instead. A flower-specific promoter was found in
...6. Gentiana triflora by transforming the promoter-GUS construct into Petunia hybrid. The promoter of chalcone synthase gene (GTCHS1) was proven to be able to direct the expression of GUS gene specifically in flowers, while the 3SS promoter activated the expression of GUS gene in all plant tissue (Kobayashi et al., 1998). When studying the promoter of bean PAL2 gene in transgenic tobacco, two classes of cis-element (AC-element and G-box), locating within – 254 bp of the transcription start site, were shown to be responsible for the tissue-specific expression (Hatton et al., 1995). Three AC-elements and a G-box were discovered and examined in this study by transforming GUS-tagged wild-type and different mutated constructs into tobacco. One example was that mutation in AC-1 did not activate the expression of GUS gene in the root tip but activated the expression in internal phloem, while the wild-type showed no GUS activity in internal phloem but strong GUS activity in the root tip (Hatton et al., 1995). These examples suggest that particular sequences in the promoters can indeed direct tissue-specific expression. Two G-box motifs were in fact found in all three promoters and therefore, there might be other TF-binding motifs that regulate the tissue-specific expression. Transforming the promoter with GUS reporter gene in all rose organs could provide some information on their organ-specificity. Besides, it would be also interesting to know the expression specificity of the two bHLH79 candidates.

Very little is known about transcriptional regulation in terpenoid biosynthesis. Excluding the researches of TFs in terpenoid indole alkaloid biosynthesis (Van Moerkercke et al., 2015, Yang et al., 2012), a few TFs were found that regulate the biosynthesis of terpenoids. A cotton (Gossypium arboreum) transcription factor GaWRKY1 was found to have a similar temporal and spatial expression pattern to a sesquiterpene synthase CAD1 [(+)-δ-cadinene synthase] (Xu et al., 2004b). In addition, this TF could interact with the W-box found in the promoter of the CAD1 gene in vitro, which could be highly activated by overexpression of this TF, showing its regulation role of CAD1 gene (Xu et al., 2004b). A MYC2 transcription factor in Arabidopsis thaliana was found to be able to bind to promoters of two sesquiterpene synthases TPS21 and TPS11 and activate their expression, resulting in the increased emission of sesquiterpenes (Hong et al., 2012). In tomato (Solanum lycopersicum), a zinc finger TF – EOT1 (Expression of Terpenoids 1) was discovered (Spyropoulou et al., 2014a). This TF had interactions with the promoter of a monoterpenoid synthase TP55 (linalool synthase) in vitro in a yeast-one-hybrid screen and in vivo in N. benthamiana (Spyropoulou et al., 2014a). Recently, in Artemisia annua, a bZIP transcription factor HY5 was found to interact with the G-box of the promoter of a monoterpenoid synthase QH6 (β-pinene synthase) in vitro (Zhou et al., 2015). By performing experiments on transgenic GUS and LUC A. thaliana with the mutation of HY5, they authors demonstrated that HY5 is essential to the rhythmic expression of QH6 (Zhou et al., 2015). According to the available knowledge so far, there is no one specific type of TFs that play role in the regulation of terpenoid synthesis, and no pattern was found in the regulatory element (e.g. G-box or W-box) that is essential to the regulation either, indicating that the regulatory mechanism of terpenoid biosynthesis is complex and diverse.

Even less is known about the transcriptional regulation of NUDX genes. Three promoters that regulated the expression of NUDX genes were identified so far in plants. A 716-bp promoter fragments of NUDX1 gene in Chrysanthemum lavandulifolium was found to be a cis-acting element, which could transactivate the GUS gene in C. lavandulifolium and C. morifolium (Huang et al., 2012). A pathogen-responsive promoter of AtNUDX5 was able to transactivate the reporter GUS gene under the attack of avirulent Pst avrRpm1 and virulent Pst strains in A. thaliana (Zhang et al., 2013). The promoter of AtNUDX7 was activated and induced the expression of GUS gene in response to ozone and pathogen stresses (Muthuramalingam et al., 2015). In addition, in this study, the TF that binds to the promoter of AtNUDX7 was discovered. The expression of the Ethylene response factor (ERF1) was in synchrony with the expression of AtNUDX7 under ozone stress and it bound to the GCC-box in the promoter of AtNUDX7 using in vitro binding assay (Muthuramalingam et al., 2015). Another TF was found to mediate the expression of a NUDX1 ortholog gene - MTH1 in human (Qiu et...
Inhibition of the TF, hypoxia-inducible factor-1 (HIF-1α), by small interfering RNA (siRNA) resulted in the decrease of MTH1 expressing and led to the accumulation of 8-oxo-dGTP and as a consequence, a bigger tumour volume (Qiu et al., 2015). More discoveries of TFs that are involved in the regulation of NUDX genes would certainly advance the understanding of their regulatory mechanisms.
References


