Mechanism of PHERES1 imprinting in Arabidopsis

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Accepted 8 January 2008

Journal of Cell Science 121, 906-912 Published by The Company of Biologists 2008
doi:10.1242/jcs.023077

Summary

Genomic imprinting is a phenomenon where only one of the two alleles of a gene is expressed – either the maternally or the paternally inherited allele. Imprinting of the plant gene PHERES1 requires the function of the FERTILIZATION INDEPENDENT SEED (FIS) Polycomb group (PcG) complex for repression of the maternal PHERES1 allele. In this study we investigated the mechanism of PHERES1 imprinting and found that PcG silencing is necessary but not sufficient for imprinting establishment of PHERES1. We provide evidence that silencing of the maternal PHERES1 allele depends on a distantly located region downstream of the PHERES1 locus. This region needs to be methylated to ensure PHERES1 expression but must not be methylated for PHERES1 repression. This mechanism is analogous to the regulation of several imprinted genes in mammals, suggesting the employment of similar evolutionary mechanisms for the regulation of imprinted genes in mammals and flowering plants.

Key words: Arabidopsis, Epigenetics, FERTILIZATION INDEPENDENT SEED genes, DNA methylation, Polycomb group proteins

Introduction

A subset of genes in mammals and flowering plants is only expressed from one of the two homologous chromosomes, depending on whether they are maternally or paternally inherited. This process is called genomic imprinting and often affects genes with essential functions for normal development (Feil and Berger, 2007). In mammals, imprinted genes regulate placental development and fetal growth, and several human diseases are linked to mutations in imprinted genes (Reik, 2007). Mechanisms to distinguish maternal and paternal alleles have been extensively investigated in mammals. Most mammalian imprinted genes are clustered in the genome and are regulated by differentially methylated imprinting-control regions (ICRs) (Edwards and Ferguson-Smith, 2007). Different DNA methylation marks are applied during germ-line formation by de novo methyltransferases and are maintained in somatic tissues by maintenance methyltransferases. ICRs are also marked by different histone modifications and can either act as insulators preventing promoter-enhancer interactions or give rise to the formation of non-coding RNAs that attract chromatin-modifying complexes (Delaval and Feil, 2004). Some imprinted genes are regulated by Polycomb group (PcG) complexes that methylate histones and establish repressive chromatin domains (Delaval and Feil, 2004).

In flowering plants, imprinting has only been detected in the endosperm, a terminal tissue that develops after fertilization of the central cell. In dicot species like Arabidopsis, the endosperm nourishes the embryo during its growth phase and is almost completely consumed during embryo development (Berger, 2003). Thus far, only four imprinted genes have been identified in Arabidopsis. Three of them [MEDEA (MEA), FWA and FERTILIZATION INDEPENDENT SEED 2 (FIS2)] are maternally expressed and paternally silenced (Vielle-Calzada et al., 1999; Kinoshita et al., 2004; Jullien et al., 2006b). The same paternally imprinted expression pattern applies to all imprinted genes that have been identified in maize (Scott and Spielman, 2006). Paternal imprinting of MEA requires activity of the evolutionary conserved FIS-PcG complex, with MEA itself being a subunit of this complex. The FIS complex mediates trimethylation of histone H3 at the lysine residue at position 27 (H3K27me3) of the paternal MEA allele causing its repression (Baroux et al., 2006; Gehring et al., 2006; Jullien et al., 2006a). Activation of the maternal MEA allele in the female gametophyte requires the 5-methylcytosine excising activity of the DNA glycosylase DEMETER (DME). DME acts antagonistically to the maintenance methyltransferase MET1 that methylates DNA at the 5-methylcytosine residue (Chan et al., 2006b), silencing of the paternal MEA allele is likely to be independent of the repeat sequences (Spillane et al., 2004). Upregulation of the FIS complex is directly associated with the PHE1 locus and FIS repressive activity is correlated with H3K27me3 modification at PHE1 (Köhler et al., 2003; Makarevich et al., 2006).
of PHE1 in mea mutants is in part responsible for the mea mutant phenotype that can be alleviated by reducing PHE1 expression (Köhler et al., 2003).

In this study, we asked whether FIS-mediated repression is sufficient for PHE1 imprinting or whether additional mechanisms are involved to repress the maternal PHE1 allele. Our data clearly show that imprinting is not a direct consequence of FIS-mediated repression but necessitates the presence of additional elements. We identified elements within the PHE1 3' region that are necessary for PHE1 imprinting and predict a model that explains how the FIS complex – together with the identified region – confers stable silencing of the maternal PHE1 allele.

Results
The maternal PHE1 allele is not reactivated in mutants that are defective in DNA methylation
We asked the question whether the FIS complex is sufficient to suppress the maternal PHE1 allele, or whether additional mechanisms cooperate with the FIS complex to silence the maternally derived PHE1 allele. Parental allele-specific DNA methylation has been found at most imprinted mammalian gene clusters and imprinted plant genes that have been examined (Köhler and Makarevich, 2006; Edwards and Ferguson-Smith, 2007). Therefore, we tested whether imprinting of PHE1 is also regulated by DNA methylation. Mutations in the maintenance MEHTYLTRANSFERASE 1 (MET1) gene cause a drastic reduction of symmetric CG methylation (Kankel et al., 2003), whereas mutations in the CHROMOMETHYLASE 3 (CMT3) gene and the de novo methyltransferases DRM1 and DRM2 affect CNG and asymmetric methylation, respectively (Cao and Jacobsen, 2002). We pollinated met1, cmt3 and drm1/drm2 double mutants with pollen of C24 wild-type plants and analyzed allele-specific expression of PHE1. As shown in Fig. 1A and supplementary material Fig. S1A and Fig. S3, in none of the mutants a reactivation of the maternal PHE1 allele was detectable and the paternal PHE1 allele remained the predominantly expressed allele. In conclusion, DNA methylation is not responsible for repression of the maternal PHE1 allele.

Lack of DNA methylation causes reduced expression of the paternal PHE1 allele
In mammals, DNA demethylation is responsible for silencing of a large number of imprinted protein-coding genes (Sleutels and Barlow, 2002). Previously we observed strongly reduced PHE1 expression levels in the decrease in DNA methylation 1 (ddm1) mutant, which is impaired in the maintenance of DNA methylation (Köhler et al., 2003). This prompted us to test whether DNA methylation is required for expression of the paternal PHE1 allele. We did not detect substantial PHE1 expression in pollen (data not shown); however, fertilization of wild-type plants with hypomethylated pollen of met1 mutants caused much lower PHE1 transcript levels in developing seeds than fertilization with wild-type pollen (Fig. 2A), indicating reduced activity of the paternal PHE1 allele. This suggests that DNA methylation is important to maintain high levels of paternal PHE1 expression, but is not necessary to repress the maternal PHE1 allele. When we tested allele-specific PHE1 expression in seeds developing after pollination with met1 pollen, we clearly detected reduced expression of the paternal PHE1 allele (Fig. 2B), which supports our conclusion that DNA methylation is required for paternal PHE1 expression. However, we also detected expression of the maternal PHE1 allele in seeds that inherited a hypomethylated paternal PHE1 allele (Fig. 2B and supplementary material Fig. S1B), suggesting that relief of repression of the maternal PHE1 allele depends on additional not-yet-known factors. Because DNA methylation appears to be important for PHE1 regulation, we asked the question which regions
Disruption of the PHE1 3′ regions causes activation of the maternal PHE1 allele

Our results suggest that DNA methylation in the identified PHE1 3′ region is important for PHE1 imprinting. In order to test this hypothesis we addressed the question whether disruption of the PHE1 3′ region disrupts PHE1 imprinting. We identified a T-DNA mutant containing a 4 kb T-DNA insertion 441 bps downstream of the PHE1 stop codon (referred to as phe1-3) (Fig. 4A). Using this line, we could test whether an insertion within the 3′ region disrupts PHE1 imprinting. We tested allele-specific expression of PHE1 in phe1-3 by performing reciprocal crosses of phe1-3 with C24 plants. Whereas expression of the paternal PHE1 allele was not affected in phe1-3 (Fig. 4B, upper panel and supplementary material Fig. S1C), we observed a drastic effect on the expression of the maternal allele when phe1-3 was used as the maternal parent (Fig. 4B lower panel and supplementary material Fig. S1C). In phe1-3 mutants, PHE1 was not maternally silenced but, in contrast to wild-type, which has only a weak expression of the maternal PHE1 allele, this allele was strongly expressed (Fig. 4B, lower panel). Surprisingly, we detected a decrease in the expression of the paternal PHE1 allele when phe1-3 was used as the maternal parent (cross phe1-3 × C24; Fig. 4B and supplementary material Fig. S1C). One possible explanation for this phenomenon might be the triploid nature of the endosperm consisting of two maternal versus one paternal genome copies. Therefore, if the two maternal PHE1 alleles become reactivated, they might outcompete expression of the single paternal PHE1 allele. We also considered the possibility that strong expression of the maternally derived PHE1 allele in phe1-3 is caused by de-repression of PHE1 in maternal sporophytic tissues of phe1-3 plants. Therefore, we tested expression of PHE1 in phe1-3 leaves. However, as shown in Fig. 4C, PHE1 remains as weakly expressed in phe1-3 leaves as in wild-type leaves. We further considered the possibility that transcripts derived from the T-DNA can influence PHE1 expression. To test this possibility, we performed northern blot analysis using probes flanking the insertion site. However, we did not obtain any expression signal with either of the probes, but detected a clear expression signal for the NEOMYCIN PHOSPHOTRANSFERASE II (NPTII) selection gene that is located within the T-DNA (Fig. 4D). Finally, we tested PHE1 imprinting in two additional transgenic lines containing a 4 kb T-DNA or a 6.6 kb Ds transposon insertion 1022 bps or 1168 bps after the PHE1 stop codon, respectively. The T-DNA in the insertion line (referred to as phe1-4) is inserted in the antisense orientation compared with the T-DNA of the phe1-3 allele, whereas insertion of the Ds element (referred to as phe1-5) is in the same orientation as phe1-3 (Fig. 4A). Consistent with the results obtained for the phe1-3 allele, we observed reactivation of the maternal PHE1 allele and a reduction of paternal PHE1 expression (Fig. 4E and supplementary material Fig. S1C), whereas we did not observe an effect on expression of the paternal PHE1 allele in those mutants (data not shown). Taken together, these results clearly demonstrate that the 3′ region of PHE1 contains elements necessary for repression of the maternal PHE1 allele.

The PHE1 downstream region is necessary for imprinting

To obtain final proof that the identified region is necessary for PHE1 imprinting, we designed a construct containing 3 kb of the PHE1 promoter sequence, the PHE1 coding region fused to a GUS reporter gene and a 3.5 kb sequence downstream of the PHE1 stop codon (referred to as PHE1S000::GUS_3′). We established transgenic plants containing this construct and tested imprinting of this
Imprinting of the PHERES1 locus

As imprinting of the PHER1 transgene depends on de novo DNA methylation of the transgenic PHER1 3′ region, we tested whether this region indeed becomes methylated when transformed into wild-type plants. Using bisulfite sequencing we analyzed a region that allowed us to distinguish between the transgene sequence and the endogenous PHER1 locus. As shown in Fig. 5, in this region we detected CG methylation at a level similar to that in wild type, as well as additional CNG methylation. Thus, the transgenic PHER1 3′ region becomes methylated de novo when transformed into wild-type plants. We tested imprinting of this transgene by performing reciprocal crosses of three independent transgenic lines with wild-type plants. Indeed, as shown in Fig. 6A, PHE13000::GUS_3′ is exclusively expressed when inherited from the paternal parent; we did not detect expression when PHE13000::GUS_3′ became inherited from the maternal parent. By contrast, when we tested expression of the PHE13000::GUS lacking the 3′ PHER1 region, we always detected maternally derived PHER1::GUS expression (Köhler et al., 2005) (Fig. 6B).

Our results suggest that DNA methylation of the paternal PHE1 allele is necessary for paternal PHE1 expression (Fig. 2). To substantiate these findings we transformed the PHE13000::GUS_3′ construct into a drm1/drm2 mutant background. DRM1 and DRM2 are necessary for de novo cytosine methylation in all known sequence contexts and are guided to their templates by small interfering (si) RNAs (Chan et al., 2004). It has previously been demonstrated that transgene sequences when transformed into a drm1/drm2 mutant background remain unmethylated owing to lack of de novo methyltransferase activity (Chan et al., 2004). Indeed, we did not detect significant levels of DNA methylation of the transgene in the drm1/drm2 mutant background (Fig. 5). Using this transformation assay allowed to directly address the question whether DNA methylation of the PHE13000::GUS_3′ transgene affects PHE1 expression and avoids secondary effects caused by global DNA demethylation. We tested allele-specific PHE1 expression by crossing wild-type plants with pollen from drm1/drm2; PHE13000::GUS_3′ transgenic lines. In none of the three tested independent transgenic lines did we detect expression of the paternally derived PHE13000::GUS_3′ construct (Fig. 6C).

Thus, DNA methylation of the 3′ region of the paternal PHE1 allele is necessary for PHE1 expression. Taken together, our data clearly demonstrate that the PHE1 downstream region contains important sequence elements necessary for imprinting of PHE1.

Fig. 4. Establishment of imprinting at the PHE1 locus is compromised in mutants whose PHE1 downstream region is disrupted. (A) Schematic overview of the location of the phe1-3-, phe1-4- and phe1-5- mutant alleles. Probes used for northern blot analysis are indicated. (B) Allelic expression analysis of PHE1 after reciprocal crosses of wild-type and phe1-3 mutant plants with the C24 accession. (C) Expression analysis of PHE1 in leaves of wild-type and phe1-3-mutant plants. (D) Northern blot analysis of RNA from leaves of wild-type and phe1-3-mutant plants with probes indicated in panel (A). (E) Allelic expression analysis of PHE1 in wild-type and phe1-4- and phe1-4-mutant plants after crosses of wild-type and mutant plants with the C24 accession. Lb, left border; Rb, right border; g, genomic DNA; ACT, ACTIN; wt, wild-type.

Fig. 5. Cytosine methylation profile of the PHE13000::GUS_3′ transgene in wild-type and drm1/drm2-mutant background compared with the methylation profile of the endogenous PHE1 locus analyzed by bisulfite sequencing. Cytosine positions relative to the translational stop codon and sequence contexts (CG and CNG, CNN not indicated) are indicated on the x-axis.
Discussion
DNA methylation is necessary for PHE1 expression

Our data demonstrate that FIS binding to the PHE1 promoter region and DNA demethylation of the 3′ region of PHE1 are both necessary and sufficient for stable PHE1 imprinting. DNA methylation in intergenic regions is often associated with transposons and repeat sequences as well as noncoding RNAs (Zhang et al., 2006). De novo methylation of repeats depends on the de novo methyltransferase DRM2 that is guided by siRNAs using the RNA-directed DNA methylation pathway (Chan et al., 2004b). Whereas DRM2-mediated de novo methylation occurs in all sequence contexts, DRM2-mediated maintenance methylation is restricted to CNG and asymmetric cytosine residues (Cao and Jacobsen, 2002). The identified direct repeats in the PHE1 3′ region are preferentially methylated on symmetric CG residues, indicating that methylation of PHE1 repeats depends on MET1 maintenance methyltransferase activity. Thus, methylation of PHE1 repeats is likely to occur through similar mechanisms as methylation of repeats in the promoter of the imprinted FWA gene. Methylation of endogenous FWA repeats depends on MET1 activity, whereas methylation of transgenic FWA repeats depends on DRM2 activity (Kinoshita et al., 2004; Chan et al., 2004b). However, in contrast to the role of DNA methylation for silencing of the paternal FWA allele, we found DNA methylation being necessary for expression of the paternal PHE1 allele. This conclusion is supported by two findings: (1) endogenous paternal PHE1 expression is reduced in a met1 mutant, and, (2) PHE1 3′::GUS 3′ is not paternally expressed when transformed into the de novo methyltransferase mutant drm1/drm2 that is deficient in de novo methylation of repeated transgene sequences during plant transformation (Chan et al., 2004b). As PHE1-repeat sequences are also methylated in tissues where PHE1 is not substantially expressed, we conclude that DNA methylation is necessary but not sufficient to determine PHE1 activity. It is possible that additional activating signals present only during seed development are necessary for PHE1 expression.

Surprisingly, we detected expression of the maternal PHE1 alleles in seeds inheriting a hypomethylated paternal PHE1 allele. One possible explanation for this finding could involve recruitment of the FIS complex to the demethylated paternal PHE1 allele. If the number of FIS complexes is limited, additional FIS target genes could cause a reactivation of silenced maternal PHE1 alleles.
Is there a difference in DNA methylation between maternal and paternal alleles? We failed to solve this question for technical reasons, as it requires DNA isolation of either female gametophytes or pure endosperm tissues. However, given the presence of active DNA-demethylating enzymes in Arabidopsis, with DME having an assigned function of demethylation in the central cell, it is possible that PHE1 becomes demethylated in the central cell of the female gametophyte. DME is necessary for MEA and FIS2 expression, and lack of DME function causes seeds to abort with a fis-like phenotype. As MEA is necessary for repression of the expression, and lack of DME function causes seeds to abort with a fis-like phenotype. As MEA is necessary for repression of the expression, and lack of DME function causes seeds to abort with a fis-like phenotype. As MEA is necessary for repression of the expression, and lack of DME function causes seeds to abort with a fis-like phenotype.

Model for PHE1 imprinting establishment

We suggest a model for PHE1 imprinting establishment in male and female gametes (Fig. 7). We propose that the 3′ region of PHE1 is differentially methylated, with the maternal allele being specifically demethylated in the central cell of the female gametophyte. If unmethylated, this region – together with the FIS complex – can block transcription in the endosperm either by forming a repressive chromatin loop or by facilitating the long range action of silencer regions. By contrast, in pollen, this region is methylated and either inhibits the formation of repressive chromatin loops or blocks the action of silencer elements by adopting insulator function. Both models are currently being tested. In met1-mutant pollen, this region is unmethylated and blocks transcription. Whether this involves the action of PcG complexes, is currently unknown. However, based on the finding that the maternal PHE1 alleles become reactivated in seeds inheriting a hypomethylated paternal PHE1 allele (Fig. 2B), we currently favor this hypothesis. After fertilization, the maternal PHE1 alleles remain unmethylated in the endosperm and targeted by the FIS complex, whereas the methylated paternal PHE1 allele is not targeted by FIS and active. This model implies that there is no reset of the PHE1 DNA methylation imprint, because demethylation is restricted to the terminally differentiated endosperm. This suggests that maternal imprinting of PHE1 relies on similar molecular mechanisms as paternal imprinting of MEA, FWA and FIS2 (Xiao et al., 2003; Kinoshita et al., 2004; Jullien et al., 2006b). Our model provides an explanation for the seemingly contradictory findings that demethylation of the paternal PHE1 allele causes paternal PHE1 repression, whereas insertions of transgene sequences do not impair PHE1 expression. We propose that the demethylated paternal PHE1 allele becomes repressed, either by formation of repressive chromatin loops or the action of silencing elements. By contrast, insertions of long transgene sequences in the 3′ PHE1 region will negatively interfere with loop formation or inhibit the long range action of silencing elements, resulting in active PHE1 expression. We failed to detect antisense transcripts generated within the 3′ PHE1 region in wild-type and met1-mutant plants (data not shown), suggesting that long antisense RNAs are not involved in PHE1 imprinting.

Similar to the situation for PHE1, several imprinted genes in mammals depend on DNA demethylation for silencing, whereas DNA methylation is necessary for expression (Sleutels and Barlow, 2002). Similar to the model proposed by Sleutels and Barlow (Sleutels and Barlow, 2002), we assume that imprinting of PHE1 evolved in two steps. First, PHE1 expression became silenced by insertion of a repetitive sequence into a regulatory region located in the 3′ region of the gene. In a second step this repetitive sequence became methylated, thereby causing restoration of PHE1 expression. Owing to a female-gamete-specific demethylation activity, the maternal PHE1 allele is silenced, whereas the paternal allele is methylated and active. This model makes two predictions, (1) removal of the insertion mutation that causes the silencing effect should reactivate the silenced allele and, (2) loss of DNA methylation should result in silencing. Indeed, both predictions are supported by the results presented in this study. We could demonstrate that removal of the PHE1 3′ region causes de-repression of the maternal PHE1 allele and loss of DNA methylation causes silencing of the paternal PHE1 allele. The effect of DNA methylation to suppress the silencing effect of transposons has long been appreciated in plants (Martienssen, 1998) and was probably used as a mechanism to achieve active PHE1 expression in male gametes.

Fig. 7. Model for the establishment of PHE1 imprinting. In the central cell of the female gametophyte the FIS-PcG complex binds to a polycomb response element (PRE) in the promoter region of PHE1. The differentially methylated region (DMR) is unmethylated in the central cell of the female gametophyte and blocks transcription together with the FIS complex, resulting in stable PHE1 repression in the endosperm. The FIS complex is absent in pollen und the methylated DMR prevents silencing activity, causing the paternal allele to be active in the endosperm. In met1-mutant pollen, the DMR is demethylated and can block PHE1 expression. Whether this involves PcG complexes is currently not known. Maternal alleles are shown in red, paternal alleles in blue.
Materials and Methods
Plant material and growth conditions
The meo-1 mutant used in this study is the meo-1 allele (Le accession) described by Grossniklaus et al. (Grossniklaus et al., 1998). The meo1 mutant studied in this study corresponds to the meo-1 allele (Col accession) described by Saze et al. (Saze et al., 2003). For all experiments with met1 mutants, only first-generation homozygous plants were used. The dml/dmr2 double mutant (Wassilewskija accession) was obtained from the Nottingham Arabidopsis Stock Centre (CS6366). The cmt3-11 allele corresponds to SALK_148381 (Col accession) (Chan et al., 2006a). The mutant alleles phe1-3, phe-1 and phe-1 correspond to SALK_023774 (Col accession), SALK_010202 (Col accession) and GT_5_108090 (Le accession), respectively. The PHE1:GUS line (Le accession) have been described by Köhler et al. (Köhler et al., 2003). Plants were grown in a greenhouse at 70% humidity and daily cycles of 16 hours light at 21°C and 8 hours darkness at 18°C. Developed gynoecia were emasculated and hand pollinated one day afteremasculating. For RNA expression analysis, three gynoecia or siliques were harvested at the indicated time points.

Plasmid constructs and generation of transgenic plants
To generate the PHE1:GUS::GUS_3 construct, a 3000 bp sequence upstream of the PHE1 translational start and the PHE1-coding region were amplified by PCR, introducing EcoRI and XmaI restriction sites. The fragment was ligated into pCAMBIA1381Xc, creating an in-frame fusion with the Barox, C., Gagliardini, V., Page, D. R. and Grossniklaus, U. (2003).

Allele-specific expression analysis
Allele-specific PHE1 expression was analyzed using the assay described by Köhler et al. (2005). The amplified products were digested with HphI and analyzed on a 2.5% agarose gel. All primers are specified in the supplementary material (Table S1).

GUS expression analysis
Staining of seeds to detect GUS activity was done as described previously (Köhler et al., 2005). Quantitative PCR was performed following the protocol of (Jacobsen et al., 2000).

RNA extraction and qPCR analysis
RNA extraction and generation of cDNAs were performed as described previously (Köhler et al., 2005). Plant material and growth conditions
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Supplementary Figure 1

A

Col x C24

met1 x C24

cmt3 x C24
drm1/2 x C24

B

C24 x Col

C24 x met1
Supplementary Figure 3

![Bar graph showing relative PHERES expression over time.

Legend:
- Col x Col
- met1 x Col

Y-axis: relative PHERES expression
X-axis: 0, 1, 2, 4 DAP (days after pollination)
### List of primers used in this study.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Gene</th>
<th>Primers</th>
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| Quantitative PCR            | *PHE1* (At1g65330) | Fwd: CGCATGTGCGGTCATCC  
                          |                             | Rev: TCCAACACGGAAACTCCCAT   |
| Quantitative PCR            | *ACT11* (At3g12110)  | Fwd: GGAACAGTGTAAGCTCACACCACATC  
                          |                             | Rev: AAGCTGTTTCTTCTACGCC   |
| Allele specific PCR         | *PHE1* (At1g65330)  | Fwd: CGCATGTCGGGTGCTACCC  
                          |                             | Rev: CGTCTCTTGATCCGACCACCTCTTCTTGGTC   |
| Northern blot               | Left border   | Fwd: GTTGATGTATTTGCTGA  
                          | SALK_023774                 | Rev: ACATGACATAGGCCTAGC     |
| Northern blot               | Right border  | Fwd: CCCACGGAAGTTAGTATG  
                          | SALK_023774                 | Rev: GGAATAATGATAATAACAGC   |
| Northern blot               |               | Fwd: CTCTGATTGGGCGGCTTTG  
                          | NPTII SALK_023774           | Rev: CGATGCTGTTGCCTTGGTGGTC |
| Southern blot               | *PHE1* (At1g65330)  | Fwd: TCTCCAAAGAGTAAACCGTA  
                          | 3' region                   | Rev: TCAGTTGTAATGACACCCAG   |
| Bisulfite sequencing        | *PHE1* (At1g65330)  | Fwd1: GTATGGAAGGTATTTTGAAG  
                          | 3' region                   | Rev1: CTTTTATATAATATTTTATACATATC |
|                             |                             | Fwd2: GATAATGATTGATGATGATG  
                          |                             | Rev2: TAACCTCATATAATTTTATAGT |
|                             |                             | Fwd3: TATTTATATTTAAGAATTAAATAGTAAA |
|                             |                             | Rev3: TCAATTATAATATAACCAAATTTATATAA |