

# The *gypsy* Insulator of *Drosophila melanogaster*, Together With Its Binding Protein Suppressor of Hairy-Wing, Facilitate High and Precise Expression of Transgenes in *Arabidopsis thaliana*

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## ABSTRACT

The variation of expression pattern exhibited by a transgene as a result of random integration, known as position effect, is, among other mechanisms, a particular challenge to reverse genetics. We present a strategy to counteract position effect in *Arabidopsis thaliana* by flanking the transgenes with the *gypsy* insulator from *Drosophila melanogaster*. In addition, Suppressor of Hairy-wing [Su(Hw)], the binding protein of the *gypsy* insulator, was coexpressed. Results indicated that the *gypsy* insulators could efficiently improve the expression levels of reporter genes driven by various kinds of promoters by 8- to 13-fold. Coexpression of the Su(Hw) protein led to a more uniform expression level of transgenes, as the coefficient of variation of expression levels was reduced further. The *gypsy*-Su(Hw) system enhanced expression levels, but did not alter the specificity of promoter activities, as experimentally evidenced by the promoters of the *PIN* and the *AFB* gene families. Interestingly, the *gypsy* insulator was also able to improve the expression of a selectable marker gene outside the insulated region, which facilitated the screen of transformants. Our system will likely decrease the number of lines that experimenters need to create and examine for a given transgene by contributing to relatively high and precise expression of transgenes in plants. Certain features of the *gypsy* insulator in *Arabidopsis* also provide new perspectives on the insulator field.

GENETIC engineering has become a routine technique for studying gene function and regulation of complex physiological networks. In plant transgenesis, the transgene is usually integrated randomly into the host genome. The expression level of the transgene may vary among independent lines due to a number of factors including the transgene copy number, RNA silencing, and transgene insertion site (BUTAYE *et al.* 2004; DE BOLLE *et al.* 2007; DE PAEPE *et al.* 2009). The variation of transgene expression has complicated phenotype characterization and necessitates the analysis of multiple transgenic lines.

Recent studies have identified sequence-specific mRNA decay by post-transcriptional gene silencing (PTGS) as the major cause in the variation of the expression level of transgenes driven by strong promoters in plants. RNA silencing was triggered if the

transcript level of a transgene surpassed a gene-specific threshold (SCHUBERT *et al.* 2004). Improperly terminated, unpolyadenylated mRNA from transgene transcriptions generated by 3' readthrough was subjected to RNA-DEPENDENT RNA POLYMERASE6 (RDR6)-mediated RNA silencing (LUO and CHEN 2007).

Variation of transgene expression induced by the copy number and the homology-based PTGS could be minimized by several approaches (BUTAYE *et al.* 2005). Screening for single-copy T-DNA transformants greatly enriches for stable and high transgene expression because PTGS is thought to be particularly triggered by multiple, complexly arranged copies of transgenes (DE BUCK *et al.* 2004; NAGAYA *et al.* 2005). In addition, the use of PTGS mutant backgrounds as the target of transformation has been proven to be of high value for a stable and high expression level of transgenes (BUTAYE *et al.* 2004; DE BOLLE *et al.* 2007). Recently, it has been shown that the CRE/*loxP* recombination system is capable of resolving complex T-DNA loci into single T-DNA inserts and producing a high frequency of single-copy T-DNA transformants (DE BUCK *et al.* 2007; DE PAEPE *et al.* 2009).

The variation of transgene expression induced by the insertion site itself is known as position effect. The surrounding host chromatin structure as well as the negative and positive regulatory elements in adjacent loci

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.110.117960/DC1>.

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are implicated in mediating position effects (KELLUM and SCHEDL 1991; PEACH and VELTEN 1991; GELVIN and KIM 2007). Although a great deal is known about position effects in *Drosophila*, there is conflicting evidence as to whether or not they exist in plants (MATZKE and MATZKE 1998; DE BUCK *et al.* 2004; NAGAYA *et al.* 2005). It has been suggested that pronounced position effects should never be detected in stable transformants that have undergone antibiotic selection, as negative position effects should also suppress the expression of the resistance marker (KIM *et al.* 2007). However, several lines of evidence have suggested that position effects should exist in plants. As in *Drosophila*, large-scale enhancer trapping using transgenes equipped with minimal promoters has provided a powerful approach for identifying tissue- and stage-specific gene expression in *Arabidopsis* (CAMPISI *et al.* 1999; LAPLAZE *et al.* 2005; LIU *et al.* 2005). Many of the proteins required for the machinery of the position effects are conserved from *Drosophila* to *Arabidopsis* (HENDERSON and JACOBSEN 2007). Genome-wide transposon tagging revealed location-dependent effects on transcription and chromatin organization in *Arabidopsis* (ROSIN *et al.* 2008). An example of a position effect that is associated with the reversal of epigenetic silencing has recently been documented in maize (SINGH *et al.* 2008).

Genes subjected to reverse genetic studies may have very subtle phenotypes that are masked by “noise” from the position effect and fall beneath the threshold of detection. In promoter analysis, position effects can be substantially deleterious if the expression of the reporter gene is influenced by particular spatial and temporal factors.

Various techniques have been developed to overcome the confounding potential of position effects. The ability of matrix attachment regions (MARs) to anchor DNA to the nuclear matrix has made MARs interesting candidates as transgene boundary elements in animals (STIEF *et al.* 1989; NAMCIU *et al.* 1998; SGOUROU *et al.* 2009) and plants (SPIKER and THOMPSON 1996; HOLMES-DAVIS and COMAI 1998; HALWEG *et al.* 2005). Several models have been proposed to explain the function of MARs. The loop-domain model suggested that MARs could induce transgenes to form independent loops that create protective environments for them (SPIKER and THOMPSON 1996). Another model suggested that MARs could inhibit transcriptional read-out and thus prevent RNA silencing of transgenes (MLYNAROVA *et al.* 2003).

MARs have not been widely applied in animal and plant transgenesis. They often consist of long DNA fragments that are not amenable to processing via the traditional recombinant DNA systems. The limited knowledge about protein factor(s) that interact with MARs precludes their wide application in transgenesis (DE BOLLE *et al.* 2007). Furthermore, while MARs have been used in conjunction with the 35S CaMV promoter in plants to achieve high-level expression of transgenes,

it is unclear whether MARs could guarantee the precise expression of transgenes driven by other promoters. Two recent reports revealed negative results of MARs in plant transgenesis (DE BOLLE *et al.* 2007; LI *et al.* 2008).

Insulators are another type of DNA boundary elements that have been attractive candidates for protection against positional effects (BELL *et al.* 2001; GERASIMOVA and CORCES 2001). One type of insulator element is thought to establish independent domains that block enhancer–promoter communication whereas a second type is postulated to create a barrier against the spread of heterochromatin. Some insulators are composite elements with separable activities, while others employ a single mechanism to confer both properties (KUHN and GEYER 2003; GASZNER and FELSENFELD 2006). Insulators that have been well studied in transgenic animals include the *gypsy* and *scs/scs'* insulators from *Drosophila* (BAROLO *et al.* 2000; SARKAR *et al.* 2006; MARKSTEIN *et al.* 2008) and the  $\beta$ -globin *HS4* insulator from chicken (WANG *et al.* 2009).

The *gypsy* insulator, originally identified from the *gypsy* retrotransposon, is one of the best-characterized insulators (GDULA *et al.* 1996; BELL *et al.* 2001; KUHN and GEYER 2003). Studies have revealed that it contains a cluster of binding sites for the Suppressor of Hairy-wing [Su(Hw)] protein, a zinc-finger DNA-binding protein (HARRISON *et al.* 1993; GASZNER and FELSENFELD 2006). The *gypsy* insulator is believed to regulate gene expression by establishing higher-order domains of chromatin structure and blocking the interference of nearby enhancers or repressors (GASZNER and FELSENFELD 2006; BUSHEY *et al.* 2008).

In a pioneering study, the insulator element *ArsI* (arylsulfatase) from sea urchin (*Hemicentrotus pulcherrimus*) was shown to suppress variations of transgene expression in cultured tobacco cells (NAGAYA *et al.* 2001). However, reports concerning the exploitation of insulators in plant transgenesis are limited. Here, we have introduced the *gypsy* insulator and its binding protein Su(Hw) into *Arabidopsis*. The transgenic vectors were constructed using GATEWAY technology based on a site-specific recombination cloning system. We found that the *gypsy* insulators improved the expression level of transgenes driven by various promoters. The *gypsy*-Su(Hw) system reduced position effects and mediated the specificity of promoter activities. Our strategy can be used to guarantee high and precise expression of transgenes in plants. Certain features of the *gypsy* insulator in *Arabidopsis* also provide new perspectives on the insulator field.

## MATERIALS AND METHODS

**Materials:** All transgenic experiments used accession Columbia-0 of *Arabidopsis thaliana* provided by the Arabidopsis Biological Resource Center (ABRC).

**Chemicals and original vectors:** Clones that contained sequences of *Su(Hw)* and the *gypsy* insulator of *Drosophila* were purchased from the *Drosophila* Genomics Resource Center. GATEWAY-compatible destination vectors (KARIMI *et al.* 2002) for promoter analysis (pHGWS7) and overexpression (pH7FWG2.0, pK7WG2D.1) were ordered from the Department of Plant Systems Biology, VIB-Ghent University (Ghent, Belgium). The vector pAVA319 contained a translation leader (TL) and was obtained from ABRC. The TOPO cloning and LR reaction kits were purchased from Invitrogen (<http://www.invitrogen.com>).

**Construction of entry vectors:** Entry vectors for promoter analysis of *Silent Information Regulator 2 (SIRT2)*, the *Pinform (PIN)* gene family, and the *Auxin Receptor F-box (AFB)* gene family and for overexpression of *Su(Hw)* were constructed using the pENTR/D-TOPO kits. The PCR primers for construction of entry vectors are listed in the supporting information, Table S1. Each entry clone was confirmed by DNA sequencing.

**Construction of destination vectors containing insulators:** The *gypsy* insulator (395 bp) was amplified from the pH-Stinger vector (BAROLO *et al.* 2000), with primers indicated in Table S1. First, one copy of the *gypsy* insulator, named I2, was inserted at the *SacI/SpaI* sites of pHGWS7 to obtain pHGWS7-I2. Then, pHGWS7-I2 was divided into a long and a short fragment by *AatII* digestion. Another *gypsy* insulator with *BstXI* sites introduced at both ends was inserted in the *AatII* site of the long fragment. This transition vector was ligated to the short fragment of pHGWS7-I2 to produce the pGYPSY vector with *gypsy* insulators flanking both sides of the *EGFP-GUS* reporter gene (Figure 1B). The TL from the vector pAVA319 was cloned into pGYPSY at the *SacI* site to produce the pGYPSY-TL vector (Figure 1C). Finally, we inserted the *Su(Hw)* overexpression cassette, driven by the 35S CaMV promoter, into the *SacI/XhoI* site of pGYPSY and pGYPSY-TL to obtain pGYPSY-*Su(Hw)* and pGYPSY-TL-*Su(Hw)* (Figure 1, D and E). The PCR primers used for the construction of destination vectors are listed in Table S2.

**Construction of expression vectors:** The LR reaction was conducted to generate different expression vectors. The system contains entry vectors of interest, their corresponding destination vectors, and the LR recombinant enzyme.

**Transformation and selection of transformed plants:** The selected expression vectors were transformed into *Agrobacterium tumefaciens* (GV3101) via electroporation. Arabidopsis plants were transformed using the vacuum infiltration method of BECHTOLD *et al.* (1993). Seeds collected from infiltrated plants were cultured on B5 medium containing hygromycin (12 mg/L) or kanamycin (30 mg/L). To estimate the number of transgene integration loci in the T1 generation, about 100–150 seeds were plated on a hygromycin medium to determine the segregation of the selectable marker gene *HPT (hygromycin phosphotransferase)*. Transgenic lines whose progeny showed Mendelian characteristics (3:1 segregation,  $\chi^2$  test) were termed “single-locus lines” (FORSBACH *et al.* 2003). The single-locus lines were then subjected to Southern blot analysis to reveal the single-copy lines. Genomic DNA was digested by *SacI* and hybridized with the HPT probe. The DNA probe was labeled and detected using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics). During the development of the *gypsy-Su(Hw)* system in Arabidopsis, single-copy lines were used for the quantitative analysis of the promoter activities of *SIRT2*, 35S, *PIN1*, and *PIN2*. During the extension of the *gypsy-Su(Hw)* system in Arabidopsis, single-locus lines were used for the qualitative analysis.

**Seed germination and plant growth measurement:** Arabidopsis seeds were surface-sterilized and cultured aseptically on 9-cm petri dishes containing Gamborg's B5 medium with 1%

(w/v) sucrose and 1% (w/v) agar. The plates were maintained at 4° for 2 days and then transferred to a culture room (23°, 100  $\mu\text{M m}^{-2}\cdot\text{s}^{-1}$  irradiance with a 16-hr photoperiod, 30–40% relative humidity). After 6 days, the plates were digitally photographed. Root and hypocotyl length was measured using magnified images. Statistical analyses of the data were performed using Microsoft Excel and Student's *t*-test.

**Histochemical GUS staining and quantitative analysis:** Six-day-old seedlings containing the reporter gene *GUS* ( $\beta$ -glucuronidase) were collected and stained at specific times at 37° in X-glucuronide (5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic) dissolved in 10 mM EDTA, 1 mM potassium hexacyanoferrate, 0.1% Triton X-100, and 100 mM phosphate buffer, pH 7.0. The GUS images were examined under a Nikon Eclipse 80i microscope with DXM1200 CCD camera and EclipseNet software (<http://www.laboratory-imaging.com>). Quantitative GUS activity was measured through the detection of the cleavage of 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) into 4-methylumbelliferon by fluorometric assay and calculated as nanomoles of 4-methylumbelliferon per minute and per milligram of total soluble proteins (JEFFERSON *et al.* 1987). The protein extraction solution contained 100 mM PBS, pH 7.0, 10 mM EDTA, 10 mM  $\beta$ -mercaptoethanol, 0.1% Triton X-100, and 140  $\mu\text{M}$  PMSF. Protein concentrations were determined by methods described by BRADFORD (1976). The GUS reaction solution contained 1 mM MUG in the protein extraction solution. The reaction was carried out in 37° for 20 min. Fluorescence was measured using a spectrofluorophotometer (RF-530IPC, Shimadzu) at 460 nm with excitation at 355 nm.

**Enhanced green fluorescent protein visualization:** Enhanced green fluorescent protein (EGFP) fluorescence and differential-interference-contrast images were visualized using a fluorescent microscope (Nikon Eclipse 80i). Band-pass filter sets for EGFP (excitation at 460–500 nm, emission at 510–550 nm) were used. Seedlings were mounted in water.

## RESULTS

**Construction of T-DNA vectors consisted of *gypsy* insulators:** To counteract position effects in the transgenesis of Arabidopsis, T-DNA destination vectors containing *gypsy* insulators of *Drosophila* were constructed. The procedure has been detailed in MATERIALS AND METHODS, and the schematic overview of these vectors is illustrated in Figure 1. The original vector for promoter analysis, pHGWS7, employed a fused *EGFP* and *GUS* as its reporter gene and the *HPT* driven by the nopaline synthase promoter (*NOS*) as its selectable marker (Figure 1A; KARIMI *et al.* 2002). In the pGYPSY vector, the expression cassette of the *GUS-EGFP* gene was located between the two *gypsy* insulators, whereas the expression cassette of the hygromycin marker gene was outside the insulated region (Figure 1B), with the intention of reducing the effects of the enhancers of the selectable marker gene on the promoters under analysis. On the basis of the pGYPSY vector, the pGYPSY-TL vector was constructed to include a translation leader to the reporter gene in anticipation of the benefit to the expression of the fusion protein (Figure 1C). *Su(Hw)* is the binding protein of the *gypsy* insulator, and it is critical to efficient activity in *Drosophila* (KUHN and



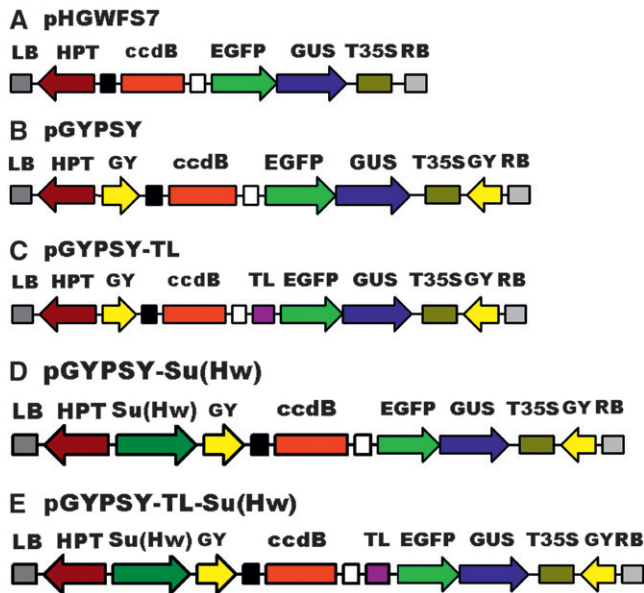


FIGURE 1.—Schematic overview of the promoter analysis vectors used in this study. The positions and relative orientations of the selectable marker gene *hygromycin phosphotransferase* (*HPT*) and the reporter gene *EGFP-GUS* are shown with respect to the right border (RB) and the left border (LB) of the T-DNA regions. The *ccdB* region was expected to be replaced by a promoter sequence by a LR reaction of the GATEWAY system. (A) The pHGWFS7 vector (KARIMI *et al.* 2002). (B) pGYPSY harbors *gypsy* insulators (GY) at both ends of the *EGFP-GUS* expression cassette based on the pHGWFS7. (C) pGYPSY-TL with a translation leader (TL) inserted into pGYPSY. (D) pGYPSY-Su(Hw) with an overexpression cassette of Su(Hw), the binding protein of the *gypsy* insulator, inserted into pGYPSY. (E) pGYPSY-TL-Su(Hw) with a Su(Hw) overexpression cassette inserted into pGYPSY-TL.

GEYER 2003). To explore the function of Su(Hw) in Arabidopsis, a Su(Hw) overexpression cassette driven by the *35S* CaMV promoter was introduced into pGYPSY and pGYPSY-TL vectors, resulting in two vectors pGYPSY-Su(Hw) and pGYPSY-TL-Su(Hw) (Figure 1, D and E).

**The *gypsy* insulator boosted the expression of reporter genes with different promoters in Arabidopsis:** The *SIRT* gene family encodes nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent protein deacetylases evolutionarily conserved from Archaeobacteria to humans (HUNT *et al.* 2004). Arabidopsis has two *SIRT* homologs: *AtSIRT1* (AT5G55760) and *AtSIRT2* (AT5G09230). We aimed to study the expression pattern of *AtSIRT2* using promoter reporter vectors pHGWFS7-*SIRT2* (the control) and pGYPSY-*SIRT2* (including the *gypsy* insulators). Single-copy and homozygous transgenic lines were studied using histochemical staining and quantitative analysis. In the GUS staining assay, results demonstrated that the expression level of  $\beta$ -glucuronidase in transgenic lines without the *gypsy* insulators varied among the lines tested even after staining for 15 hr, which presented a substantial technical challenge to interpreting results

and limited its efficacy as a reporter system (Figure 2A). In contrast, when the *gypsy* insulator was introduced, the staining pattern was more reproducible among the lines, and the threshold of detection was reached in only 3 hr (Figure 2B; Figure S1). A quantitative fluorometric assay revealed that GUS activities in transgenic lines with insulators were  $10.20 \pm 0.71$ -fold higher than in the uninsulated ones (Figure 2C,  $n = 15$ ).

To examine whether the effectiveness of the *gypsy* insulator was dependent on the type of promoter, various well-characterized promoters were tested. The *35S* CaMV promoter remains the most frequently used promoter in plant transformation. The *PIN1* promoter drives an auxin efflux carrier gene important for proper embryogenesis, vascular bundle formation, and many other developmental processes, while the *PIN2* promoter drives another auxin efflux carrier gene that affects gravitropism of Arabidopsis roots (PAPONOV *et al.* 2005). The *35S* CaMV promoter is constitutive, the *PIN1* promoter is tissue-specific, and the *PIN2* promoter is tissue-specific and inducible. Quantitative analysis of the *35S*, *PIN1*, and *PIN2* promoter activities alone or in the presence of insulators revealed that the magnitude of improvement by the *gypsy* insulators was  $9.41 \pm 0.58$ ,  $8.13 \pm 0.56$ , and  $13.7 \pm 0.69$ , respectively (Figure 2C). These data suggested that the *gypsy* insulator might improve the expression of reporter genes driven by different types of promoters.

**The variability of transgene expression was further reduced by coexpression of Su(Hw):** The *gypsy* insulator contains 12 binding sites for Su(Hw), a protein factor proven to be pivotal for proper insulation function in *Drosophila* (GDULA *et al.* 1996; BELL *et al.* 2001; KUHN and GEYER 2003). However, there is no homolog of Su(Hw) in the Arabidopsis genome. Hence, we investigated the effects of Su(Hw) coexpression upon the efficacy of the *gypsy* insulator in the transgenesis of Arabidopsis.

Initially, we overexpressed the Su(Hw)-EGFP fusion construct in conjunction with the *35S* promoter (Figure 3A) to verify the subcellular localization of the Su(Hw) protein in Arabidopsis. As shown in Figure 3B, EGFP fluorescence was detected in the nucleus, indicating that the localization pattern of Su(Hw) protein in plant was identical to that observed in *Drosophila* (HARRISON *et al.* 1993).

We then investigated the role played by Su(Hw) in two different modes of expression. In the *cis* expression mode, the T-DNA contained a promoter analysis region and a Su(Hw) overexpression cassette [pGYPSY-Su(Hw), Figure 1D]. In the *trans* expression mode, one T-DNA contained a promoter analysis region with hygromycin resistance as the selection marker (pGYPSY, Figure 1B), while the other T-DNA contained a Su(Hw) overexpression cassette with kanamycin resistance as the selection marker (Figure 3A). In the latter case, homozygous Arabidopsis plants overexpressing Su(Hw)

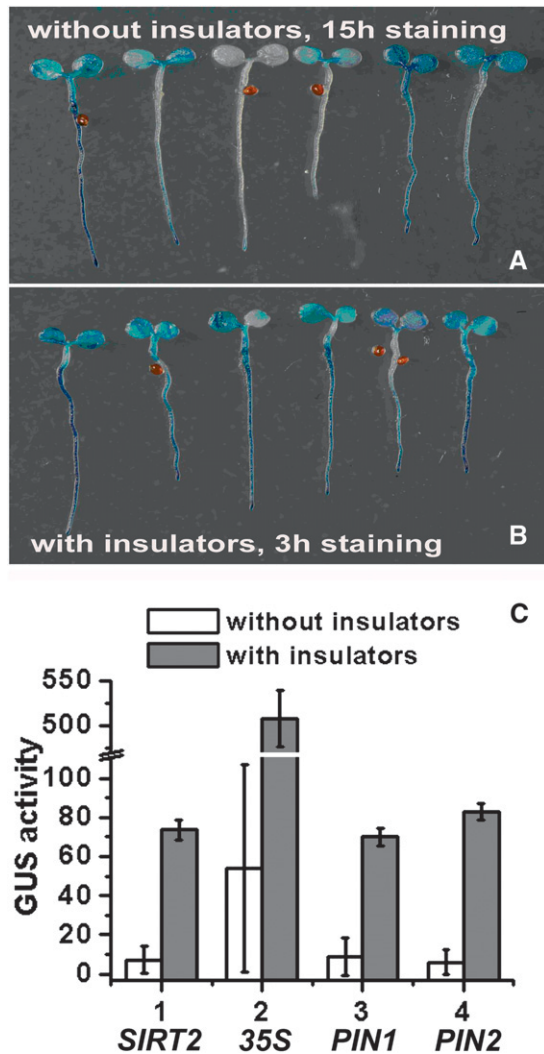


FIGURE 2.—The *gypsy* insulator improved the expression of reporter genes with different promoters. (A) Histochemical GUS staining indicated the promoter activity of *Silent Information Regulator 2* (*AtSIRT2*) in transgenic lines lacking the *gypsy* insulators (pHGWF57-*SIRT2*). The expression level varied among the lines tested, even after staining for 15 hr. Six lines are shown. (B) Histochemical GUS staining indicated the promoter activity of *AtSIRT2* in transgenic lines including the *gypsy* insulators (pGYPSY-*SIRT2*). The staining pattern was less variable (staining for 3 hr). Six lines are shown. (C) Quantitative GUS assay of promoter activities of *AtSIRT2*, 35S cauliflower mosaic virus (CaMV), *Pinform1* (*PIN1*; auxin efflux carrier), and *Pinform2* (*PIN2*) in transgenic lines with (using vector pGYPSY) or without (using vector pHGWF57) the *gypsy* insulators. The GUS activity was calculated as nanomoles of 4-methylumbelliferone per minute per milligram of protein. Each value was the mean of 15 single-copy transgenic lines. Error bars represent standard deviation.

were generated first and provided the transgenic backgrounds for the second transformation.

Four classes of transgenic lines ( $n = 15$  each) were created for promoter analysis of *AtSIRT2*. As shown in Figure 3C, the GUS activities for uninsulated lines (pHGWF57-*SIRT2*) were lower and more variable than that observed for the insulated lines (pGYPSY-*SIRT2*).

The variation of GUS activities was reduced when the Su(Hw) protein was overexpressed in the *cis* [pGYPSY-Su(Hw)-*SIRT2*] or the *trans* modes [pGYPSY-*SIRT2* plus 35S::Su(Hw)] (Figure 3C). The coefficient of variation (standard deviation/mean  $\times 100\%$ ) for GUS activities of the four classes of transgenic lines was 95.1%, 31.8%, 21.3%, and 19.0%, respectively, indicating that the *gypsy* insulator acting with the Su(Hw) was able to reduce the coefficient of variation by approximately fivefold.

**The *gypsy*-Su(Hw) system does not alter the specificity of promoter activities of the *PIN* and *AFB* gene families:** The above results demonstrated that the *gypsy* insulator and its binding protein Su(Hw) were able to boost the expression of transgenes and reduce the variability of the expression of transgenes in Arabidopsis. We next sought to determine whether this system altered the specificity of plant promoter activities.

There are eight members of the *PIN* family encoding auxin efflux carriers that are crucial to polar auxin transport (BLILOU *et al.* 2005). We constructed a set of promoter analysis vectors for this gene family, from *PIN1* to *PIN8*, using the pGYPSY-TL-Su(Hw) construct, where the *gypsy* insulators flanked the transgenes and the Su(Hw) protein was expressed in the *cis* manner. In 6-day-old seedlings, GUS activity in cotyledon, hypocotyl, lateral root, and primary root and EGFP signal in root tips were examined (Figure 4A). As expected, transgene expression variability was minimal among different transgenic lines for all eight promoters, which facilitated the comparison of expression patterns of the different promoters. In root tips, *PIN1* was strongly expressed in vascular tissue, whereas *PIN2* was expressed in the outer cortical cells and epidermis cells. *PIN3* was transcribed in the vascular cells and notably at the basal end of the provascular cells. *PIN4* was restricted to the vascular cells and principally to the quiescent center and auxin peak region. *PIN7* was present in the vascular cells and was strongly expressed in the columella cells. *PIN6* was weakly expressed in provascular cells, whereas *PIN5* and *PIN8* were undetectable in root tips (Figure 4B).

The *TIR1/AFB* gene family encodes proteins related to auxin responses (DHARMASIRI *et al.* 2005a). As a subunit of the E3 ubiquitin ligase SCF<sup>TIR1</sup>, TIR1 functions as a receptor for plant hormone auxin. After binding of auxin to TIR1, ubiquitination and degradation of AUX/IAA proteins, a class of transcriptional repressors in auxin signaling, is triggered (DHARMASIRI *et al.* 2005b; KEPINSKI and LEYSER 2005). Using the *gypsy*-Su(Hw) system, we generated a set of promoter analysis lines for the *TIR1/AFB* gene family.

The expression patterns of the *TIR1/AFB* gene family members during certain stages of embryogenesis were examined. As shown in Figure 5A, at the late heart stage, the *TIR1* promoter exhibited a stronger expression pattern in shoot primordium than in root

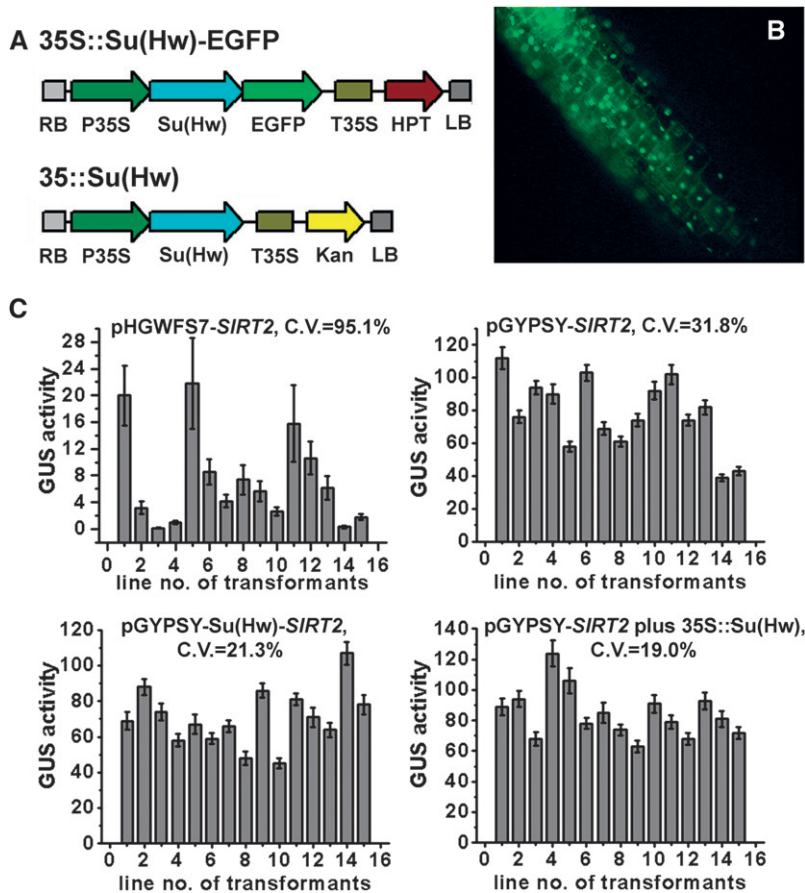


FIGURE 3.—Variability of reporter gene expression driven by *AtSIRT2* (*Silent Information Regulator 2*) promoter was reduced when Su(Hw) was expressed in a *cis* or a *trans* manner. (A) Schematic for the vectors that overexpress the Su(Hw)::GFP fusion and the Su(Hw) proteins. (B) Nuclear localization of the Su(Hw)::GFP fusion in the hypocotyls of 6-day-old transgenic seedlings. (C) Comparison of GUS activities in four classes of transgenic lines: pHGWF57-SIRT2, the control; pGYPSY-SIRT2, with the *gypsy* insulators; pGYPSY-Su(Hw)-SIRT2, the Su(Hw) protein coexpressed in the *cis* mode; and pGYPSY-SIRT2 plus 35S::Su(Hw), the Su(Hw) protein coexpressed in the *trans* mode. The coefficient of variation of each treatment is indicated. The GUS activity was calculated as nanomoles of 4-methylumbelliferone per minute per milligram of protein. Each value was the mean of 15 single-copy transgenic lines. Error bars represent standard deviation.

primordium. In contrast, at the torpedo stage, it exhibited a stronger expression in root primordium than that in shoot primordium. At the mature embryo stage, *TIR1* was strongly expressed throughout the whole embryo. *AFB1* was expressed in the provascular bundle at the mature embryo stage, and its expression at the heart and torpedo stages was low. In contrast, *AFB2* was highly expressed in precotyledons from the heart stage to the mature embryo stage. At the late heart and torpedo stages, *AFB3* promoter produced striking EGFP signal in shoot primordium and provascular bundles, while at the mature embryo stage, its expression was more widespread, similar to that observed for the *TIR1* promoter. For both *AFB4* and *AFB5* promoters, weak signals on the paraxial side of two precotyledons at the torpedo stage were detected (Figure 5A).

We then examined the expression patterns of these *TIR1/AFB* genes in 6-day-old seedlings using GUS staining (Figure 5B). For all six promoters, variation of expression pattern among different transgenic lines was less detectable, which indicated that position effects were largely reduced. Taking advantage of higher intensity signals and lower variation, the more subtle differences in expression features of *TIR1/AFB* genes in the cotyledon, hypocotyl, lateral root, mature root region, and root tip were readily discernible (Figure 5B).

#### The *gypsy* insulator improved the expression of a selectable marker gene outside the insulated region:

The expression cassette of the hygromycin marker was outside the insulated region, although its *NOS* promoter was adjacent to one of the insulators (Figure 1B). We observed an improved hygromycin resistance in transgenic plants with the *gypsy* insulators. To characterize the hygromycin resistance capacity, we measured the primary root length of 6-day-old seedlings. Data indicated that the mean values of root length for transgenic lines with or without insulators were similar when germinated on medium without antibiotics (with insulators:  $2.25 \pm 0.09$  cm,  $n = 19$ ; without insulators:  $2.26 \pm 0.11$  cm,  $n = 19$ ; Figure 6A). However, as seedlings grown in antibiotic-containing medium, the root length for the insulated lines ( $1.45 \pm 0.23$  cm,  $n = 19$ ) was on average threefold greater than that measured for the uninsulated lines ( $0.47 \pm 0.32$  cm,  $n = 19$ , Figure 6A). Root length of transgenic lines without insulators fluctuated more greatly than their counterparts in response to antibiotic exposure: the coefficient of variation for uninsulated and insulated lines was 68.1% and 15.8%, respectively.

To rule out the possibility that increased hygromycin resistance in insulated transgenic lines was due to activities of the *AtSIRT2* promoter, we introduced a protein coding sequence of At1g68450 (a VQ-domain-containing protein



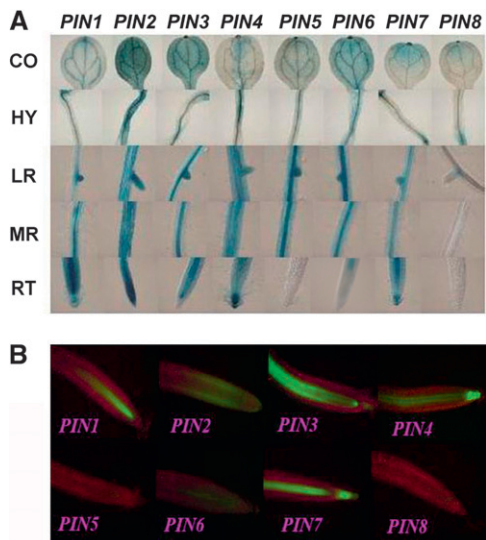


FIGURE 4.—The *gypsy*-Su(Hw) system did not alter the specificity of promoter activities of the *Pinform* (*PIN*; auxin efflux carrier) gene family. Promoter analysis lines for this gene family were generated using the pGYPSY-TL-Su(Hw) construct where the *gypsy* insulators flanked the transgenes and the Su(Hw) protein was expressed in a *cis* manner. (A) The GUS staining pattern in cotyledon (CO), hypocotyl (HY), lateral root (LR), mature root (MR), and root tip (RT) of 6-day-old transgenic seedlings. (B) The EGFP fluorescence images for the expression patterns of the *PIN* family promoters in root tips of 6-day-old Arabidopsis seedlings.

with unknown function). Results for pHGWFS7-Atlg68450 and pGYPSY-Atlg68450 constructs are shown in Figure 6B. Again, when seedlings were grown on antibiotic-free medium, the root length of transgenic

lines with insulators was similar to that of uninsulated ones (with insulators:  $2.24 \pm 0.16$  cm,  $n = 19$ ; without insulators:  $2.23 \pm 0.14$  cm,  $n = 19$ ), but exhibited an approximately threefold difference on antibiotic-infused medium (with insulators:  $1.39 \pm 0.26$  cm,  $n = 19$ ; without insulators:  $0.48 \pm 0.28$  cm,  $n = 19$ ). All data considered, the *gypsy*insulator improved the expression of a selectable marker outside the insulated region.

DISCUSSION

To overcome position effects in the transgenesis of Arabidopsis, we adopted the *gypsy*insulator of *Drosophila* together with its binding protein Su(Hw) in Arabidopsis. The transgenic vector system described here has several features. First, previous approaches using animal MARs or insulators to create transgenic plants have focused on *cis*-acting elements only. Our transgenic vectors have been combined with relevant *cis*-acting elements with their cognate *trans*-acting factors. Second, previous studies dealt with strong promoters and were concerned with the expression level only. Our goal was to maximize the expression level and also the specificity of promoter activities. Finally, the GATEWAY high-throughput system was employed, allowing for the construction of T-DNA vectors with high speed and reliability.

We found that, in Arabidopsis, the *gypsy* insulators were able to boost the expression of reporter genes driven by various kinds of promoters, such as *AtSIRT2*, *35S*, *PIN1*, and *PIN2*, by ~8- to 13-fold (Figure 2). To

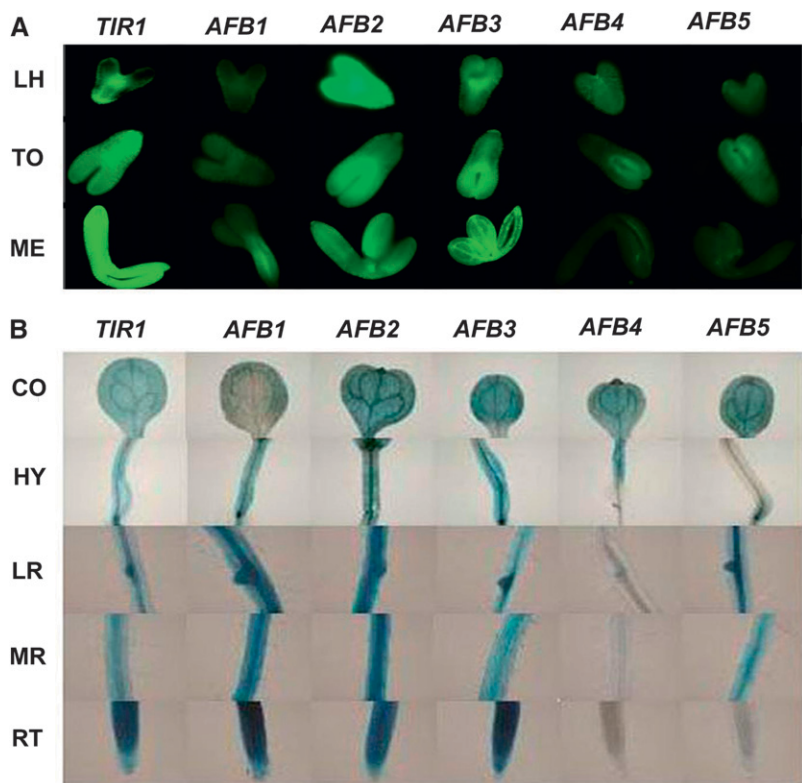


FIGURE 5.—The *gypsy*-Su(Hw) system facilitated the examination of the promoter activities of the *Transport Inhibitor Response 1/Auxin Receptor F box* (*TIR1/AFB*) gene family. Promoter analysis lines for this gene family were generated using the pGYPSY-TL-Su(Hw) construct in which the *gypsy* insulators flanked the transgenes, and the Su(Hw) protein was expressed in a *cis* manner. (A) The EGFP images of the expression pattern of the *TIR1/AFB* gene family during embryogenesis at the late heart (LH), torpedo (TO), and mature embryo (ME) stages. (B) The GUS images of the expression pattern of the *TIR1/AFB* gene family in cotyledon (CO), hypocotyl (HY), lateral root (LR), mature root (MR), and root tip (RT) of 6-day-old seedlings.

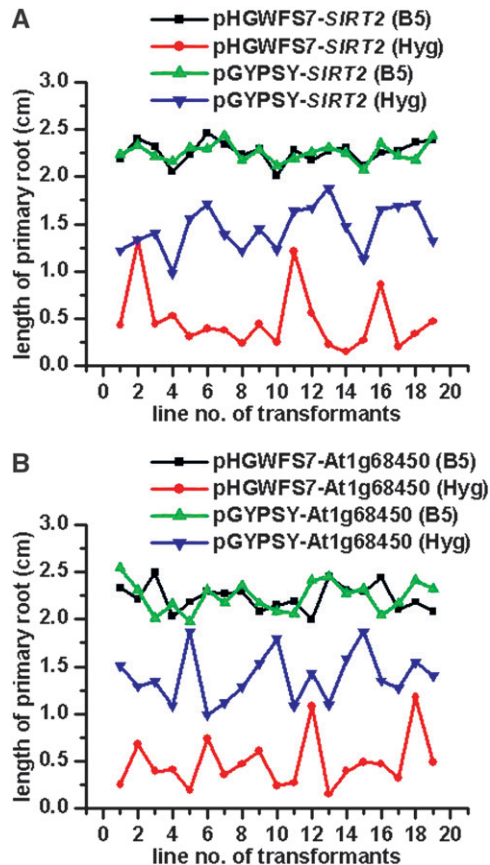


FIGURE 6.—Transgenic plants with *gypsy* insulators exhibited improved hygromycin resistance. The promoter analysis vectors pHGWFS7 (control) and pGYPSY (with *gypsy* insulators) included a hygromycin resistance marker. Single-locus transgenic lines were cultured on hygromycin (Hyg) or control (B5) medium. (A) Comparison of pGYPSY-SIRT2 with pHGWFS7-SIRT2. *Silent Information Regulator 2* (*SIRT2*) is a promoter sequence of AT5G09230. (B) Comparison of pGYPSY-At1g68450 with pHGWFS7-At1g68450. At1g68450 is a protein-coding sequence of the corresponding gene. The root lengths of 6-day-old seedlings were measured. Each treatment was carried out on 19 transgenic lines. Error bars represent standard deviation.

ensure proper function of the *gypsy* insulator in Arabidopsis, the Su(Hw) protein was overexpressed in a *cis* or *trans* manner (Figure 3A). Interestingly, the Su(Hw) co-expression led to a more uniform expression level of a transgene, as the coefficient of variation of observed expression levels was reduced further (Figure 3C), but not to a general increase in the expression level.

We also found that the *gypsy*-Su(Hw) system reduced position effect but did not alter the specificity of promoter activities of the *PIN* and the *TIR1/AFB* gene family (Figures 4 and 5). Promoter reporter lines and immunocytochemical analysis data for *PIN1*, *PIN2*, *PIN3*, *PIN4*, and *PIN7* have been previously documented (BLILOU *et al.* 2005), as has *TIR1*, *AFB1*, *AFB2*, and *AFB3* (DHARMASIRI *et al.* 2005a). During the writing of this article, the expression pattern of *PIN5* was reported (MRAVEC *et al.* 2009). Our results for the promoter activities of the *PIN*

and the *AFB* gene families were in agreement with the published data. This suggested that the *gypsy* insulators did not act as tissue-specific or time-specific enhancers directly and that they were applicable to precise gene expression in Arabidopsis. The enhanced gene expression properties and the specificity of promoter activities make our transgenic approach a promising candidate technique for effective plant reverse genetics.

The mechanism mediating the function of the *gypsy* insulator has not yet been completely elucidated, but several models have been proposed in *Drosophila*. The transcriptional model and the structural model are two of the most discussed models (KUNH and GEYER 2003; GASZNER and FELSENFELD 2006; BUSHEY *et al.* 2008). The former emphasized the competition between insulators and enhancers/repressors, and the latter stressed the formation of a chromatin loop with the help of insulators. In addition, the *gypsy* insulator is believed to facilitate the assembly of the transcriptional complex to improve the expression of transgenes in *Drosophila* (GOLOVNIN *et al.* 2005). As insulators are known to block both the repressive and the activating influences of the surrounding environment, the pervasive boosting effects of *gypsy* insulators observed in *Drosophila* transgenesis suggest that, on balance, transgenes in *Drosophila* are under repressive influences (MARKSTEIN *et al.* 2008).

In *Drosophila*, the Su(Hw) protein is necessary for chromatin loop formation and enhancer/repressor blockage (GERASIMOVA and CORCES 2001; KUNH and GEYER 2003). In Arabidopsis, the *gypsy* insulators themselves can reduce transgene variations efficiently by minimizing those transgenic lines with low-level expression (Figure 2). These findings implied that an Arabidopsis protein was binding to the *gypsy* insulator and was providing insulator activity. However, expression of the Su(Hw) protein had some effects. The variability in transgene expression among individual lines was reduced further (Figure 3C). Perhaps the Su(Hw) protein of *Drosophila* acted as a better insulator protein than the Arabidopsis counterpart. Since there is no homolog of Su(Hw) in the Arabidopsis genome, further investigation using the yeast one-hybrid technique to identify the putative protein factor(s) recruited by the *gypsy* insulator promises to clarify the mechanism. A better understanding of these processes would provide a new perspective on the function of the *gypsy* insulator and enhance the insulator field in general.

The chromatin loop model cannot explain all of the findings from our studies. The insulator improved the expression of a selectable marker gene, although its expression cassette was not located between the two insulators (Figure 6). It would be helpful to know if protection by the insulators represents a block of a spread of silencing or some other effect of the *gypsy* insulators as discussed below.



Position-effect variegation is a well-documented concept in *Drosophila*, but very little is known about it in *Arabidopsis*, where the 35S promoter of virus origin has been extensively employed in transgenesis. The variation of the expression level of a transgene driven by this strong and constitutive promoter has been attributed largely to a gene-specific RNA-silencing mechanism (SCHUBERT *et al.* 2004; LUO and CHEN 2007). The impact of position effects on the variation of the expression pattern of a transgene driven by a tissue- or stage-specific plant promoter was less documented. Here, we show that, similar to that in *Drosophila*, the *gypsy* insulator, together with its binding protein Su(Hw), facilitate high and precise expression of transgenes in *Arabidopsis*. Since we had not directly detected the epigenetic status of the transgenes with and without the *gypsy* insulators, interpretations other than the shielding of position effects could not be excluded. The *gypsy* insulator upstream from the promoter region of transgenes might facilitate the assembly of the transcriptional complex, as suggested in *Drosophila* by GOLOVNIN *et al.* (2005). Increasing evidence suggests that insulators have evolved from promoters (RAAB and KAMAKAKA 2010). The *gypsy* insulator downstream from the terminator region of transgenes might ensure correct termination of reporter transcripts, because it was documented that RNA silencing triggered by mRNA 3' readthrough was a major cause of reduced expression of transgenes in plants (LUO and CHEN 2007). However, terminator activity for the *gypsy* insulator was recently tested in *Drosophila*, where it was found not to function (SILICHEVA *et al.* 2010). It is also possible that transgenes with *gypsy* insulators integrated in distinct genomic regions that might be more permissive to gene expression. Further work is required to address these questions.

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# GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.110.117960/DC1>

**The *gypsy* Insulator of *Drosophila melanogaster*, Together With Its Binding Protein Suppressor of Hairy-Wing, Facilitate High and Precise Expression of Transgenes in *Arabidopsis thaliana***

**Wenjing She, Weiqiang Lin, Yubin Zhu, Yong Chen, Weiyuan Jin, Yanjun Yang, Ning Han, Hongwu Bian, Muyuan Zhu and Junhui Wang**

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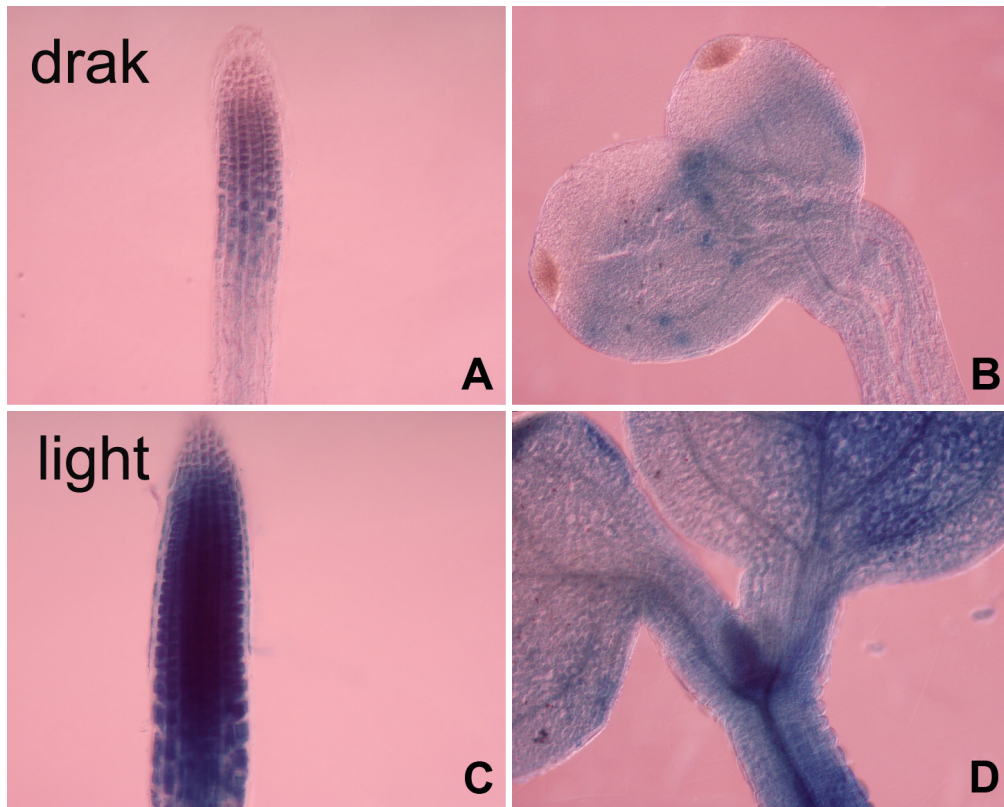


FIGURE S1.—The expression pattern of *AtSIRT2* (*Silent Information Regulator 2*, a putative NAD<sup>+</sup>-dependent protein deacetylase) as revealed by GUS ( $\beta$ -glucuronidase) staining. For promoter analysis, a T-DNA vector pGYPSY containing *gypsy* insulators was used. Six-day-old *Arabidopsis* seedlings grown in dark and light were stained for 3 h and then photographed.

TABLE S1

## Oligonucleotide primers used for construction of entry vectors

Sequence name	Primers (up/dn)	Nucleotide sequence (5'-3') (up/dn)
Su(Hw)-N	Su-N-up	caccatgagtgcctccaaggagag
	Su-N-dn	agctttctcttggcctcacg
Su(Hw)-over <i>SIRT2</i>	Su-o-up	caccatgagtgcctccaaggagag
	Su-o-dn	tcaagctttctcttggcctca
(AT5G09230)	<i>SIRT2</i> p-up	aactgcaggagaacaaaatcgacgtatg
	<i>SIRT2</i> p-dn	catgccatggaagcctaataaatcgag
35S <i>CaMV</i>	35S-up	caccatctctctcgtaaaccgatcc
	35S-dn	tctcgcacctgagactttcaacaagggt
<i>PIN1</i> (AT1G73590)	<i>PIN1</i> p-up	caccaccgaatagagaagccaagca
	<i>PIN1</i> p-dn	agagagaggggaagagagagaag
<i>PIN2</i> (AT5G57090)	<i>PIN2</i> p-up	cacctgaaagtagtgaagtggcca
	<i>PIN2</i> p-dn	gagagagaaagaagaagaaa
<i>PIN3</i> (AT1G70940)	<i>PIN3</i> p-up	caccgttaagccaccagtagccatc
	<i>PIN3</i> p-dn	aagcaacggtgtcggaggaagaag
<i>PIN4</i> (AT2G01420)	<i>PIN4</i> p-up	caccgagagcctcatgaaggagag
	<i>PIN4</i> p-dn	gtgggttttgagtttagtgagaaga
<i>PIN5</i> (AT5G16530)	<i>PIN5</i> p-up	caccgaatgaatgaatgagagaa
	<i>PIN5</i> p-dn	ggttttatcagaaaaatagaatgt
<i>PIN6</i> (AT1G77110)	<i>PIN6</i> p-up	caccaaactctacgttttgccaattaac
	<i>PIN6</i> p-dn	cttttcttgcctcttcttctct
<i>PIN7</i> (AT1G23080)	<i>PIN7</i> p-up	caccaagaggataaacccgac
	<i>PIN7</i> p-dn	tttgagtgtgaaaccgtaaggaa
<i>PIN8</i> (AT5G15100)	<i>PIN8</i> p-up	caccgctttttttctatttttctt
	<i>PIN8</i> p-dn	tgaaaacaacatggtagatcgcgagc

<i>TIR1</i>	<i>TIR1</i> p-up	caccttcatcaatcgattatatat
(AT3G62980)	<i>TIR1</i> p-dn	tgcggccaataaacctcgagatctc
<i>AFB1</i>	<i>AFB1</i> p-up	caccgaattacgatcaacaaaacg
(AT4G03190)	<i>AFB1</i> p-dn	agtgagagagagagaggggggaag
<i>AFB2</i>	<i>AFB2</i> p-up	caccccatctaacaacgatataagtc
(AT3G26810)	<i>AFB2</i> p-dn	tgaagaagattgatgagagaggaga
<i>AFB3</i>	<i>AFB3</i> p-up	cacctacgaattaatcgagatggaa
(AT1G12820)	<i>AFB3</i> p-dn	gaacagaggaattgacttcaagctt
<i>AFB4</i>	<i>AFB4</i> p-up	caccagagagaaagaggaaaggca
(AT4G24390)	<i>AFB4</i> p-dn	atccagaatttatgagctcaggtgc
<i>AFB5</i>	<i>AFB5</i> p-up	cacctacgaattaatcgagatggaa
(AT5G49980)	<i>AFB5</i> p-dn	gaacagaggaattgacttcaagctt

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**TABLE S2****Oligonucleotide primers used for construction of destination vectors**

Sequence name	Primers (up/dn)	Nucleotide sequence (5'-3') (up/dn)
In1	In1-up	actcatccagacgtctggactagtcacgtaataagtgcggtgaa
	In1-dn	ttattccacacgtgtggaagcttaattgatcggctaaatggtatg
In2	In2-up	taatgagctctactcgagcacgtaataagtgcggtgaa
	In2-dn	cgcgtctagacaagatctaattgatcggctaaatggtatg
TL	TL-up	aaagttgcagtgccggccctatcgttcgtaaatggtga
	TL-dn	ccagagctctgggtcacgtaataagtgcggtgaa