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Original Paper

Induction of double flowers in *Pharbitis nil* using a class-C MADS-box transcription factor with Chimeric REpressor gene-Silencing Technology

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Abstract The Chimeric REpressor gene-Silencing Technology (CRES-T) system is a novel reverse genetic method using a chimeric transcriptional repressor fusing an EAR transcriptional repression domain called SRDX. We sought to change the flower shape of *Pharbitis nil*, a model ornamental flower, using an *Arabidopsis* transcription factor fused with SRDX. For the first trial modulating flower shape we transformed with the class-C MADS-box transcription factor AGAMOUS (AG) fused with SRDX (*AGSRDX*). Defects in class-C genes cause double flowers in *Arabidopsis* and *Pharbitis*. However, when *AGSRDX* was expressed under the CaMV 35S promoter (*p35S*), the transgenic *Pharbitis* bore a malformed flower with a protruding pistil. We then used *DUPLICATED* (*DP*), one of the class-C genes in *Pharbitis*. The *p35S :: DPSRDX*-introduced callus were difficult to regenerate during transgenic steps, but occasionally made a perfect double flower bud showing severe growth defects. The flower buds never developed to flower opening stage. These results indicate that CRES-T is functional in *Pharbitis* but even using a conserved transcription factor, some species-specific variation might exist. To avoid these unwanted effects, we recruited inducible promoters to control expression of the chimeric transcription repressors in combination with the DNA-binding domain of GAL4 in yeast fused with SRDX and the GAL4 upstream activator sequence (UAS). Normal regeneration was observed by inducible repression of *DPSRDX* during *in vitro* redifferentiation, and the double-flowered *Pharbitis* was generated. We successfully induced a non-transformant (NT)-like flower in *DPSRDX*-expressing double-flowered transformants. Our approach will enable us to breed transgenic horticultural plants with inducible fertility.

Key words: Chimeric repressor, CRES-T, double flower, inducible promoter, *Pharbitis nil.*

In higher plants, adjustment of development, differentiation, and responses to the environment are controlled strictly by transcription factors, which often form large gene families in which family members include strongly conserved DNA-binding domains (Riechmann et al. 2000). This structural redundancy obstructs the functional analysis of plant transcription factors. Even if the expression of one transcription factor is controlled, that influence is not often shown in the plant phenotype. To solve this problem, Takagi's group developed a novel gene silencing technology, called CRES-T (Chimeric REpressor gene-Silencing Technology), in which a chimeric repressor is produced by fusion of a transcription factor to the plant-specific ETHYLENE-RESPONSIVE ELEMENT-BINDING FACTOR (ERF)-associated amphiphilic repression (EAR)-like motif repression domain (Hiratsu et al. 2002; Ohta et al. 2001). The repression domains of ERF and TFIIIA-type zinc finger repressors of transcription that include SUPERMAN (SUP) (Bowman et al. 1992) contain the EAR motif (Hiratsu et al. 2002; Ohta et al. 2001). The EAR motif is able to convert a transcriptional activator into a strong repressor, and the repressive activity of the EAR motif repression domain is dominant over both intra- and intermolecular activational activities (Hiratsu et al. 2002; Ohta et al. 2001). The chimeric repressors derived from various transcription factors dominantly suppress the expression of the respective target genes, and the resultant transgenic plants exhibit loss-of-function phenotypes specific for the target genes at high frequency (Fujita et al. 2005; Hiratsu et al. 2003; Iida et al. 2007; Ikeda et al. 2009; Koyama et al. 2007;

Abbreviations: CaMV, *Cauliflower mosaic virus*; CRES-T, Chimeric REpressor gene-Silencing Technology; NT, non-transformant
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Matsui et al. 2004, 2005; Mitsuda et al. 2005, 2006; Tohge et al. 2005).

Mutant analyses of *Arabidopsis thaliana* and *Antirrhinum majus* have revealed that a number of genes that regulate flower development encode transcription factors. These transcription factors are categorized into ABC genes, which specify floral organ identity (Bowman et al. 1989; Coen and Meyerowitz 1991; Coen et al. 1990; Goto and Meyerowitz 1994; Jack et al. 1994; Mandel et al. 1992; Yanofsky et al. 1990). Most ABC genes encode MADS-box transcription factors. One of the most striking phenotype is the mutation of the *AGAMOUS* (*AG*) gene in *Arabidopsis*. Stamens of the *ag* mutant are converted into petals, and the fourth whorl bears another *ag* flower, resulting in an indeterminate flower with a repetitive pattern of sepals, petals and petals (Yanofsky et al. 1990).

In this report, we describe the application of CRES-T to the *Arabidopsis* transcription factor *AG* (Yanofsky et al. 1990) and its homologue in *Pharbitis*, namely *DUPLICATED* (*DP*) (Nitasaka 2003), which are involved in the regulation of stamen and carpel identity, with the objective of generating new traits in horticultural plants. *Pharbitis nil* (*Ipomoea nil* [L.] Roth), the Japanese morning glory, was first introduced into Japan from China during the Nara era (710–794) and has long been cultivated as a horticultural plant. Most of the existing mutants were selected during the late Edo era (1603–1868), and these mutant lines are still maintained. Japanese geneticists analyzed some of these mutants in the 1910s (Imai 1930, 1938). Based on recent molecular studies of these mutations, most are thought to be induced by Enhancer/Suppressor-mutator (En/Spm)-related transposable elements of the *Tpn1* (transposable element from *Pharbitis nil* 1) family (Iida et al. 1999, 2004). In a *dp* mutant, an E_n/Spm-related transposable element of *P. nil* (Tpn-botan) was inserted in the second intron of *DP* (Nitasaka 2003). Mutants of this species exhibit particularly rich and varied floral colors, patterns and floral form compared to other model plants.

Although *P. nil* is a typical short-day plant, cv. Violet is an especially sensitive genotype and can be induced to flower by a single 16-h exposure to continuous darkness, whereas plants of cv. Violet grown under continuous light cannot be induced to flower (Imamura 1967; Vince-Prue and Gressel 1985). Therefore *P. nil* cv. Violet has been used as a model plant for studies of floral color, floral form and photoperiodic induction of flowering. Previously, we reported a reliable transformation protocol mediated by *Agrobacterium tumefaciens* containing virGN54D, called a 'ternary system', and plant regeneration for *P. nil* cv. Violet (Kikuchi et al. 2005).

CRES-T system is a powerful tool for genetic recombination in plants and the *Cauliflower mosaic virus* (CaMV) 35S promoter-driven chimeric repressor dominantly suppresses the target genes. However, in some cases, growth defects and decreased fertility are observed. Thus, we developed new technology to control the expression of the transgene, with the aim of realizing genetic recombination of floricultural plants. The GAL4DB transcription factor of yeast was used, and vectors in which *GAL4DBSRDX* were expressed by inducible promoters were constructed. The *GAL4DBSRDX* was originally developed to compare the strength of repression motifs and was used only for transient gene expression assay (Hiratsu et al. 2004). We applied this *GAL4DBSRDX* for controlling gene expression in stable transformants as the key component, and developed the GAL4SRDX/UAS system driven by inducible promoters (alcohol-inducible and heat-shock promoters) represses the overexpression of the transgene under the control of *35S promoter : GAL4UAS*.

The Alc gene expression system, which is based on a regulon from *Aspergillus nidulans* (Felenbok et al. 1988; Pateman et al. 1983; Sealy-Lewis and Lockington 1984), and the heat-shock inducible promoter (Matsuhara et al. 2000; Takahashi et al. 1992) were used as inducible promoters. The alcR transcriptional regulator is expressed with the CaMV35S promoter such that, in the presence of ethanol, ALCR induces expression of any gene fused to a modified alcR promoter (Caddick et al. 1998). On the other hand, the heat-shock inducible promoter *HSP18.2* from *Arabidopsis*, encoding a heat-shock protein, is indicated to function as a strong inducible system in plants (Takahashi et al. 1992; Matsuhara et al. 2000). These inducible promoters have been used to construct controllable gene expression systems in plant cells. In this report, we describe the construction and phenotype of transgenic *Pharbitis* using a CRES-T system and the application in combination of these inducible promoters. It is expected that utilization of the GAL4SRDX/UAS system driven by inducible promoters (alcohol-inducible and heat-shock promoters) could be applied for genetic engineering of horticultural plants because GAL4SRDX could repress the growth defects and decreased fertility caused by an ectopic overexpressing transgene.

Materials and methods

Plant materials and growth conditions

Seeds of *Pharbitis nil* cv. Violet (Marutane Co., Kyoto, Japan) and a *dp-1* mutant Q0426 (kindly provide by Dr. Nitasaka) were germinated on wet vermiculite. Seedlings were grown at 24±1°C with illumination by continuous cool-white fluorescent light (60 mmol m^{-2} s^{-1}, FL 40SS W/37 lamps; Matsushita Electronics Co., Tokyo, Japan).

Construction of plasmids for chimeric repressor-overexpressor

The *AGSRDX*-overexpressor construct (*pBCKK-35S-AG-RD*) was used (Mitsuda et al. 2006). To construct the chimeric repressor, *DPSRDX*, the coding sequence of *DUPLICATED* (*DP*) (AB006182) cDNA, excluding the stop codon, was amplified from a *Pharbitis* apical bud cDNA. The amplified fragment was cloned into the *Sma*I site of the *p35SSRDXG* vector, in frame to the region that encodes the SRDX repression domain (LDLDLELRLGFA) from SUPERMAN (Hiratsu et al. 2002, 2003, 2004). The *p35SSRDXG* vector contains a CaMV 35S promoter followed by an Ω translation enhancer sequence, the SRDX repression domain sequence, *NOS* terminator, and the attL1 and attL2 Gateway recombination sites (Invitrogen Corp., Carlsbad, CA, USA) outside the regions of the 35S promoter and the nopaline synthase (*NOS*) terminator in the pUC119 vector. The transgene cassette was transferred into the destination vector *pBCKK* (Fujita et al. 2005) using the Gateway LR clonase reaction (Invitrogen). The *pBCKK-35S-DP-RD* construct was formed from a destination vector, *pBCKK*, and an entry clone, *p35S-DP-RD*, using the Gateway LR clonase reaction (Invitrogen).

Reverse transcriptase-polymerase chain reaction

To investigate the expression profiles in NT plants and mRNA accumulation in transformants, total RNAs were isolated using the Get Pure RNA Kit (Dojindo, Kumamoto, Japan). First-strand cDNA was synthesized from 1 μg of each RNA sample in a 20 μl reaction solution using a Super Script® First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Reactions were carried out on a thermal cycler, starting denaturation at 94°C for 2 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. The *PnUBQ* gene was used as an internal standard. The primers used in this experiment are listed in Supplemental Table 1. The PCR products were separated on 1.5% agarose gels.

Construction of plasmid for inducible repression vectors

Inducible repression vectors using GAL4SRDX and GAL4UAS transcription systems were constructed with the MultiSite Gateway® Three-Fragment Vector Construction Kit (Invitrogen). The combined genetic elements *p35S-GAL4UAS* (in pDONR™ 221-P4-P1r; between B4 and B1 in Figure 3C), *DPSRDX* or *LUCIFERASE* (*Luc*) (in pENTR-D-TOPO; between B1 and B2 in Figure 3C), and *35S-T-NOS-T-GAL4SRDX-inducible promoter, Alc* promoter system (*p35S::alcR* and *AlcA* promoter from binSRNACatN [pALC] or *HSP18.2* promoter from pTT101 [pHSP]) (in pDONR-P2r-P3; between B2 and B3 in Figure 3C) were merged and introduced via a MultiSite LR clonase reaction into a destination vector (pKm43GW) (Karimi et al. 2005) carrying the *attR4-ccdB-attR3* Gateway cassette, yielding the expression clones *pKmAGD* (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX*), *pKmHGD* (*pHSP::GAL4SRDX;p35S:GAL4UAS::DPSRDX*), *pKmAGL* (*pALC::GAL4SRDX;p35S:GAL4UAS::Luc*) and *pKmHGL* (*pHSP::GAL4SRDX; p35S:GAL4UAS::Luc*), respectively.

The coding sequence of *DP* and *Luc*, excluding the stop codon, were amplified from each cDNA clone, and cloned into the entry vector pENTR/D-TOPO (Invitrogen). The genetic element *p35S-GAL4UAS* was amplified from *p35S:GAL4UAS-TATA-LUC* vector using the attB4-flanked primer, 5′-GGGGACTGCTTTTTTGTACAAACTTGGATCTAGTAACATAGATGACACCGC-3′ and the attB1r-flanked primer, 5′-GGGGACTGCTTTTTTGTACAAACTTGGGTCGACTGTAATTGTAAATAGTAATTG-3′, and cloned into pDONR™221-P4-P1r (Invitrogen) by BP clonase™ (Invitrogen). To combine the genetic fragments of *35S-T*, *GAL4SRDX-NOS-T, Alc* promoter system (pALC) or the heat-shock promoter (pHSP), the overlapping sequences were included in the oligonucleotides carrying the 5′ and 3′ gene-specific sequences designed for PCR amplification of the corresponding fragments. The genetic element *35S-T-NOS-T-GAL4SRDX-inducible promoter* (*Alc* promoter system [pALC] or *HSP18.2* promoter [pHSP]) was amplified from the combined PCR products using the attB2r-flanked primer, 5′-GGGGACAGCTTTCTTGTACAAAGTGGGGTCACTGGATTTTGGTTTTAGG-3′ (for *Alc* and heat-shock promoters) and attB3-flanked primers, 5′-GGGGACAACTTTGTATAATAAAGTTGAATTCCCATGGAGTCAAAGATTC-3′ (for *Alc* promoter system), and 5′-GGGGACAACTTTGTATAATAAAGTTGTTCTCTTCATTTCTCTTTCTTTC-3′ (for Heat-shock promoter), and cloned into pDONR™-221-P2r-P3 (Invitrogen) with BP clonase™ (Invitrogen).

Transformation of Pharbitis nil

The pBCKK-35S-AG-RD, pBCKK-35S-DP-RD, pKmAGD, pKmHGD, pKmAGL and pKmHGL expression vectors were transformed into *Pharbitis nil* cv. Violet using *A. tumefaciens* strain LBA4404 (*virG* N54D) as previously described (Kikuchi et al. 2005).

Table 1. Plasmids used for transgenic *Pharbitis* in this study

transgenic plants plasmid		transgene
p35S::AGSRDX	pBCKK-35S-AGRD	*p35S::AGSRDX*
p35S::DPSRDX	pBCKK-35S-DPRD	*p35S::DPSRDX*
AGD	pKmAGD	*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX*
AGL	pKmAGL	*pALC::GAL4SRDX;p35S:GAL4UAS::Luc*
AL	pKmAL	*pALC::Luc*
HGD	pKmHGD	*pHSP::GAL4SRDX;p35S:GAL4UAS::DPSRDX*
HGL	pKmHGL	*pHSP::GAL4SRDX;p35S:GAL4UAS::Luc*
HL	pKmHL	*pHSP::Luc*

pBCKK (Fujita et al. 2005); pKm: pKm43GW (Karimi et al. 2005); *pALC*: *p35S::alcR* and *pAlcA* from binSRNACatN (Caddick et al. 1998); *pHSP*: *pHSP18.2* from pTT101 (Takahashi et al. 1992).

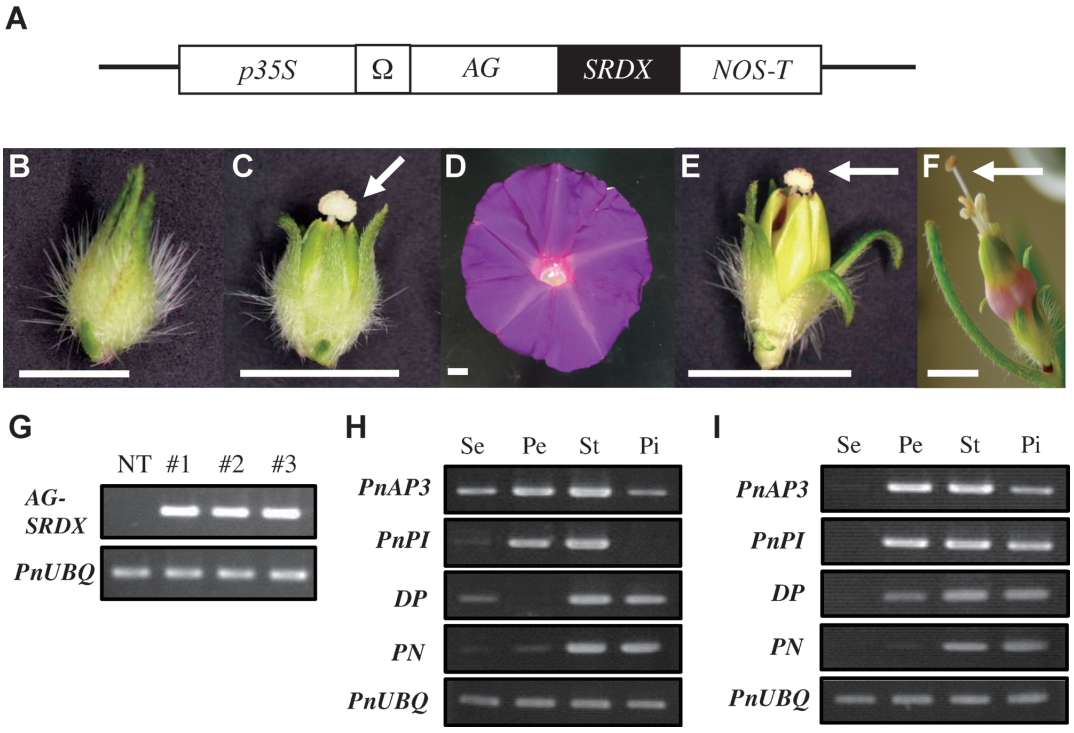

Figure 1. Functional analysis of *p35S::AGSRDX* in *Pharbitis*. (A) Schematic representation of the *p35S::AGSRDX* construct. *35Spro*, *Ω*, *SRDX*, and *NOS-T* represent the *Cauliflower mosaic virus* (CaMV) 35S promoter, the translational enhancer sequence from *Tobacco mosaic virus*, repression domain (LDLDLELRLGFA) from *Arabidopsis* SUPERMAN, and a nopaline synthase terminator, respectively. (B) A flower bud of non-transformant (NT) *Pharbitis*. (C) A flower bud of *p35S::AGSRDX* transformant. (D) A flower of NT *Pharbitis*. (E, F) The B-type mutant-like flower of *p35S::AGSRDX*. (E) Strong phenotype line of *p35S::AGSRDX*. (F) Weak phenotype line of *p35S::AGSRDX*. Arrows indicate a projecting pistil. (G–I) Expression analysis by reverse transcriptase-polymerase chain reaction (RT-PCR). *PnUBQ* indicates expression of the gene for ubiquitin, used as an internal control. (G) The expression of *AGSRDX* in NT and in three independent T_1 transgenic lines (numbers indicated above gel). Total RNA was extracted from 1.5-cm-long young leaves of each plant. (H) Expression of *PnAPETALA3 (PnAP3), PnPISTILLATA (PnPI), DP*, and *PN* in NT tissues. Total RNA was extracted from floral organs (Se: sepal, Pe: petal, St: stamen, Pi: pistil). (I) Expression of *PnAP3, PnPI, DP*, and *PN* in *AGSRDX*. All scale bars represent 8 mm.

Ethanol induction

In vitro cultured transgenic T_1 shoots were treated with absolute ethanol vapor for 30 min in the plastic container every two weeks. For plants cultured in soil, ethanol treatment was performed by spraying 0.1% ethanol onto apical shoots, or the tip of the vine of mature plants was dipped into 1% (v/v) ethanol solution.

Heat-shock induction

In vitro cultured transgenic T_1 shoots were heat-shock-treated in a plant-growth chamber at 39°C for 2 h per day under long-day (14 h light/10 h dark) conditions. For plants cultured in soil, the heat-shock treatment was performed in a plant-growth chamber under identical conditions. These plants were cultured for one month.

Results

Expression of chimeric repressors derived from AG in Pharbitis resulted in a class-B mutant-like phenotype

In *Arabidopsis*, the phenotypes of transgenic plants under the control of the CaMV 35S promoter (*p35S::AGSRDX*) were very similar to those of *ag* mutants (Mitsuda et al. 2006; Yanofsky et al. 1990). Flowers of the transgenic plants were composed of a repeated structure of sepals instead of functional stamens and carpel (Mitsuda et al. 2006). In the case of *Pharbitis p35S::AGSRDX* transgenic plants, 33% of independent T_1 transgenic lines (9 of 27 lines) were similar to the *Arabidopsis* weak *apetala3* (*ap3*) mutant (a MADS-box class-B mutant; Jack et al. 1992), which had extra short green petals and a projecting pistil (Figure 1C, E, F) compared to non-transformant (NT) flowers (Figure 1B, D). Because these transgenic lines were male sterile, we could not obtain seeds. We confirmed the expression of *AGSRDX* in all 27 *p35S::AGSRDX* transgenic lines; expression in three independent T_1 transgenic lines is shown in Figure 1G. To quantify the mRNA of *PnAP3* (an *AP3* homologue), *PnPI* (a *PISTILLATA* homologue), *DP* (an *AG* homologue) and *PN* (an *AG* homologue), which are all homologues of MADS-box genes in *Pharbitis nil*, in floral organs of NT *Pharbitis* (Figure 1H) and *p35S::AGSRDX* transgenic *Pharbitis* (Figure 1I), mRNAs from each floral whorl were isolated and analyzed by RT-PCR. Figure 1H shows *PnAP3* expression from whorl 1 (sepal: Se), whorl 2 (petal: Pe),

whorl 3 (stamen: St), and whorl 4 (pistil: Pi). Transcripts of the two B-function genes *PnPI* and *PnAP3* were detected mainly in whorl 2 and whorl 3, but very weak expression in whorl 1 was also observed (Figure 1H). An identical result was observed for the tobacco MADS-box gene *NTDEF* (Davies et al. 1996), but this expression pattern differed from those of other B-function MADS-box genes, *AP3*, *PI*, *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) (Jack et al. 1992; Sommer et al. 1990; Trobner et al. 1992). In contrast, *DP* transcripts were found mainly in whorl 3 and whorl 4. This expression pattern follows observations from other class-C MADS-box genes, *AG* and *PLE*; their transcripts were only found in floral buds, but especially in whorl 3 and whorl 4 (Bradley et al. 1993; Yanofsky et al. 1990). However, weak expression of *DP* and *PN* transcripts were also observed in whorl 1 and whorl 2 (Figure 1H).

Ectopic over-expression of *AGSRDX* resulted in repression of *PnAP3* expression in whorl 1, but enhanced *PnPI* expression in whorl 4 and *DP* expression in whorl 2 (Figure 1I). These results indicate that *AG* acts as a B-function gene in *Pharbitis*, and in some cases the function of the chimeric repressor is not conserved in other plant species.

Expression of chimeric repressors derived from DP in Pharbitis resulted in a double flower

To construct double flower of *Pharbitis*, the chimeric repressor of *DP* (*DPSRDX*), which is a homologue of *AG* in *Pharbitis*, under the control of the CaMV 35S promoter (*p35S::DPSRDX*), was introduced as a transgene in *Pharbitis* (Figure 2A). Non-transformant *Pharbitis* has one pistil and five stamens in a flower bud (Figure 2B). However, the *p35S::DPSRDX*-introduced callus was difficult to regenerate during transformation, but occasionally a perfect double flower bud showing severe developmental defects was formed, which had a phenotype similar to that of *dp* mutants (Figure 2C, D). The flower buds were composed of a repeated structure of sepals and petals instead of functional stamens and carpel (Figure 2C, D). The flower buds never developed to the flower opening stage. The *dp* mutant phenotype is similar to *agamous* (*ag*) of *Arabidopsis* (Yanofsky et al. 1990) and *plena* (*ple*) (Bradley et al. 1993) of *Antirrhinum* (Nitasaka 2003). Both genes encode class-C MADS-box genes and the gene responsible for the *dp* mutation is also thought to be a member of the class-C MADS-box gene family (Figure 2D). In *Pharbitis nil*, in the course of cloning the *DP* gene, cDNA clones containing another C-function MADS-box gene, designated *PEONY* (*PN*), were isolated (Nitasaka 2003). The amino acid sequences of DP and PN were compared with those of other C-function MADS-box genes; DP is related to *Antirrhinum majus* FAR and *Arabidopsis* AG, and PN is related to *Antirrhinum majus* PLE (Nitasaka

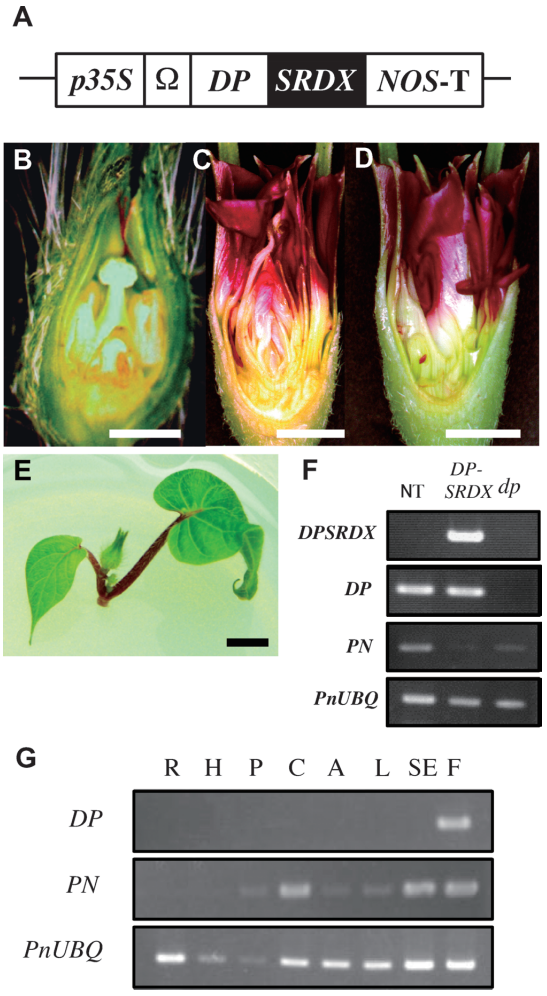

Figure 2. Functional analysis of *p35S::DPSRDX* in *Pharbitis*. (A) Schematic representation of the *p35S::DPSRDX* construct. *35Spro*, *Ω*, *SRDX*, and *NOS-T* were same as Figure 1. (B) Cross-section of an non-transformant (NT) flower bud. (C) Cross-section of a *p35S::DPSRDX* flower bud, in which the stamens and carpel are transformed into petals and a new floral bud, respectively. The inner flower has the same structure. (D) Cross-section of a *dp* mutant flower bud shows similar structure to *p35S::DPSRDX*. (E) A regenerated *p35S::DPSRDX* shoot formed on somatic embryo culture. (F, G) Expression analysis by RT-PCR. *PnUBQ* was used as an internal control. (F) Expression of *DPSRDX, DP*, and *PN* in NT, *p35S::DPSRDX,* and *dp* mutant. Total RNA was extracted from 1.5-cm-long young flower buds of each plant. (G) Expression of *DP* and *PN* in NT tissues. Total RNA was extracted from several tissues (R: root, H: hypocotyl, P: petiole, C: cotyledon, A: apical bud) from six-day-old seedlings, leaf (L) from adult plants, somatic embryo (SE) from the transformation process, and flower bud (FB) from adult plants. All scale bars represent 3 mm.

2003). The function of PN is not clear because its mutant is not known, but this gene might have similar functions to *FAR* (Nitasaka 2003). The *p35S::DPSRDX Pharbitis* showed severe growth defects and died during regeneration (Figure 2E). We compared the mRNA amount of *DPSRDX*, *DP* and *PN* in floral buds of *DPSRDX* transgenic *Pharbitis* and a *dp* mutant (Figure 2F). In transgenic *DPSRDX* plants, *PN* expression was

decreased remarkably, as in the *dp* mutant (Figure 2F). This result indicates that *PN* might be a target of *DP*, and *DPSRDX* repressed expression of *PN* (Figure 2F). To reveal the cause of the growth defects in *DPSRDX Pharbitis*, we confirmed the expression of internal *DP* and *PN* in a variety of organs of NT *Pharbitis* (Figure 2G). Although *DP* expression was observed only in floral buds, *PN* was expressed in the floral bud and various other vegetative organs. From this result, it is likely that *PN* has a function in vegetative growth in addition to C-function in floral buds, ectopic over-expression of *DPSRDX* resulted in the repression of *PN* in various other vegetative organs, and growth defects are caused by loss of *PN*.

Construction of inducible repression vectors

To avoid growth defects in *DPSRDX Pharbitis,* we used inducible promoters to control the expression of *DPSRDX*. The GAL4DB transcription factor of yeast was used, and the vectors expressing *GAL4DBSRDX* by inducible promoters were constructed, as GAL4SRDX represses the overexpression of *DPSRDX* under control of *p35S::GAL4 UAS* (Figure 3B, C). We used MultiSite Gateway technology (Invitrogen) for construction of the inducible repression vector. It was developed for the simultaneous cloning of multiple DNA fragments in a versatile format (Cheo et al. 2004; Magnani et al. 2006; Sasaki et al. 2004), and plant binary vectors have been devised for the MultiSite Gateway protocol (Karimi et al. 2005). The functional part of the MultiSite Gateway destination vector (pKm43GW) is the attR4-attR3 cassette, which can recombine with three Gateway donor vectors carrying the appropriate attL sites (Figure 3C) (Karimi et al. 2005). Combined genetic elements *p35S-GAL4UAS* in pDONR^{TM} 221-P4-P1r, *DPSRDX* and *Luc* in pENTR-D-TOPO, and *35S-T-NOS-T-GAL4SRDX-inducible promoter* (*Alc* promoter [*pALC*] or heat-shock promoter [*pHSP*)) in *pDONR-P2r-P3* were merged and introduced via a MultiSite LR clonase reaction into a destination vector (pKm43GW) (Karimi et al. 2005), yielding the expression constructs pKmAGD, pKmHGD, pKmAGL and pKmHGL respectively (Figure 3C).

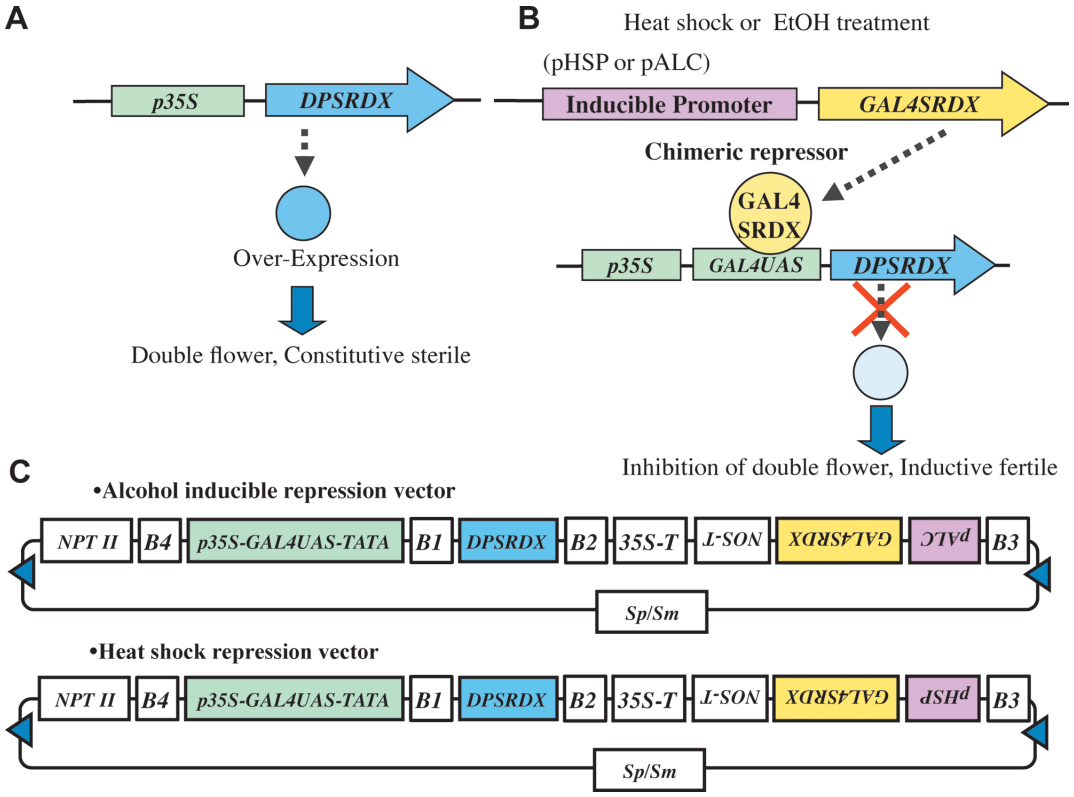

Figure 3. Construction of inducible repression vectors. (A) Schematic diagram of ectopic overexpressing *DPSRDX*, driven by CaMV 35S promoter. *DPSRDX* induces double flowers but constitutive sterility. (B) Schematic diagram of repression of overexpressing *DPSRDX* by inducible expression of *GAL4SRDX*. *GAL4SRDX* was driven by the alcohol-inducible promoter system or a heat-shock promoter at a suitable time, that allows inhibition of double flower and inductive fertility. (C) Schematic representation of the Gateway binary expression vectors for alcohol-inducible repression and heat-shock-inducible repression of ectopic overexpression *DPSRDX*. *35S*, *GAL4UAS*, *TATA*, *GAL4SRDX*, *Alc Pro*, *HSP Pro*, *35S-T*, *NOS-T*, *NPTII*, *Sp/Sm*, represent CaMV35S promoter, GAL4DB binding sequence from yeast, TATA-box the GAL4 DNA-binding domain fused SRDX (repression domain from *Arabidopsis* SUPERMAN), the alcohol-inducible promoter system (*p35S::alcR* and *pAlcA*) from *Aspergillus nidulans* (Caddick et al. 1998), HSP18.2 promoter from *Arabidopsis* (Takahashi et al. 1992), CaMV 35S terminator, a nopaline synthase terminator, kanamycin resistance selectable marker, and spectinomycin/streptomycin resistance selectable marker, respectively.

GAL4SRDX-induced AGD and HGD transgenic plants showed normal growth and double flowers

We generated transgenic *Pharbitis* plants with *AGD* (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX*) and *HGD* (*pHSP::GAL4SRDX;p35S:GAL4UAS::DPSRDX*) designed for inducible repression of *DPSRDX* or *Luc* (used as an expression control; *AL* and *HL* shown in Table 1) by GAL4SRDX. The *p35S::DPSRDX*-transformed callus were difficult to regenerate during transgenic steps, as mentioned above, and repression of *DPSRDX* during regeneration is necessary for regeneration of *DPSRDX* transgenic *Pharbitis*. Thus, we investigated the inductive condition during regeneration, because we had no definite information on a suitable inductive condition for *Pharbitis* with ethanol treatment or heat-shock treatment. At first, in order to confirm *Alc* promoter system and *HSP* promoter activity, *AL* (*pALC::Luc*) and *HL* (*pHSP::Luc*) and *in vitro* cultured T_1 transgenic callus was used for the luciferase assay. Ethanol vapor treatment is convenient for the culture dish, and Luc activity was detected by ethanol vapor treatment (data not shown). However, long ethanol vapor treatment (overnight or for 1 h) caused severe damage to transgenic callus. Thus, we chose ethanol vapor treatment for 30 min. *HSP18.2* is a heat-shock inducible natural promoter in *Arabidopsis* (Takahashi et al. 1992). *HSP18.2* promoter activity in *Pharbitis* was characterized by incubation at a variety of temperatures (33, 35, 37, 39, or 41°C) with Luc activity. In *Pharbitis*, *HSP18.2* promoter activity was hardly detected at 25, 33, 35, and 37°C. But was strongly detected at 39°C and was less reduced at 41°C (data not shown). Therefore, during regeneration, we treated *AGD* (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX*) transforming cultures with absolute ethanol vapor for 30 min in the plastic container every two weeks, and *HGD* (*pHSP::GAL4SRDX;p35S:GAL4UAS::DPSRDX*) transforming cultures with 39°C for 2 h every day, we successfully regenerated *AGD* and *HGD* transgenic *Pharbitis* plants without compromising vegetative growth. Ethanol treatment or heat-shock treatment were effective for regeneration of transformants compared with the non-treated condition. Resultant rate of regeneration in *DPSRDX* transgenic *Pharbitis* plants *(AGD* and *HGD)* was similar to that of the luciferase control transgenic plant (*AGL* and *HGL*) (data not shown).

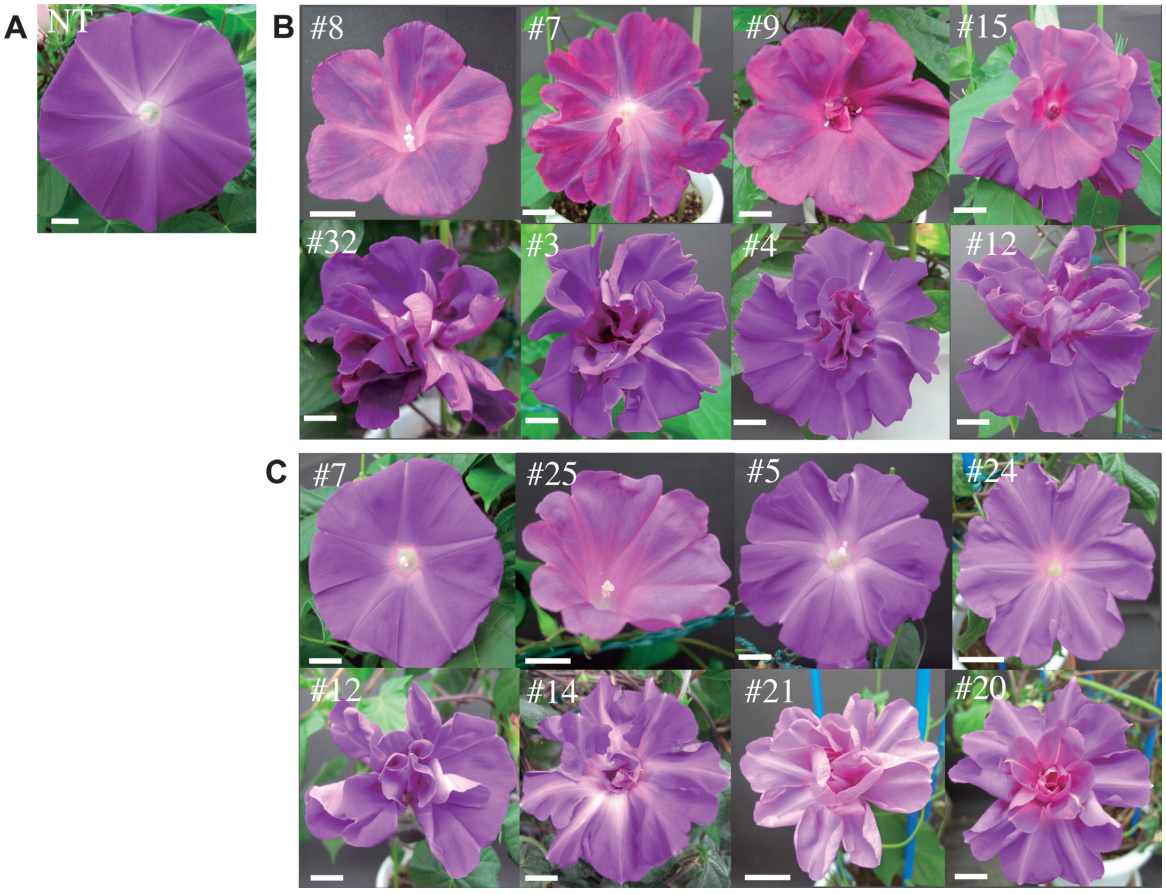

Figure 4. Appearance of *DPSRDX* induced double flowers. (A) Non-transformant (NT) flower. (B) Flower of *AGD* (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX*). The photographs depict flowers from eight independent T_1 transgenic lines. (C) Flower of *HGD* (*pHSP::GAL4SRDX;p35S:GAL4UAS::DPSRDX*). The photographs depict flowers from eight independent T_1 transgenic lines. All scale bars represent 1 cm. These photographs are arranged in the order of severity of the phenotype.

The 58 independent T_1 transgenic lines of *AGD (pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX)* and the 30 independent T_1 transgenic line of *HGD (pHSP::GAL4SRDX;p35S:GAL4UAS::DPSRDX)* were examined under normal cultivation conditions. Most of these transgenic plants bore double flowers similar to the *ag* mutant of *Arabidopsis* (Yanofsky et al. 1990) and *dp* mutant of *Pharbitis* (Nitasaka 2003) (Figure 4B: line #15, #32, #3, #4, #12; 4C: #12, #14, #21, #20). The double flower phenotype was observed in 22.4% (13 lines) of *AGD* lines and 43.3% (13 lines) of *HGD* lines. The other plants exhibited relatively normal flowers as in NT (Figure 4A) *Pharbitis* (Figure 4B: #8, #7; 4C: #7, #25, #5, #24). Detailed morphological examination revealed that some of the flowers displayed notched and frilled petals (Figure 4B: #8, #7; 4C: #7, #25, #5, #24). The same floral phenotypic patterns were observed in same transgenic lines, in which weak phenotype lines (Figure 4B: #8, #7; 4C: #7, #25, #5, #24) and strong phenotype lines (Figure 4B: #15, #32, #3, #4, #12 ; 4C: #14, #12, #21, #20) were observed. However, intermediate phenotype lines (Figure 4B: #9) were observed both with NT-like and double flowers. The number of petals was increased in extremely strong phenotype lines (Figure 4B: #3, #4, #12; 4C: #20, #21).

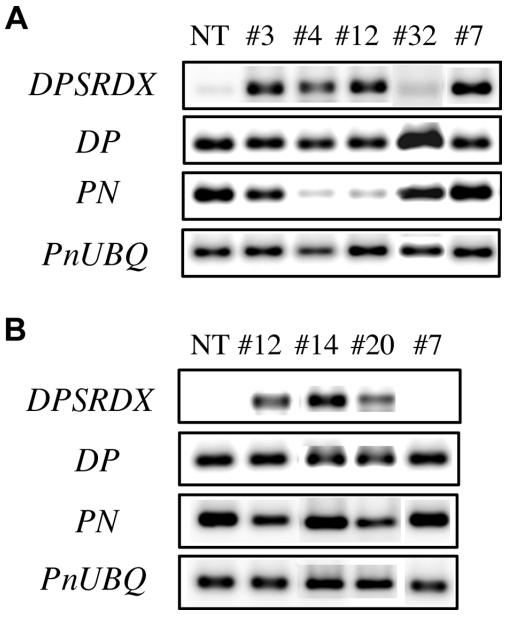

Figure 5. Expression analysis of *DPSRDX* induced double flowers. Expression of *DPSRDX*, endogenous *DUPULICATED* (*DP*), and *PEONY* (*PN*) in non-transformant (NT) and T_1 transgenic lines (numbers above gel) were determined by RT-PCR. *PnUBQ* was used as an internal control. (A) Transgeneic plants of *AGD* (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX).* (B) Transgeneic plants of *HGD* (*pHSP::GAL4SRDX;35S:GAL4UAS::DPSRDX*).

Expression level of DPSRDX does not correlate with severity of the phenotype

To observe the correlation of phenotype with *DPSRDX* and *PN* (another class C-function MADS-box gene mentioned above) transcription level, we performed RT-PCR on *AGD* (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX*) and *HGD* (*pHSP::GAL4SRDX;p35S:GAL4UAS::DPSRDX*) transgenic *Pharbitis* plants. Expression of *DPSRDX* was detected in most flower buds of the *AGD* and *HGD* transgenic *Pharbitis* plants (Figure 5A, B). However, the phenotype of *AGD* lines #3, #4, #12 and #32 was similar to that of the *ag* mutant of *Arabidopsis* (Yanofsky et al. 1990) and *dp* mutant of *Pharbitis* (Nitasaka 2003) (Figure 4B), and the *DPSRDX* expression level was variable. In particular, *AGD* line #32 showed a low expression level of *DPSRDX* (Figure 4B) but bore perfect double flowers. In contrast, *AGD* line #7 showed high expression of *DPSRDX* (Figure 5B) but bore NT-like single flowers (Figure 4B). Similar results were obtained for *HGD* lines #12, #14 and #20 (Figure 5B), which bore double flowers (Figure 4C). However, *DPSRDX* expression was not detected in *HGD* line #7 (Figure 5B), yet it bore a single-flowered phenotype similar to NT (Figure 4C). Although the transcription level of endogenous *DP* was unaffected by *DPSRDX*, *PN* expression was reduced in the flower buds of *AGD* transgenic lines #3, #4, #12, and #32, which resulted in development of double flowers (Figure 5A). *PN* expression was maintained in NT and line #7, similar to NT (Figure 5A). Identical results were obtained for *HGD* transgenic lines #12, #14, and #20 (Figure 5B). Accumulation of *PN* transcripts showed good correlation with severity of the phenotype.

Repression of overexpressing DPSRDX by alcohol treatment results in a NT-like flower phenotype

In an attempt to reveal whether GAL4SRDX was induced by ethanol treatment and could repress the overexpressing *DPSRDX* transcription, we examined the inductive conditions of *GAL4SRDX*. *AGD (pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX)* transgenic *Pharbitis* were treated by absolute ethanol vapor, 0.1% (v/v) ethanol spray, or 1% (v/v) ethanol dipping. Mature plants obtained from five *AGD*-containing *Pharbitis* lines and a NT were treated with ethanol vapor during 8 h dark period for two weeks in a 40 L plastic bag. For vaporous induction, 1.5 ml microcentrifuge tubes filled with absolute ethanol were regularly placed next to the plants in the soil. However, we could not find any phenotypic changes in *AGD* (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX*) transgenic plants in response to ethanol vapor treatment (data not shown). The apical shoot of five *AGD Pharbitis* lines and a NT (control) was directly sprayed with 0.1% (v/v) ethanol. In *AGD* transgenic line #4 the double-flowered phenotype was partially repressed (Figure 6B, E, H, K), compared to *AGD* line #3 without ethanol treatment (Figure 6A, D, G, J). However, other

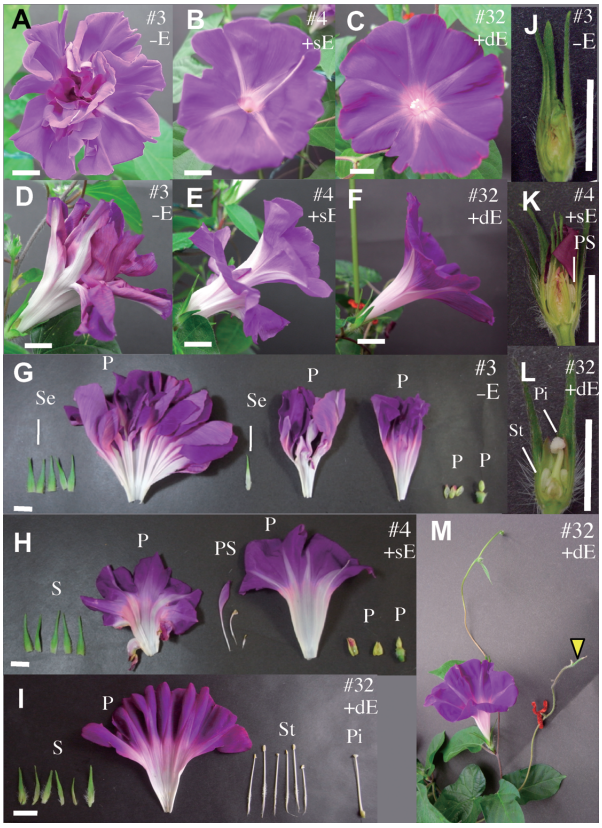

Figure 6. Repression of overexpressing *DPSRDX* by ethanol treatment resulted in a non-transformant-like flower phenotype. (A, D, G, J) *AGD (pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX)* line 3 (#3) without ethanol treatment (−E). (B, E, H, K) *AGD* line 4 (#4) with 0.1% ethanol spray (+sE). (C, F, I, L) *AGD* line 32 (# 32) with 1% ethanol dipping treatment (+dE). (A, B, C) Face view of the flower. (D, E, F) Side view of the flower. (J, K, L) Cross-section of flower buds. Se: sepal, P: petal, PS: petal-like stamen, St: stamen, Pi: pistil. (G) A mature flower of *AGD#3* dissected into organs. (H) A mature flower of *AGD#4* partially restored reproductive organs with 0.1% ethanol spray dissected into organs. (I) A mature flower of *AGD#32* restored reproductive organs with 1% ethanol dipping treatment dissected into organs. (M) *AGD#32* plant one month after ethanol treatment. Arrowhead indicates the shoot subjected to 1% ethanol dipping treatment (+dE). All scale bars represent 1 cm.

AGD lines showed no typical phenotypic changes in response to 0.1% ethanol spraying (data not shown). In addition, the shoot tip of mature plants obtained from three *AGD* lines was dipped in 1% (v/v) ethanol solution. As with the ethanol dipping treatment, *AGD* transgenic line #32 showed a flower phenotype similar to NT (Figure 6C, F, I, L). The flower of the transgenic plant was composed of six sepals, six petals, six stamens, and one pistil (Figure 6I). One month after ethanol dipping treatment, *AGD* line #32 formed a NT-like flower in an axillary bud of the same plant (Figure 6M). The apical bud of the shoot treated with ethanol dipping was killed (Figure 6M).

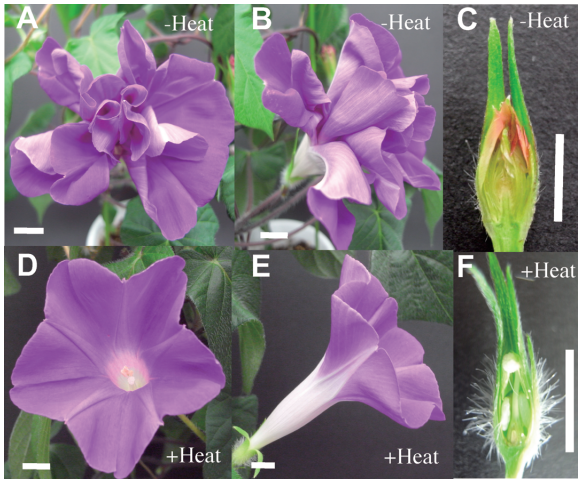

Figure 7. Repression of overexpressing *DPSRDX* by heat-shock treatment resulted in a non-transformant-like flower phenotype. All photographs represent *HGD (pHSP::GAL4SRDX; p35S:GAL4UAS::DPSRDX)* line 12 (*#12*). (A, B, C) *HGD#12* without heat-shock treatment. (D, E, F) *HGD#12* with heat-shock treatment (39°C for 2 h daily for one month). (A, D) Face view of the flower. (B, E) Side view of the flower. (C, F) Cross-section of flower buds. All scale bars represent 1 cm.

Repression of overexpressing DPSRDX by heat-shock treatment results in a NT-like flower phenotype

The *HSP18.2* promoter (*pHSP*) is strongly activated in all organs of the plant, except in seeds, in response to a shift of temperature to 35–37°C in *Arabidopsis* and 37–38°C in *Petunia* (Takahashi et al. 1992). *pHSP::Luc (HL)* transgenic *Pharbitis* were used to determine a suitable heat-shock treatment. Observation of Luc activity of *HL* transgenic *Pharbitis* at 33–41°C showed that the strongest induction of Luc activity occurred at 39°C (data not shown). Thus, *HGD#12* (*pHSP::GAL4SRDX;p35S::GAL4UAS::DPSRDX* line 12) and *HGL#26* transgenic T_1 lines were exposed to 39°C for 2 h every day for one month. At a normal cultivation temperature (25°C, LD), *HGD#12* had completely double flowers (Figure 4C, 7A–C). After heat shock treatment, *HGD#12* produced morphologically NT-like flowers (Figure 7D–F). This result indicates that heat-shock-induced GAL4SRDX could repress the double-flower phenotype by repressing *DPSRDX*.

Repression of DPSRDX by GAL4SRDX induced pollen formation

Some flowers of *AGD* (*pALC::GAL4SRDX;p35S::GAL4UAS::DPSRDX)* and *HGD* (*pHSP::GAL4SRDX; p35S::GAL4UAS::DPSRDX)* plants treated with ethanol or heat-shock produced morphologically NT-like flowers (Figures 6I, L, 7). The *PN* expression level in a flower bud of *AGD*#32 was similar to that of NT (Figure 8A). Moreover, the anther of the *AGD*#32 flower contained morphologically normal pollen grains (Figure 8B).

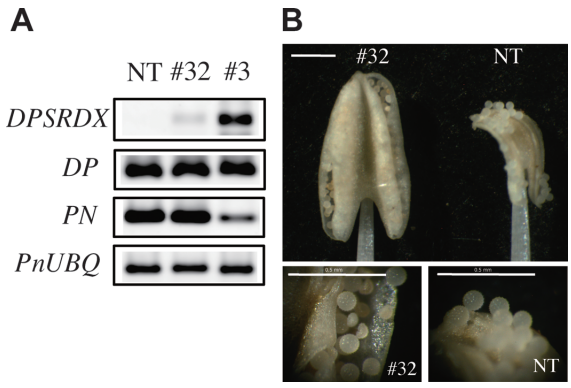

Figure 8. Repression of overexpressing *DPSRDX* by ethanol treatment restored *PN* expression and non-transformant (NT)-like flower phenotype with morphologically normal pollen grains. (A) Expression of *DPSRDX*, endogenous *DUPULICATED* (*DP*), and *PEONY* (*PN*) in young flower buds of NT, *AGD* (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX)* line 32 (#32) and *AGD* line 3 (#3) were examined using RT-PCR one month after 1% ethanol dipping treatment. (B) Comparison of mature anther of a *p35S:GAL4UAS::DPSRDX-Alc pro::GAL4SRDX* (*AGD#32*) plant and NT. No pollen grains were released in *AGD#32*. All scale bars represent 0.5 mm.

However, ethanol treatment of *AGD*#32 reduced anther dehiscence compared with NT (Figure 8B), and we could not prove that these recovered flowers were fertile.

Discussion

The CRES-T system is a powerful tool for genetic engineering of horticultural plants, including *Torenia fournieri, Chrysanthemum morifolium, Gentiana triflora*×*G. scabra, Cyclamen persicum, Eustoma grandiflorum,* and *Pharbitis nil* (Mitsuda et al. 2008). However, there are some exceptions to CRES-T system**.** In *Arabidopsis*, *AGSRDX* transgenic plants under the control of the CaMV 35S promoter (*p35S::AGSRDX*) are very similar to those of *ag* mutants (Mitsuda et al. 2006; Yanofsky et al. 1990). Flowers of the transgenic plants were composed of a repeated structure of sepals instead of functional stamens and a carpel (Mitsuda et al. 2006). In the case of *p35S::AGSRDX* transgenic *Pharbitis* plants, independent T_1 transgenic lines were similar to the *Arabidopsis* weak *ap3* mutant (MADS-box class-B mutant; Jack et al. 1992), which had extra short green petals and a projecting pistil (Figure 1C, E, F) compared to NT flowers (Figure 1B, D). These results indicate that *AG* act as a B-function gene in *Pharbitis*. Although growth defects were observed in *p35S::DPSRDX* transgenic *Pharbitis*, occasionally a perfect double flower bud developed. In addition, we also observed that *p35S::DPSRDX* expressed in *Arabidopsis* showed a B-function mutant-like phenotype (data not shown). These results indicated that the functions of *AGSRDX* and *DPSRDX* were converted in *Pharbitis* and *Arabidopsis*. Expression analysis showed that ectopic overexpression of *AGSRDX* resulted in repression of *PnAP3* expression in whorl 1 (sepal: Se), but also enhancement of *PnPI* expression in whorl 4 (pistil: Pi) and *DP* expression in whorl 2 (petal: Pe) (Figure 1I). Although we have no definite information on molecular mechanisms of *p35S::AGSRDX* expression in *Pharbitis*, it seems reasonable to suppose that these alterations of gene expression might be involved in the B-function mutant-like phenotype of *p35S::AGSRDX* in *Pharbitis*. These results indicated that the chimeric repressor of *Arabidopsis* is not an 'all-round player' and, in some cases, each plant species requires its own chimeric repressor generated from its own transcription factors.

One other factor is important for utilization of the CRES-T system. Growth defects and decreased fertility are sometimes caused by ectopic overexpression of a chimeric repressor. In this study, we observed growth defects in *p35S::DPSRDX* transgenic *Pharbitis* during *in vitro* culture for regeneration (Figure 2E), and flower buds of *p35S::DPSRDX* transgenic *Pharbitis*, similar to the *agamous* (*ag*) mutant (Yanofsky et al. 1990) of *Arabidopsis* and *plena* (*ple*) (Bradley et al. 1993) of *Antirrhinum*. The *dupulicated* (*dp*) mutant (Nitasaka 2003) of *Pharbitis* exhibits a complete transformation of reproductive organs to perianth organs, which results in male- and female-sterile flowers (Figure 2C). Such a drastic floral phenotype renders it difficult or impossible to self-pollinate lines and cross them with other lines for breeding. To solve this problem, there are three approaches: the first is to control the overexpressing transgene using a GAL4SRDX/UAS system driven by inducible promoters; the second is to control expression of the transgene by inducible promoters; and the third is to control expression of the transgene by organ-specific or stage-specific promoters. A GAL4SRDX/UAS system driven by inducible promoters may be advantageous for application in the genetic engineering of floricultural plants, because such a system allows for the control of transgene expression as the need arises; for instance, genetically modified (GM) floricultural potted plants always show the drastic floral phenotype without any treatment whenever they are cultivated by consumers. However, if the suitable natural promoter for transgene expression in the flower is known, we would be a step closer to application of the system for GM floricultural plants. In this case, two further points need to be resolved: improvement of the culture system for vegetative propagation, and selection of a suitable organ-specific or stage-specific promoter. Although we are trying to utilize the floral meristem-specific *PnAP1 (Pharbitis nil APETALA1)* promoter at present, we could not obtain transgenic *Pharbitis* with perfect double flowers using *DPSRDX* (data not shown). In addition, we attempted to propagate plants vegetatively by cuttings. A different approach such as tissue culture is needed for

vegetative propagation.

Therefore, we developed the GAL4SRDX/UAS system driven by inducible promoters (alcohol-inducible and heat-shock promoters) as a new gene-silencing technology for repression of overexpressing transgenes. The EAR-like motif repression domain of SUPERMAN (SRDX) was fused downstream of the coding sequence for the DNA-binding domain of the GAL4 protein (GAL4DB), under the control of the enhancer sequence of the CaMV 35S promoter (p35S-GAL4DB-RD) (Hiratsu et al. 2004). Transient assay in *Arabidopsis* demonstrated that GAL4SRDX repressed expression of the luciferase reporter gene, which was driven by the enhancer sequence of the CaMV 35S promoter and five copies of the GAL4 binding site (Hiratsu et al. 2004). We used this GAL4SRDX/UAS system for the construction of a plant binary vector to control the overexpressing transgene.

We used MultiSite Gateway technology (Invitrogen) and constructed the expression clones pKmAGD (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX*), pKmHGD (*pHSP::GAL4SRDX;p35S:GAL4UAS::DPSRDX*), and the control expression constructs pKmAGL and pKmHGL, which carry the *Luc* reporter gene. We produced transgenic *Pharbitis* plants with pKmAGD, pKmHGD, pKmAGL and pKmHGL. In these transgenic plants, *DPSRDX* or *Luc* mRNA could be overexpressed constitutively by the CaMV 35S promoter without alcohol or heat-shock treatment. Although *p35S::DPSRDX* transgenic plants did not survive during regeneration, GAL4SRDX induced by alcohol and heat-shock treatment made it possible to produce efficiently *DPSRDX* transgenic plants. Furthermore, *AGD* (*pALC::GAL4SRDX;p35S:GAL4UAS::DPSRDX*) and *HGD* (*pHSP::GAL4SRDX;p35S:GAL4UAS::DPSRDX*) transgenic lines showed a variety of variable flower phenotypes, including double, frilled, and notched-petal flowers (Figure 4). The 58 independent T_1 transgenic lines of *AGD* and the 30 independent T_1 transgenic line of *HGD* were examined under normal cultivation conditions; 22.4% (13 lines) of *AGD* and 43.3% (13 lines) of *HGD* transgenic lines exhibited a repeated structure of sepals and petals instead of functional stamens and carpel, which is a phenotype similar to that of the *ag* mutant of *Arabidopsis* (Yanofsky et al. 1990) and *dp* mutant of *Pharbitis* (Nitasaka 2003) (Figure 4B: #15, #32, #3, #4, #12; 4C: #12, #14, #20, #21). Although the *DPSRDX* expression level differed among the lines, the phenotype and *DP* transcript level were not correlated (Figure 5). In contrast, the *PN* transcript level showed good correlation with phenotype severity. This indicates that the male- and female-sterile phenotype of the transgenic plants might be unaffected by *DPSRDX* transcription level, but rather caused by accumulation or activation of DPSRDX, and a candidate target for DPSRDX might be *PN*. We have no definite information on the chimeric repressor at the protein level, because it is difficult to make specific antibodies for detection of the chimeric repressor, owing to the functional domain of the chimeric repressor (SRDX) consisting of a short amino acid sequence (LDLDLELRLGFA).

We demonstrated control of expression of the transgene (*DPSRDX*) via a conditional promoter (either an alcohol-inducible or heat-shock-inducible promoter) and GAL4SRDX/UAS system. Transgenic plants that expressed *DPSRDX* were male and female sterile lacking both male and female organs. To provide a basis for production of hybrid plants in breeding, expression of the transgene, which is related to fertility, must be repressed to recover fertility. In this study, we succeeded in recovering NT-like single flowers with GAL4SRDX induced by alcohol or heat-shock treatment from a part of double-flowered *DPSRDX*-expressing transgenic plants (AGD#32, Figure 6C, F, I, L) and HGD#12, Figure 7D–F). The mRNA accumulation of DPSRDX in AGD#32 and HGD#12 transgenic T1 lines were lower than other double-flowered *DPSRDX*-expressing transgenic lines (Figure 5A, B). Recovering NT-like single flowers of these lines is probably due to low accumulation of *DPSRDX* mRNA, and it is easy to stop the *DPSRDX* expression by GAL4SRDX. In order to make practical use of this inducible repression system in breeding, we need to establish suitable inductive conditions for alcohol and heat-shock treatment. In this regard, we are studying Luc activity in different inducible conditions using *AGL*, *HGL, AL* and *HL* transgenic lines. CRES-T is a very effective method for controlling the plant floral phenotype because chimeric repressors can function dominantly to overcome the activity of functionally redundant transcription factors.

It is expected that utilization of the GAL4SRDX/UAS system driven by inducible promoters (alcohol-inducible and heat-shock promoters) could be applied for genetic engineering of horticultural plants. For example, in *Arabidopsis thaliana*, several families of transcription factors promote adaxial or abaxial cell identity in leaves and other lateral organs. The adaxial-promoting *PHABULOSA (PHB), PHAVOLUTA*, and *REVOLUTA* Class II homeodomain/leucine zipper transcription factors (*HD-ZIPIIIs*) are expressed in the adaxial domain of lateral organs and the central region of the shoot apical meristem. By contrast, members of the abaxial-promoting *KANADI* family (*KAN1-3*) are expressed in the abaxial domain of organs in a pattern that is complementary to the *HD-ZIPIIIs* (Emery et al. 2003; Eshed et al. 2001; Kerstetter et al. 2001; McConnell et al. 2001; Otsuga et al. 2001). *p35S::PHBSRDX, p35S::KAN1SRDX, p35S::KAN2SRDX* transgenic *Pharbitis* each displayed a valuable floral phenotype (Ono et al. unpublished). However, these transgenic

Pharbitis also showed a male- and female-sterile phenotype, and thus it is difficult to utilize the plants for breeding. In the case of *p35S::PHBSRDX, p35S::KAN1SRDX*, and *p35S::KAN2SRDX* transgenic *Pharbitis,* an inducible *GAL4SRDX* gene repression system driven by inducible promoters (alcohol-inducible and heat-shock promoters) might be effective for repression of overexpressing transgenes. Furthermore, establishment of the GAL4SRDX/UAS system allows more profound functional gene analysis by directed expression of the transgene in other species in the future.

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