A new location to split Cre recombinase for protein fragment complementation

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Summary

We have previously described a recombinase-mediated gene stacking system in which the Cre recombinase is used to remove lox-site flanked DNA no longer needed after each round of Bxb1 integrase-mediated site-specific integration. The Cre recombinase can be conveniently introduced by hybridization with a cre-expressing plant. However, maintaining an efficient cre-expressing line over many generations can be a problem, as high production of this DNA-binding protein might interfere with normal chromosome activities. To counter this selection against high Cre activity, we considered a split-cre approach, in which Cre activity is reconstituted after separate parts of Cre are brought into the same genome by hybridization. To insure that the recombinase-mediated gene stacking system retains its freedom to operate, we tested for new locations to split Cre into complementing fragments. In this study, we describe testing four new locations for splitting the Cre recombinase for protein fragment complementation and show that the two fragments of Cre split between Lys244 and Asn245 can reconstitute activity that is comparable to that of wild-type Cre.

Introduction

A long-term aim of this laboratory has been to develop site-specific gene stacking to ease the introgression of transgenes from transformable lines to elite field cultivars (Hou et al., 2014). Each step of the reiterated gene stacking scheme involves using the Bxb1 integrase to direct the insertion of new DNA into a genomic Bxb1 attachment site (attP or attB site), followed by introduction of the 343aa Cre recombinase to delete the 34 bp lox-site flanked DNA no longer needed after site-specific integration. While the Bxb1 integrase can be transiently introduced along with the integrating DNA, the Cre recombinase is most conveniently introduced from hybridization to a cre-expression line. Maintaining an efficient cre-expressing line over many generations, however, could potentially be a problem as high expression of cre in petunia and tomato has been associated with crinkled leaves and/or reduced fertility (Que et al., 1998; Cappoose et al., 2003). As recombinases are DNA-binding proteins, it remains possible that high production of these proteins could interfere with normal chromosome activities. To insure against this possibility, we considered a split-cre approach as shown in Figure 1, in which the integrating vector brings along a portion of cre, and after integration, the rest of cre can be subsequently introduced from hybridization.

Figure 2a lists the various reports on splitting Cre into two peptides. The first approach is based on protein fragment complementation, where Cre activity is reconstituted from separate peptides. Casanova et al. (2003) first reported testing various pairs of nonfunctional N-terminal and C-terminal Cre fragments at break points between aa160 and aa203 and found one pair, aa1-196/aa182-343, that reconstituted activity in a transient assay in CV1-5B monkey cells, but at only 33% efficiency compared to wild-type Cre. Seidi et al. (2007) also reported success with a split-Cre pair at the same region, aa1-194 and aa180-343, and with up to 68% excision efficiency in a transient assay in COS-7 monkey cells. More recently, Wen et al. (2014) reconstructed Cre from the pair aa1-59/aa60-343 with and without the SV40 NLS and in an excision assay of A. rhizogenes-mediated transformation of tobacco root hair, up to 67% of wild-type Cre efficiency was reported.

Uses of heterodimerizing peptides to facilitate the reassembly of split-Cre complementing peptides have also been tested. Jullien et al. (2003) reported reconstituting the pairs aa19-59 and aa60-343 and aa19-104/106-343, in which each N-terminal fragment (N-fragment) was fused to FKBP12 (FK506 binding protein) while each C-terminal fragment (C-fragment) was fused to FRB (binding domain of the FKBP12-rapamycin-associated protein). Upon addition of rapamycin that induces FKBP12/FRB interaction, Cre activity was reconstituted in both a transient and a stable excision assay in a Rat2/CALNLZ cell line. In the transient assay, the aa19-104/106-343 pair showed higher recombination activity, whereas in the stable excision assay, the aa19-59/aa60-343 pair was more efficient. In a study by Xu et al. (2007), each N- and C-fragment of the aa1-190/aa191-343 pair was fused to antiparallel leucine zippers. In transient and transgenic mouse cells, excision efficiencies were about 30% compared to wild-type Cre. Maruo et al. (2008) tested parallel binding modules and reported that a Zip(+)(−) synthetic leucine zipper based on the vitellogenin-binding protein from a chicken b-ZIP family was most efficient among three heterodimerizing peptide pairs of aa19-59/aa60-343 tested in Cos-7 monkey cells and in immature mouse neurons, but its efficiency was not compared to wild-type Cre. Seidi et al. (2009) used the AP-1 transcription regulatory proteins blun and bFos in a transient monkey COS7 cell assay that showed...
Figure 1 Reconstitution of Cre activity for removing DNA no longer needed after site-specific integration. Scheme for recombinase-mediated gene stacking shows the chromosomal target construct with an attP site (a) recombining with an integrating vector (b) that also includes a C-Cre peptide encoding gene. After site-specific integration from attP recombination with the M2 distal attB site to yield the configuration shown in (c), the construct encoding N-Cre peptide (d) is introduced by genetic hybridization to yield the expected configuration shown in (e). M1, M2 and M3 represent marker genes, and G1 and G2 represent trait genes. Symbols of recombination sites defined in legend. Note that the M3-N-cre fragment is also excised in the F1 (e).

blun-Cre (aa-1-194) and bFos-Cre (aa-184-343) yielded 23% deletion efficiency compared to wild-type Cre. Finally, Hirrlinger et al. (2009) reported using the coiled coil domain of the yeast transcriptional activator GCN4 on the split-Cre pair aa19-59/aa60-343 in transgenic mice and found 26% deletion efficiency.

In contrast to reconstituting activity from protein fragment complementation, split inteins can reconstitute a whole Cre protein by splicing together separate protein peptides. The first report of using split inteins to reconstitute Cre was described in a patent issued to Dupont (Yadav and Yang, 2007). The split inteins from *Cyanobacterium synechocystis* DnaE reconstructed Cre from the pair of aa1-155/aa156-343 fragments. Using this same approach, Wang et al. (2012) reconstructed Cre peptides aa19-59 and aa60-343 and reported high Cre activity in mouse brain tissue. Han et al. (2013) tested the same *Ssp DnaE* split intein on the Cre pair aa1-190/aa191-343 and obtained excision activity in tobacco. Based on the number of leaf explants resistant to the herbicide Basta, up to 77% of wild-type Cre efficiency was report. More recently, Ge et al. (2016) tested five Cre pairs (aa1-128/aa129-343, aa1-153/aa154-343, aa1-190/aa191-343, aa1-232/aa233-343 and aa1-281/aa282-343) in a transient tobacco explant assay and concluded that the best pair for split-intein reconstruction of Cre was aa1-232/aa233-343. In transgenic *Arabidopsis*, this split-intein reconstruction of Cre was reported to be near 100% efficiency in F1 hybrids.

In terms of location flexibility to split Cre, as well as restoration of Cre activity, the split-intein approach to reconstitute a whole Cre protein would seem preferable to complementation activity with separated peptides. However, as an important aim for developing our ‘open-source’ gene stacking system was to permit the freedom to operate for commercial crop improvement (Ow, 2016), we had to consider the intellectual property constraints of the Dupont intein-Cre patent that is in effect until 2023 (Yadav and Yang, 2007). With the Cre fragment complementation approach, there have been two patent applications filed based on the work of Jullien et al. (2003) and Xu et al. (2007) that claim the use of specific heterodimerizing peptides along with specified split-Cre locations, namely after aa 59, 104 and 190 (Gu and Xu, 2009; Herman and Jullien, 2004). As issued patents of these were not found, it seems likely that these applications have been abandoned. Surprisingly, however, despite prior art on splitting Cre after aa 59, a Chinese patent was issued recently based on the work of Wen et al. (2014) that claimed splitting Cre after aa 59 (Gao et al., 2015). Given the freedom-to-operate uncertainty, we thought it would be preferably to create our own version of Cre fragment complementation.

As indicated in Figure 2a, previous reports have led to relatively few break points that can yield Cre fragment complementation, and they are all within loops that connect alpha-helical segments or beta-sheets. Four Cre pairs were split at the region between aa180-196 within loops linking beta-sheets 1, 2 and 3; another four pairs split after aa59 between alpha-helixes B and C, and a single report of splitting at aa104-106 between alpha-helixes D and E (Figure 2b). Likewise, we thought it might be possible to split the protein within the loop connecting beta-sheets 4 and 5. Additionally, because it was convenient to generate C-fragments that begin with ATG start codon, we also included splitting Cre after Asn96, Val116 and Asp298 (Figure 2b), even though each of them disrupts an alpha-helical segment. Here, we report that the break point between Lys244 and Asn245 indeed yields two peptides that can undergo Cre fragment complementation activity in *E. coli* and in transgenic *Arabidopsis*. In *Arabidopsis*, the recombination efficiency by hybrid-reconstituted Cre was comparable to that of wild-type Cre and was obtained without the need of heterodimerizing peptides.

**Results**

**Testing new split-Cre pairs for protein fragment complementation in *E. coli***

To examine whether the 343 amino acid Cre recombinase can be functionally reconstituted when split at regions other than the three previously reported, we chose to split Cre after Asn96 (Figure 2a, location 1), Val116 (location 2), Lys244 (location 3) and Asp298 (location 4). Locations 3 and 4 are within the C-terminal end of the protein that have not been previously tested (Figure 2a). Locations 1, 2 and 4 were also chosen because each could be split such that the C-terminal fragment begins with
an ATG start codon, bypassing the need to add another amino acid to the C-terminal peptide, which was the case for splitting at location 3 (after Lys244). Based on the Cre X-ray crystal structure (Guo et al., 1997) of five alpha-helical segments (A-E) connected by short loops in its amino-terminal domain and nine alpha-helical segments (F-N) along with five beta-sheets (1–5) in its carboxy-terminal domain (Figure 2b), our location 1 cut is located in alpha-helix D between Asn96 and Met97 resulting in peptide 1N (1–96 aa) and 1C (97–343 aa) fragments. Location 2 is in alpha-helix E between Val116 and Met 117 yielding 2N (1–116 aa) and 2C (117–343 aa) fragments. Location 3 cut is between the 4th and 5th beta-sheets after Lys244 resulting in 3N (1–244 aa) and 3C (245–343 aa) fragments, and location 4 is in the K alpha-helix between Asp298 and Met299 to yield 4N (1–298 aa) and 4C (299–343 aa) peptides (Figure 2b). By mixing N-terminal and C-terminal fragments of the different pairs, a total of 10 pairs, including six overlapping ones, were tested for Cre fragment complementation in E. coli (Figure 2b). For use as a positive control, we also generated a fifth split-Cre pair of aa1-192/aa181-343 because this region has been reported to work by four prior studies (ATG start codon added before aa181; referred to as 5N/5C).

Each N-terminal and C-terminal gene fragment was expressed in pETDuet and pRSFDuet vectors to produce the ampicillin-resistant pETDuet-N and kanamycin-resistant pRSFDuet-C series of constructs, respectively (Figure 3a). A third plasmid that is chloramphenicol resistant, pACYCDuet-inv served as a reporter for site-specific recombination. This construct has a hygromycin coding region (hpt) flanked by oppositely oriented lox sites such that site-specific recombination inverts the hpt fragment to produce a predicted molecular structure detectable by PCR. For a positive control, the full-length cre gene was expressed from pETDuet-Cre. All three plasmid types (pETDuet, pRSFDuet and pACYCDuet) are compatible for co-propagation in the recA1 homologous recombination impaired E. coli strain DH5α (Grant et al., 1990).

In the absence of site-specific inversion, PCR products of pACYCDuet-inv can be detected using primer pairs 1 + 2 and 3 + 4, but not from primer pairs 1 + 3 and 2 + 4 (Figure 3a). If inversion takes place, which is a reversible event, PCR products can also be detected using primer pairs 1 + 3 and 2 + 4. As expected from the controls, 90% of the colonies derived from pACYCDuet-inv showed site-specific inversion when cotransformed by pETDuet-Cre, whereas none was found when cotransformed with the pETDuet empty vector. For the cotransformation of N and C pairs, which requires the co-introduction of three plasmids instead of two for the controls, the 5N/5C (aa1-192/aa181-343) pair indeed showed site-specific inversion in 67% of the colonies. This confirms earlier reports of reconstitution of Cre activity when split at the region between aa181-196.

Figure 2 Linear depiction (a) and X-ray crystal structure (b) of Cre recombinase. (a) Split-Cre fragments that can reconstitute Cre activity through protein fragment complementation shown with grey bars. Split-Cre fragments for reconstruction of whole Cre protein through protein splicing shown with blue bars. Numbers refer to the aa residues. Fusion to the following: NLS = nuclear localization sequence; LZ = leucine zipper; SLZ = synthetic leucine zipper; bJun and bFos = AP-1 transcription regulatory proteins; CCD = coiled coiled domain of the yeast transcriptional activator GCN4; FKBP12 = FK506 binding protein; FRB = binding domain of the FKBP12-rapamycin-associated protein. Intein– from Cyanobacterium synechocystis Spp DnaE. ATG added to N-terminus of all C-Cre fragments shown. (b) X-ray crystal structure from Guo et al. (1997) showing the 14 alpha-helical segments (A-N) and five beta-sheets (1–5). Arrows point to relevant aa residues. Blue lettering, previously tested locations; red lettering, the four new locations tested in this study.
For the pairs corresponding to locations 1, 2, 3, 4, only 3N/3C (aa1-244/aa245-343) scored positive for reconstituted Cre activity. This may not be surprising as locations 1, 2 and 4 disrupt alpha-helical segments. However, among the six overlapping pairings of N- and C-fragments, reconstituted Cre activity was found in 2N/1C (aa1-116/aa96-343). It is interesting to note that 2N includes alpha-helix D, while 1C includes alpha-helix E, and the overlap provides a full set of alpha-helical segments. However, the same logic does not hold for the other overlapping pairs, 3N/1C, 3N/2C, 4N/1C, 4N/2C and 4N/3C. Given the positive results of 3N/3C and 2N/1C, we advanced them to the next step of testing for Cre fragment complementation in plant cells.

Reconstitution of Cre activity in plant transient assays
For scoring site-specific recombination in plant cells, a reporter construct was initially used in which the gus (β-glucuronidase) coding region is prevented from expression by an upstream blocking DNA that is itself flanked by a set of directly oriented lox sites (pMR1, Figure 3a). The hpt coding region along with a nos terminator (polyA) region was used as the blocking DNA. Cre-mediated excision of the blocking DNA permits gus expression from the CaMV 35S RNA promoter (P35S) in one of the excision products, and β-glucuronidase activity can be visually detected by blue staining. N- and C-fragments, and the full-length cre gene, were also expressed in separate constructs transcribed by P35S (pMM23 series constructs, Figure 3a). Microparticle bombardment (biolistics) was used to deliver the N- and C-fragment-expressing constructs along with the reporter construct into onion epidermal cells. Blue spots were visible 12 hours after bombardment with both the 3N/3C and 2N/1C pairs, with 3N/3C nearly as efficient for recombination as the wild-type Cre control. Surprisingly, our positive split-Cre control pair 5N/5C was less effective. To examine whether heterodimerizing peptides could further enhance their reconstitution of activity, leucine zippers of transcription factors were added to both 3N/3C and 2N/1C, with Myc to the C-terminus of the N-Cre fragment and Max to the N-terminus of the C-
Cre fragment. However, neither leucine zipper-containing pairs were found to be as effective as the nonleucine zipper progenitors.

Although the visual assay concluded that the 3N/3C and 2N/1C pairs work, a second test was conducted in Arabidopsis protoplasts to assess their relative recombination proficiency. Several modifications were made to the excision assay. First, for ease in quantitation, we switched to using luc (firefly luciferase gene) as a reporter. Second, in preparation of further testing in transgenic plants in which recombination activity in the nucleus is scored, an SV40 NLS was added to the N-terminus of the N-fragment and to the C-terminus of the C-fragment. Third, to reduce the cotransfer of constructs from 3 to 2, the corresponding C-cre fragment of each pair followed by two rice ubi1 terminators (Tubi1) was used as the blocking DNA (pCambia-C-luc, Figure 4a). For the control experiment with the wild-type Cre, the luc reporter construct uses as the blocking DNA a bar (basta resistance) gene instead of C-cre (construct not shown).

Twenty hours after polyethylene glycol (PEG)-mediated uptake of DNA into Arabidopsis protoplasts, the cells were assayed for luc expression as an indication of site-specific excision of the blocking DNA. Compared to wild-type Cre, 2N/1C and 5N/5C showed 27% and 69% activity, respectively. However, 3N/3C was about 2.5-fold more active than wild-type Cre (Figure 4b). As in the β-glucuronidase assay in onion cells, the leucine zipper additions to 3N/3C and 2N/1C reduced rather than increased recombination.

Cre-fragment complementation in transgenic Arabidopsis

Having shown that the NLS-linked 3N/3C pair is the most active in the two transient assays, we sought to test for recombination of chromosomal DNA. The constructs pCambia-3N and pCambia-3C-luc were separately transformed into Arabidopsis. Putative single-copy lines were screened in T1 plants by qPCR, followed by testing for a 3:1 segregation of the transgene among T2 seedlings. These two assays narrowed the number of putative single-copy lines to 6 for pCambia-3N and 12 for pCambia-3C-luc. From these 18 lines, a total of 27 crosses were conducted between pCambia-3N and pCambia-3C-luc plants. The F1 plants from these crosses were PCR genotyped to determine the plants that harboured deletion of the C-cre-\textsuperscript{p35S}-\textsuperscript{p35S}-luc fragment, which would fuse \textsuperscript{p35S} to luc. PCR primer pair e+f (Figure 4a) should...
yield either a 2.3-kb product prior to excision or a 0.7-kb band after excision. As shown in Figure 4c, detection of the 0.7-kb PCR product was found in a representative sampling of F1 plants. Moreover, this correlated with expression of luc. All 27 independent crosses yielded progeny that showed reconstitution of Cre activity as defined by the PCR and luciferase assays. Both pCambia-3N and pCambia-3C-luc constructs, as determined by PCR pairs a + b and c + d, respectively, were detected in the F1 plants that showed excision. In contrast, plants that failed to show excision lacked either pCambia-3N or pCambia-3C-luc DNA in their genomes (Figure 4c).

Ten representative recombination-junction PCR products from the progenies of the 27 independent crosses were sequenced, and all showed precise site-specific recombination. Additionally, Southern blots were conducted on the F1 and F1 backcrossed (BC1) plants to rule out the possibility that the PCR data had arisen from PCR-mediated recombination of templates with a common lax site. EcoRI and BstEII cleave just inside the border of the transgene and should yield a luc-hybridization band of ~4.3 kb before recombination and ~2.5 kb after recombination (Figure 4a). As shown in a Southern blot (Figure 4d) on plants previously found by PCR to have undergone excision, some lines (lines 9, 18) show multiple hybridizing bands to the luc probe p1. This suggests that their genomes harbour other imperfectly integrated T-DNA copies (Figure 4d). In some other lines (lines 10, 12), a faint before excision band (~4.3 kb) was also detected in addition to the excision-specific product, suggesting that the excision was not complete. However, in the remaining four lines (lines 2, 5, 15 and 33), only a single ~2.5-kb excision-specific band was detected to indicate a homogenous recombination event. To rule out the possibility that the internal EcoRI-BstEII fragment may represent the excision of more than a single pCambia-C-luc derived T-DNA, the genomic DNA of lines 2, 5, 15 and 33 were cleaved with SphI and hybridized to luc probe p2. As SphI cleaves only once within the T-DNA, p2 should detect the T-DNA left border band. As shown in Figure 4e, a single border band >1.6 kb was indeed detected in these four lines, confirming that each line harbours only a single T-DNA copy. However, it was surprising to find that lines 15 and 33 each showed a band of approximate the same size, thereby raising the possibility that they may be clonal due to a mix-up of the seeds. If that were the case, then we can only state that three of seven lines (rather than four of eight lines) examined showed efficient deletion of a single-copy T-DNA.

Testing transmission of the excision event

Given that the data show efficient recombination in somatic cells, we sought to test whether the recombination event could transmit to the next generation. For the F1 plants that showed excision, the N-fragment locus (3N) and C-fragment locus (3C) should be hemizygous (NnCc, lower case lettering indicating the absence of transgene) and four gamete types should be possible: NC, Nc, nCc and nc. In an outcross to wild-type (nc), this would yield progeny with the following genotypes: NnCc, NnCc, nNcC and nncC. If recombination took place within germ-line cells, then we would expect to recover plants with the nNcC genotype but with an excision event at the C locus. Progenies from 16 of 27 F1 to wild-type backcrosses were randomly chosen for PCR detection of the excision event (~0.7 kb e + f PCR product) as well as for the 3N and 3C fragments (a + b and c + d PCR products, respectively). However, among 50 progeny from each backcross that were positive for the excision event, all harboured the 3N locus (data not shown). Hence, we conclude that the plants that harbour the excision configuration are genotypically NnCc and that the co-assortment of both the 3N and 3C loci permitted another round of reconstituted Cre-mediated site-specific recombination in the somatic cells of the backcross progenies.

Discussion

In our recombinase-mediated gene stacking scheme, Cre is used to delete DNA no longer needed after each site-specific integrations step, such as selectable markers and plasmid backbones. Cre-mediated excision of lax-flanked DNA is generally efficient, but depends on the cre-expression donor line used in the genetic cross. In recent work, we found that a cre line used previously was less efficient than we had expected, resulting in all F1 plants chimeric with a mixture of cells with or without recombination (Hou et al., 2014). We had suspected that the cre line might have become less effective over time. Although gene silencing over the generations could have occurred with any transgene, there is some reason to suspect that high expression of a DNA-binding recombinase could interfere with normal chromosome activities. Hence, we sought to create cre lines with controllable cre DNA activity. Chemical induction (Joubes et al., 2004; Zuo et al., 2001) and heat induction of cre expression have been described (Cuellar et al., 2006; Hoff et al., 2001; Khatri et al., 2011; Wang et al., 2005; Zhang et al., 2003). However, we favour using a split-cre system as it should be possible, as illustrated in Figure 1d, to flank the M3-N-cre DNA with directly oriented lax sites. With an inducible cre system, keeping a cre line over the generations might be difficult, as any leaky expression of cre would excise itself.

What prompted us to find a new split-Cre pair instead of using those described in the literature is due to our desire to keep the recombinase-mediated gene stacking system as an open-source system with freedom to operate (Chen and Ow, 2017; Ow, 2016). The split-intein patent (Yadav and Yang, 2007) has broad claims that are difficult to invent around. For the Cre fragment complementation approach, at least three applications were found that claim specific locations by which Cre can be split into two (Herman and Jullien, 2004; filed 2002; Gu and Xu, 2009; filed 2009; Gao et al., 2015; filed 2013). At least one of these applications has since been issued (Gao et al., 2015).

Under these circumstances, we undertook the task to a search for a new location for Cre fragment complementation. The positive outcome from this research is that we indeed found a new split-Cre pair that can reconstitute activity that is comparable to the wild-type Cre control. Unlike other studies that relied exclusively on scoring for recombination through reporter gene expression, or by PCR, both of which cannot detect the percentage of substrates that have not undergone recombination, we followed up our initial analysis with a Southern analysis which showed that from eight randomly selected progenies, the excision-specific hybridizing band was found in seven of them, and with only faint or undetectable hybridization for the band representing the lack of excision. This shows that the Cre pair brought together by hybridization was highly effective at the F1 generation.

Yet, despite the high efficiency of recombination in somatic cells, the one surprising outcome of this study was that backcrossed progenies that showed excision invariably harboured both the N-cre and C-cre loci. This suggests that the excision events were generated de novo from the reconstitution...
of N-cre and C-cre in the backcrossed progeny generation. Both N-cre and C-cre genes were transcribed by the CaMV 35S RNA promoter and this promoter has previously been used to express cre to cause excision that can be transmitted through the germ-line, although the efficiency varies depending on the particular cre donor line. It remains possible that a lower level of expression was caused by having both N-cre and C-cre transcribed by the same promoter. Whatever the reason, it remains a next engineering challenge to test different promoters, including germ-line-specific promoters for more effective transmission of the recombination event (Li et al., 2007; Mlynarova et al., 2006; Van Ex et al., 2009; Verweire et al., 2007). As we have now progressed to implementing the recombinase-mediated gene stacking system in rice (Li et al., 2016), future testing of these germ-line-specific promoters will be conducted on this crop.

**Experimental procedure**

**DNA constructs**

Standard recombinant DNA methods were used throughout (Sambrook and Russell, 2001). Primer and DNA linker sequences shown in Table S1.

pETDuet-Cre: The cre gene was PCR amplified (Biolabs Phusion® High-Fidelity DNA Polymerase) from pMM23 (Qin et al., 1994) using primers N-F and C-R and inserted between EcoRI and KpnI restriction sites of pETDuet (pETDuet™-1, Novagen).

pETDuet-N series constructs: 1N-cre fragment was PCR amplified from pMM23 using primers N-1F and 1N-R, 2N-cre used primers N-2F and 2N-R, 3N-cre with primers N-3F and 3N-R, 4N-cre with primers N-4F and 4N-R, and 5N-cre with primers N-5F and 5N-R. Each of the N-cre DNA was inserted between EcoRI and AvrII restriction sites of pETDuet (pETDuet™-1, Novagen).

pRSF-Duet-C series constructs: 1C-cre fragment was PCR amplified from pMM23 using primers 1C-F and C-R, 2C-cre fragment amplified using primers 2C-F and C-R, 3C-cre fragment with primers 3C-F and C-R, 4C-cre fragment with primers 4C-F and C-R, and 5C-cre fragment with 5C-F and C-R. Each of the C-cre fragments was inserted between EcoRI and KpnI restriction sites of pACYCD (pACYCDuet™-1, Novagen).

pACYCDuet-in: The hpt gene was PCR amplified from pZH36 (ZG Han, Ow lab, unpublished) using primers Ihl-F and Ihl-R with overhanging box sites to create the lox-hpt-(inverted Iox), which was then inserted between EcoRI and AvrII restriction sites of pACYCD (pACYCDuet™-1, Novagen).

pMM23-N series and pMM23-C series constructs: 2N-cre, 3N-cre, 5N-cre, 1C-cre, 3C-cre and 5C-cre fragments were PCR amplified with same or analogous primers for the pETDuet-N and pETDuet-C series plasmids but with KpnI and Sphl restriction sites (N-F1, 2N-R1, 3N-R1, 5N-R1 for N-cre fragments and 1C-F1, 3C-F1, 5C-F1 and C-R1 for C-cre fragments) for inserting between P::gus (KpnI) and P::nos (Sphl) of pMM23.

pMM23-LZ series constructs: 2N-LZ cre and 3N-LZ cre fragments were PCR amplified from pMM23 using primers N-F2 and 2NLZ-R or 3NLZ-R for insertion into the intermediate vector pMD18-T (Takara) in between KpnI and XhoI. Afterwards, a Myc (Ayer and Eisenman, 1993) linker (Beijing AUGCT Biotechnology Co., Ltd.) was inserted between the XhoI site and the vector-derived Sphl site of pMD-18T (C-terminus of the N-cre fragment) to make 2NLZ and 3NLZ. Finally, 2NLZ and 3NLZ fragments were retrieved by cleavage with KpnI and Sphl and inserted between P::gus (KpnI) and P::nos (Sphl) of pMM23.

pMM23-Cre series constructs: 1C-LZ-cre and 3C-LZ-cre fragments were PCR amplified from pMM23 using primers C-R2, 1CLZ-F or 3CLZ-F for insertion between the EcoRI and Sphl sites of pMD18-T (Takara). Afterwards, a Max Ayer and Eisenman, 1993) linker (Beijing AUGCT Biotechnology Co., Ltd.) was inserted between the KpnI and EcoRI sites of pMD-18T (N-terminus of the cre fragment) to make 1CLZ and 3CLZ. Finally, 1C-LZ-cre and 3C-LZ-cre fragments were retrieved by cleavage with KpnI and Sphl and inserted between P::gus (KpnI) and P::nos (Sphl) of pMM23.

pMR1: An Xhol-gus-Sphl fragment was PCR amplified from pZH36 (ZG Han, Ow lab, unpublished) using primers Gus-F and Gus-R for insertion into a T-vector (Takara) between the corresponding sites. An hpt fragment (with nos terminator) was also PCR amplified from pZH36, using primers Ihl-F and Ihl-R with overhanging box sites to create a KpnI-lox-hpt-lox-Xhol site for insertion upstream of the gus gene between the lox-hpt-lox-gus fragment was then retrieved by cleavage with KpnI and Sphl for insertion between P::gus (KpnI) and P::nos (Sphl) of pMM23.

pCambia-N series constructs: 2N-cre, 3N-cre, 5N-cre, were PCR amplified from pMM23 with Nns-F and 2N-R2, 3N-R2 or 5N-R2 primers (Nns-F included a Kozak sequence, ATG and a SV40 NLS). 2N-Lox and 3N-Lox fragments were PCR amplified from pMM23-NLZ series constructs with Nns-F and Nis-R. Each fragment was inserted between P::gus (BglII) and P::nos (BstEII) of pCAMBIA1301 (http://www.cambia.org).

pCambia-C-luc series constructs: C-cre fragments were PCR amplified from the pMM23-C series constructs with primers C-R3 and either 1CNs-F, 3CNs-F or 5LZF-F that incorporated a KpnI site, a Kozak sequence, lox, ATG, and a SV40 NLS to form a KpnI-lox-cre-lox-Ndel fragment into pMD-18T (Takara). A first T::gus fragment was PCR amplified from pZH36 using primers T::gus-F and T::gus-R with and inserted downstream of lox-C-cre between Ndel and PsII sites. A second T::gus fragment was PCR amplified with primers T::gus-F2 and T::gus-Lox-R to create a PsII-T::gus-Lox fragment to insert behind the first T::gus fragment to create lox-C-cre-P::gus-T::gus-Lox linkage within pMD-18T, upon which the KpnI-BglII fragment was transferred to pCambia1301. Finally, a BglII-luc-BstEII fragment amplified by primers luc-F and luc-R from pYWP72 (Yau et al., 2011) was inserted in place of the gus gene in pCambia1301.

pCambia-bar-luc: A KpnI-lox-bar-Ndel fragment was PCR amplified from pZH210B (ZG Han, Ow lab, unpublished) using primers Bar-F and Bar-R to replace the KpnI-lox-C-cre-Ndel fragment in pCambia-C-luc.

pCambia-Cre: A BglII-cre-BstEII fragment was PCR amplified from pMM23 using Nns-F and C-R4 to insert between P::gus (BglII) and P::nos (BstEII) of pCAMBIA1301.

**E. coli assay**

E. coli DH5α (F- endA1 galV44 thi-1 recA1 relA1 gyrA96 deoR rupG φ80dlacZΔM15 lacZΔ2Yam-argF)U169 hsdR17(θ- mcrA, rK-) (Grant et al., 1990) was used throughout for recovery of recombinant molecules. For the experiment shown in Figure 3b, 100 μL competent cells were transformed with 100 ng of each plasmid construct, and resistant colonies were scored on plates containing 34 mg/L chloramphenicol, 100 mg/L ampicillin and if needed 50 mg/L kanamycin.

**Transient expression in onion epidermal cells**

DNA was purified using Tiangene® High Pure Maxi Plasmid Kit. Onion inner epidermis was placed onto MS medium plates...
(supplemented by 200 μM of D-sorbitol and 200 μM D-mannitol) and subjected to microparticle bombardment as described (Altpeter et al., 1996). Afterwards, the plates were incubated in the dark for 14–20 h and the onion epidermis stained for the Gus activity (Jefferson et al., 1987).

**Transcript expression in Arabidopsis protoplasts**

Protoplasts were isolated from 10 to 20 leaves of 3- to 4-week-old A. thaliana (cv. Columbia) plants before flowering and transformed by PEG as described (Yoo et al., 2007), using for each sample ~1.5 × 10^6 protoplasts (200 μL volume) and 10 μg of each DNA construct (Tiangene® purified). Luciferase activity was assayed by the DualLuciferase Reporter Assay System (Promega) using a GloMax Multi JR detection Luminometer (Promega) on overnight resting protoplasts and normalized to total protein (Bradford assay kit, Thermo Scientific®).

**Transgenic Arabidopsis**

The constructs shown in Figure 4a were introduced into Agrobacterium strain GV3101 through floral-dip transformation of A. thaliana (cv. Columbia) plants as described (Clough and Bent, 1998). T1 hygromycin-resistant transformants were selected on an MS medium with 40 μg/L hygromycin. For N-cre plants that lacked a cotransformed reporter gene, N-cre expression was checked by quantitative real-time PCR using F: 5’ ATTTGGCAGAACGA AAACCGCT 3’ and R: 5’-ATCAGCTACACAGAGACGG-3’ primers. Total RNA was extracted using Hipure plant RNA mini kit (MAGEN). Reverse transcription was conducted using a PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA).

**Copy number estimation by quantitative real-time PCR**

Total DNA was extracted using HiPure Plant DNA Midi Kit (Magen, D3162) according to the manufacturer’s recommendation. DNA was diluted to 100 μg/μL by ddH₂O, and 3 μL was used for PCR amplification. Quantitative RT-PCR reactions were performed in 384-well blocks using the Go Taq® qPCR Master Mix (Promega, A6001). The hpt gene was measured against AtOXS1 used as an internal positive single-copy gene control. Primers used for hpt: 5’-TCGTCATCCAGTTGCC-3’ and 5’-TC GGTCAATACACTAGTGGC3’; for AtOXS1: 5’-ACTGTCAGCAGAAATCCCGT 3’ and 5’-GGTTCTCAGACTGACCC CGT 3’ PCR reactions were 28 cycles, 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Relative quantification for copy number of T-DNA insertion was calculated by dividing the Cq values of hpt to Cq values of AtOXS1.

**Southern hybridization**

Southern blots were performed as described previously (Hou et al., 2014). DNA was further purified through NucleoBond AX100 columns (Genopure plasmid Midi kit, Roche, Germany) and concentration determined by a 2000c spectrophotometer (Thermo scientific, Wilmington). 32P-probed membranes were exposed to a phosphor screen for 24 h and detected with Typhoon FLA 9500 (IP: 635 nm, PMT: 500 V, Pixel size 200 μm).

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Supporting information

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

Table S1. Primer sequences.