A novel strategy for promoting homoplasmic plastid transformant production using the barnase–barstar system

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Abstract Plastid transformants form biofactories that are able to produce extra proteins in plastids when they are in a homoplasmic state. To date, plastid transformation has been reported in about twenty plant species; however, the production of homoplasmic plastid transformants is not always successful or easy. Heteroplasmic plants that contain wild-type plastids produce fewer target proteins and do not always successfully transfer transgenes to progeny. In order to promote the generation of homoplasmic plants, we developed a novel system using barnase–barstar to eliminate wild-type plastids from heteroplasmic cells systematically. In this system, a chemically inducible cytotoxic barnase under a plastid transit signal was introduced into nuclear DNA and barstar, which inhibits barnase, was integrated into plastid DNA with the primary selection markers aminoglycoside 3′-adenylyltransferase (aadA) and green fluorescence protein (GFP) gene. As expected, the expression of the plastid barnase was lethal to cells as seen in leaf segments, but barstar expression in plastids rescued them. We then investigated the regeneration frequency of homoplasmic shoots from heteroplasmic leaf segments with or without barnase expression. The regeneration frequency of homoplasmic-like shoots expressing barnase–barstar system was higher than that of shoots not expressing this. We expect that the application of this novel strategy for transformation of plastids will be supportive to generate homoplasmic plastid transformants in other plant species.

Key words: barnase, barstar, heteroplasmy, homoplasmy, plastid transformation.

Introduction
Plastid transformation is considered to be an important tool for creating plant biofactories that can produce large amounts of useful proteins. The technique of plastid transformation in higher plants was developed in tobacco (Svab et al. 1990). As about 10,000 plastid DNAs are present in each plant cell, useful extra proteins generally accumulate at higher levels in plastid, rather than nuclear transformants (Bock 2007; Daniell et al. 2009; Maliga 2003; Scotti et al. 2012). It is thought that homologous recombination of plastid DNA occurs only infrequently in a few plastids that receive a transformation vector by particle bombardment (Daniell et al. 2002; Maliga and Bock 2011). Therefore, untransformed wild-type plastid DNAs normally remain in antibiotic-resistant transformants that are regenerated in the primary selection step (Ye et al. 2003). Such cells, containing both wild-type and transformed plastid DNAs, are described as heteroplasmic. In addition, plants with chimeric tissues, which contain both wild-type and plastid-transformed cells are generally obtained in primary regenerate shoots. However, homoplasmic plastid transformants, which contain only transformed plastids, have been obtained successfully through repeated regeneration selection steps in mainly Solanaceous plants such as tobacco (Ruf et al. 2001; Svab and Maliga 1993; Verma and Daniell 2007), potato (Nguyen et al. 2005), and eggplant (Singh et al. 2010). On the other hand, it has been difficult to generate even heteroplasmic plastid transformants in other plant species. In Arabidopsis and rice, selection using aminoglycoside 3′-adenylyltransferase (aadA) does not work as sufficiently as it does in tobacco, resulting in the production of heteroplasmic T0 plants only (Lee et al. 2006; Sikdar et al. 1998).

Abbreviations: GFP, green fluorescence protein; aadA, aminoglycoside 3′-adenylyltransferase gene; estradiol, 17β-estradiol.
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Homoplasmic plastid transformants are more advantageous than heteroplasmic or chimeric plastid transformants, as they produce high levels of excess protein. Moreover, transgene transmission to progeny by maternal inheritance occurs in homoplasmic plants, although transgenes in heteroplasmic plants are not always transferred to progeny (Cheng et al. 2010; Khan and Maliga 1999; Lee et al. 2006). Plastid DNA in regenerating shoot apical meristem may be derived from a small number of copies selected through a stochastic process during cell division (Lutz and Maliga 2008). In order to obtain homoplasmic plastid transformants systematically, we aimed to develop a novel selection system to exclude wild-type plastids selectively from heteroplasmic or chimeric cells. Therefore, we applied the barnase–barstar system for plastid transformation. Barnase and barstar are derived from Bacillus amyloliquefaciens (Hartley 1988) and barnase, which encodes a ribonuclease, has been used to produce male and female sterile plants due to its cytotoxicity (Abe et al. 2018; Gardner et al. 2009; Mariani et al. 1990). Conversely, barstar, which is a specific inhibitor of barnase, has been used to restore fertility or inhibit leaky expression except for reproductive organs in tobacco, tomato, oilseed mustard, wheat and rice (Abe et al. 2018; Burgess et al. 2002; Jagannath et al. 2002; Kempe et al. 2013; Mariani et al. 1992). We introduced barnase under a plastid transit signal into nuclear DNA in order to exclude wild-type plastids and used a 17 β-estradiol (estradiol)-based gene induction system (Zuo et al. 2000) to control barnase gene expression. Barstar was introduced into plastid DNA to restore damage by inhibiting the ribonuclease activity of barnase in plastids. In this report, we demonstrate that the barnase–barstar system is able to promote the generation of homoplasmic plastid transformants in tobacco.

Materials and methods

Vector construction

The nuclear vector for the barnase–barstar system (Figure 1), pLexBn (Figure 2A), was constructed as follows. The coding region of the barnase gene, which codes ribonuclease from Bacillus amyloliquefaciens, containing the first CAT1 intron of the castor bean (Ricinus communis L.) (Abe et al. 2018; Kobayashi et al. 2006), was amplified using bnF-SalI (5′-gtgtcgacgcacaggttatcaacact-3′) and bnR-PacI (5′-gtttaattaattatctgatttttgtaaaggtc-3′) before being cloned into pCR2.1-TOPO® (Invitrogen, USA) and named pBnSP. A chloroplast transit peptide of RbcS1A derived from Arabidopsis thaliana (Chiu et al. 1996) was amplified with AtTPF-XhoI 5′-agctcgagatggcttcctctatgctctc-3′ (the underlined section indicates an additional XhoI site) and AtTPR-SalI 5′-ttgtcgacagttacctctcgcagt-3′ (the underlined section indicates a SalI site) before being cloned into pCR2.1-TOPO®. Then, fragments of the plastid transit peptide were digested by XhoI and SalI and connected to the XhoI/SalI site of pBnSP and named pNtpBn. XhoI/PacI fragments digested from pNtpBn were inserted into the XhoI/PacI site of pER8 (Zuo et al. 2000) and named pLexBn. pLexBn was introduced to Agrobacterium tumefaciens EHA105 (Hood et al. 1993) for transformation.

The plastid transformation vector pNtagBS was constructed as follows. The coding region of the barstar gene was amplified with bsFBamHI 5′-ggtcgagttcatgaaaaaagctgtgtaa-3′ and
bsRBamHI (5′-ccccggatcccttaagaaagtatgatggtgatg-3′), cloned into pCR2.1-TOPO®, and named pBS. BamHI fragments digested from pBS were inserted into the BamHI site of pPrrn-Trbc (Okuzaki and Tabei 2012) and named pPrrn-Bs-TrbcL. A NolI/BsiWI fragment digested from pPrrn-Bs-TrbcL was inserted into the NotI/BsiWI site of pNtag (Okuzaki and Tabei 2012) and named pNtagBs (Figure 3). pNtag and pNtagBs vector DNA for plastid transformation was extracted using a Qiagen HiSpeed Plasmid Maxi Kit (Qiagen, Japan).

**Nuclear transformation of pLexBn**
Sterile tobacco (*Nicotiana tabacum*, petit Havana SR1) plants were grown according to the protocol reported previously (Okuzaki and Tabei 2012). pLexBn was integrated into tobacco using *Agrobacterium* (Horsch et al. 1985) and selected with a medium containing 50 mg l⁻¹ hygromycin (FUJIFILM Wako Pure Chemical Industries, Ltd. Corporation, Japan) and 500 mg l⁻¹ Carbenicillin (FUJIFILM Wako Pure Chemical Industries, Ltd. Corporation). Insertion of pLexBn into these hygromycin-resistant plants was confirmed by DNA blotting analysis, followed by selection by PCR analysis using barnase-specific primers, bnF-Sall and bnR-Paci, and TaKaRa ExTaq polymerase (TaKaRa Bio, Japan).

**Plastid transformation of pNtagBS**
pNtagBS and pNtag were integrated into both wild-type tobacco and pLexBn-integrated lines by particle bombardment, according to the method described in a previous report (Okuzaki and Tabei 2012). We selected several regenerated plants, which were expected to be homoplasmic or heteroplasmic lines, by observing green fluorescent protein (GFP) fluorescence using a Leica MZ16FA fluorescence microscope (Leica Microsystems, Germany) with a GFP2 filter (480-nm excitation filter/510-nm barrier filter) (Supplementary Figure S1).

**DNA blotting analysis**
Total DNAs were extracted from the tobacco leaves using ISOPLANT II (Nippon Gene Co. Ltd., Tokyo, Japan). Total DNA (7 µg) from pLexBn-integrated or wild-type plants was digested with HindIII, fractionated on a 0.8%-agarose gel, and transferred to a positively-charged nylon membrane (Roche Diagnostics, Basel, Switzerland). Detection was performed according to standard protocols with a DIG Luminescent Detection Kit (Roche Diagnostics). Specific DNA probes for the *hpt* gene were prepared using the PCR DIG Labeling Mix (Roche Diagnostics) and Primestar GXL DNA polymerase (TaKaRa Bio) with specific primers (5′-gagcctgacctattgcatctc-3′/5′-gtacttctacacagccatcg-3′). Total DNA in 2.5 µg of plastid transformants was digested with BglII and detected with the 5′ region of trnI-specific probes according to protocol described by Okuzaki and Tabei (2012).

**Semi-quantitative RT-PCR analysis**
Total RNA was extracted from 0.1 g tobacco leaf using an RNeasy plant mini kit (Qiagen), according to manufacturer’s protocol. First-strand cDNA was synthesized from 500 ng of total RNA using the PrimeScript RT reagent kit (TaKaRa Bio), oligo-dT primer, and random 6 mers, according to the manufacturer’s instructions. First-strand cDNA was used as
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The template for PCR with TaKaRa Ex Taq DNA polymerase. PCR amplification of barnase and Act9, which is nuclear DNA-encoded control gene, was performed for 28, 30 or 32 cycles at 95°C for 15 s, 55°C for 15 s, and 72°C for 20 s, using the primer sets Bn-RTF1/BnRTR1 (5′-caaaagggaaccttgcagac-3′/5′-ccggtctgaatttctgaagc-3′) and Act9F/Act9R (5′-ctattctccgctttggacttggca-3′/5′-aggacctcaggacaacggaaacg-3′) (Cortleven et al. 2009). PCR amplification of barstar and Ntrps3, which is plastid-encoded control gene, was performed for 23 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s using the primer sets BsF1/BsR1 (5′-cagaagtatcagcgacctccac-3′/5′-gtatgatggtgatgtcgcagc-3′) and Ntrps3F/Ntrps3R (5′-ggggaaccctaccttctctg-3′/5′-ccgaaaactgaacattgctg-3′) (Cortleven et al. 2009). PCR amplification of trnI-trnA, which is target for plastid transformation (upper figure). Image of transformed plastid DNA with the pNTag (Okuzaki and Tabei 2012) construct (middle figure) or the pNTags construct (bottom). trnI region, a flanking region around the trnl gene of tobacco plastid DNA (Accession No. Z00044: 103417-105335); trnA region, a flanking region around the trnA gene of tobacco plastid DNA (Accession No. Z00044: 105330-106944); P1, ribosomal RNA operon promoter (Accession No. Z00044: 102550-102725) connected with 5′UTR of gene 10 from T7 phage; T1, rbcL terminator; P2, psbA terminator; T2, rps16 terminator (Okuzaki and Tabei 2012). P1-barstar-T1 was inserted between the GFP expression cassette and trnA region.

Observation of protoplast cells

Leaf pieces (2×5 mm) were soaked in protoplast formation medium for 2–6 h, according to a procedure (personal communications from Dr. Masaki Endo). The protoplast formation medium contained 0.25% (w/v) Macerozyme R-10 (Yakult Pharmaceuticals, Tokyo, Japan), 1% (w/v) Cellurase Onozuka R-10 (Yakult Pharmaceuticals), 0.5 M mannitol and 6.8 mM CaCl₂, and sterilized using a Millex G5 0.22 μm filter unit (Merck Millipore, Billerica, MA, USA) after adjusting the pH to 5.6. Then, soaked leaf pieces were mixed with MS liquid medium and observed using an Olympus VANOX-T (Olympus Corporation, Japan) with a B-filter/FITC (470–490 nm excitation, 515-nm barrier filter).

Estradiol treatment during leaf segment culture

Six leaf pieces (5 mm×5 mm) from each of the pLexBn-integrated and wild-type plants were cultured on the regeneration culture on MSBN medium (Okuzaki and Tabei 2012) with or without 5 μM estradiol (FUJIFILM Wako Pure Chemical Industries, Ltd. Corporation) for 4 weeks by replacing the media every 2 weeks.

Regeneration test of secondary shoots of heteroplasmic-like plastid transformants

The regeneration frequency of the homoplasmic-like plastid transformants was examined as follows. Eight leaf segments were made from two leaves of heteroplasmic-like Bn/GFP or Bn/Bs (Table 1) lines and equally divided each four segments were cultured with or without the addition of 10 μM estradiol to MSBN medium containing spectinomycin. The media were replaced every 2 weeks. Then, all regenerated shoots were transferred onto MS medium with spectinomycin to count...
the number of homoplasmic-like or heteroplasmic-like plastid transfomers by observing their GFP fluorescence using a fluorescence microscope (Leica MZ 16FA, Leica Microsystems, Germany) with a GFP2 filter (480-nm excitation filter/510-nm barrier filter). To show photographs of whole plates, several photographs were taken and combined with MosaicJ plugin for ImageJ software (Thévenaz and Unser 2007).

Results

Inhibiting the growth of leaf segments by barnase expression

We constructed two transformation vectors for introducing the barnase–barstar system working at plastids (Figure 1). Barnase was integrated by the binary vector pLexBn to express plastid transit barnase under an estradiol induction system (Zuo et al. 2000) (Figure 2A). Fourteen lines of pLexBn-integrated tobacco plants were generated using Agrobacterium transformation and the success of estradiol-based barnase induction and its effect on plastids were evaluated by observing the growth of calli. Tobacco leaf segments from the transformed T₀ lines were cultured with or without estradiol for 4 weeks. The results showed that wild-type leaf segments enlarged due to cell proliferation and produced calli on the merge of the leaf pieces (Figure 2B) that could be produced adventitious shoots both with and without the presence of 5 µM of estradiol. However, six pLexBn-integrated lines (Bn#5, #7, #8, #9, #13, and #14) did not enlarge and produce vigorous calli that produce adventitious shoots. Instead, damage could be observed on the margins of the leaf segments under estradiol treatment, while calli and adventitious shoots were produced in the absence of estradiol (Figure 2B). However, another eight lines of pLexBn-integrated tobacco (#1–4, 6, and #10–12) did not grow normally either with or without estradiol. This may have been due to the unexpected leaky expression of barnase. Therefore, we chose the six pLexBn-integrated lines (Bn#5, #7, #8, #9, #13 and #14) that seemed to express barnase in response to estradiol treatment. Bn#7 and Bn#9 were predicted to contain a single copy of the transgene, while the other lines (Bn#5, #8, #13, and #14) were predicted to contain multiple copies of the transgene (Figure 2C). We chose Bn#7 and Bn#9 lines for the plastid transformation and further experiments.

Restoration of growth inhibition due to barstar expression in plastids

The barstar integration vector (pNtagBs) was constructed to include a barstar expression cassette and aadA and GFP expression cassettes (Figure 3A). pNtagBs was introduced into the clonally propagated Bn#7 (T₀), Bn#9 (T₀), and wild-type plants (Figure 3A). Plastids transformed with pNtagBs were produced in Bn#7 and wild-type plants but not in Bn#9 plant. As a control of the barstar-integrated material, pNtag (Okuzaki and Tabei 2012), which contained both aadA and GFP expression cassettes, was also integrated into the Bn#7. Consequently, we generated six nuclear/plastid genotypes through spectinomycin selection, as listed in Table 1, including wild-type (WT/WT), WT/GFP, WT/Bs, Bn/WT, Bn/Bs, and Bn/GFP. After observing the GFP fluorescence in the leaves, we found both homoplasmic-like and homoplasmic-like plastid transfomers (including partially chimeric) from WT/Bs and Bn/Bs. While heteroplasmic-like plants conferred chlorophyll autofluorescence signal partially or whole surface of leaves mixed in the GFP fluorescence, homoplasmic-like plants conferred GFP signal over the whole surface of the plants without the chlorophyll autofluorescence signal (Supplementary Figure S1). These homoplasmic-like plants were found to contain only transformed plastid DNA, which was detected by DNA blotting analysis (Figure 3B), whereas both wild-type and transformed plastid DNA were detected in heteroplasmic-like leaves (Figure 3C). It suggested that there was correlation between observed GFP fluorescence ratio on the surface of leaves and the ratio of transformed plastid DNA. Then, to evaluate the restoration effect of barstar on growth inhibition by barnase, leaf segments of homoplasmic Bn/Bs, which contained barstar in plastid DNA, produced calli and adventitious buds in the same manner as the WT/Bs and WT/GFP (Figure 4A). The estradiol treatment regeneration test showed that there were no effects on the regeneration efficiency of WT/GFP and WT/Bs control plastid transfomers with or without estradiol treatment (Figure 4A). However Bn/GFP showed serious growth inhibition with estradiol treatment for barnase induction, leaf segments of Bn/Bs, which contained barstar in plastid DNA, produced calli and adventitious buds in the same manner as the WT/Bs and WT/GFP (Figure 4A). Next, we observed the chloroplasts in leaf cells of each of the four genotypes following estradiol treatment. The size of chloroplasts in the protoplast cells derived from estradiol-treated leaves of Bn/GFP were smaller than those in WT/GFP cells (Figure 4B). Nevertheless, the chloroplast sizes in estradiol-treated Bn/Bs were as same as those in WT/GFP cells (Figure 4B). In addition, WT/ Bs chloroplasts looked similar to those in WT/GFP cells.
Then, gene expression of barnase in Bn/GFP and Bn/Bs leaf segments after estradiol treatment was checked by RT-PCR analysis (Figure 5A). We observed that the similar levels of barnase was expressed in both Bn/GFP-1, 3 and Bn/Bs-6, 7 leaves with estradiol treatment, and the barstar was expressed in Bn/Bs-6, 7. Slight expression of barnase was also seen in the absence of estradiol treatment in all the tested lines, Bn/GFP-1, 3 and Bn/Bs-6, 7 (Figure 5B), suggesting that Bn#7 used for plastid transformation, which has one copy of pLexBn integration, had slightly leaky expression of barnase in the background. This might also have caused mild delay of growth in Bn/GFP without estradiol treatment (Figure 4A).

**Regeneration of homoplasmic-like plastid transformants using the barnase–barstar system**

In order to evaluate whether the barnase–barstar system in plastids promoted the generation of homoplasmic transformants, we examined the efficiency of producing homoplasmic plants from primary-derived heteroplasmic plastid transformants (Figure 3C) in the secondary regeneration step using Bn/Bs and WT/Bs lines. Four independent heteroplasmic-like leaves from plastid transformants, Bn/Bs-1, 4, 5, and 6 and WT/Bs-1, 2, 3, and 5, were selected by observing GFP fluorescence level on each leaf and cultured on MSBN medium with or without 10 μM estradiol. Appropriate heteroplasmic-like leaves were obtained from these lines but not from other lines including Bn/Bs-7 lines. Regenerated shoots were
cut independently from cultured leaf discs and placed in MS medium before the number of homoplasmic shoots were counted by observing GFP fluorescence (Figure 6A, Table 2). Based on the correlation between observed GFP fluorescence level and the rate of transformed plastid DNA in Figure 3B and 3C, shoots conferring the green GFP signal over the whole surface were determined to be homoplasmic-like plants and shoots conferring the red autofluorescence signal over part of the surface including chimeric plants were determined to be heteroplasmic-like plastid transformants (Figure 6B). We observed more homoplasmic-like secondary regenerated shoots from Bn/Bs plants treated with estradiol. The secondary regenerated shoots from Bn/Bs lines treated with estradiol were more compact than that from the Bn/Bs line cultured without estradiol and WT/Bs cultured with and without estradiol. These regenerated shoots rooted and grew vigorously after removal of estradiol from the culture medium. We determined the regeneration frequency of the homoplasmic-like plants to be 34.8 ± 12.9% in Bn/Bs plants treated with estradiol and 13.8 ± 5.5% in those not treated with estradiol (Figure 7). In contrast, the average regeneration frequencies of homoplasmic-like plants were 6.2 ± 5.1% and 2.9 ± 2.0% for WT/Bs lines treated with or without estradiol, respectively (Figure 7). Though we considered that statistical analysis is not suitable because of large standard errors, the regeneration frequency of Bn/Bs plants treated with estradiol has tendency to be higher than without estradiol or that for WT/Bs plants treated with and without estradiol. Several homoplasmic-like plants regenerated from the Bn/Bs or WT/Bs lines were grown in closed greenhouse and set seeds. Progenies from homoplasmic-like plants totally contained inherited transformed plastid DNA; however, progenies from

![Figure 6. Observation of homoplasmic-like secondary regenerated shoots with or without estradiol.](image)

![Figure 7. Regeneration frequency of homoplasmic-like secondary shoots from heteroplasmic-like leaf segments.](image)

**Table 2. Number of homo- or heteroplasmic secondary shoots regenerated from leaf segments of heteroplasmic primary plastid transformants with or without estradiol.**

| Line     | Estradiol treatment | No. of regenerated secondary shoots | Homoplasmic (a/b %) | Heteroplasmic | Total |
|----------|---------------------|-------------------------------------|---------------------|--------------|-------|
| Bn/BS-1  | −                   | 4 (28.6)                           | 10                  | 14           |
|          | +                   | 12 (54.6)                          | 10                  | 22           |
| Bn/BS-4  | −                   | 1 (8.3)                            | 11                  | 12           |
|          | +                   | 3 (17.6)                           | 14                  | 17           |
| Bn/BS-5  | −                   | 1 (3.4)                            | 28                  | 29           |
|          | +                   | 2 (8.0)                            | 23                  | 25           |
| Bn/BS-6  | −                   | 3 (15.0)                           | 17                  | 20           |
|          | +                   | 13 (59.1)                          | 9                   | 22           |
| WT/BS-1  | −                   | 3 (21.4)                           | 11                  | 14           |
|          | +                   | 1 (8.3)                            | 11                  | 12           |
| WT/BS-2  | −                   | 0 (0)                              | 18                  | 18           |
|          | +                   | 0 (0)                              | 18                  | 18           |
| WT/BS-3  | −                   | 0 (0)                              | 17                  | 17           |
|          | +                   | 0 (0)                              | 18                  | 18           |
| WT/BS-5  | −                   | 1 (3.3)                            | 29                  | 30           |
|          | +                   | 1 (3.2)                            | 30                  | 31           |

Homoplasmic shoots: shoots conferred GFP fluorescence in cells over whole leaves. Heteroplasmic shoots: shoots conferred mixture of GFP fluorescence and chlorophyll autofluorescence.
several heteroplasmic-like plants did not always contain inherited transformed plastid DNA (Supplementary Figure S2).

Discussion

The barnase–barstar system promoted the generation of homoplasmic-like plants

Here we have demonstrated a novel system for promoting the production of homoplasmic plastid transformants using barnase and barstar. The effective cytoplasmic use of the barnase–barstar system has been reported in many plant species (Burgess et al. 2002; Jagannath et al. 2002; Kempe et al. 2013; Mariani et al. 1992) and the system has also been shown to work in the mitochondria of yeast (Mireau et al. 2003). However, in this study we have demonstrated for the first time the use of barnase–barstar in chloroplasts. As expected, the induction of barnase expression led to the growth inhibition of cultured leaf segments in pLexBn-integrated Bn#7 (Figure 2B) and other lines (data not shown). Next, we integrated a barstar expression cassette into plastid DNA of clonally propagated Bn#7 to inhibit barnase ribonuclease activity in plastids. Chloroplasts in the protoplasts derived from the growth-inhibited leaf segments of the Bn#7 (Bn/WT) line showed extreme damage. In contrast, leaf segments of plastid transformed Bn/Bs lines grown normally with or without the induction of barnase and chloroplasts from Bn/Bs lines inducing barnase appeared the same as the control, WT/GFP, and WT/Bs lines (Figure 4). We therefore concluded that the barnase/barstar system could function in plastids and went on to investigate whether the barnase–barstar system could promote the generation of homoplasmic plastid transformants. Therefore, several heteroplasmic-like plastid transformed Bn/Bs lines or WT/Bs lines that were obtained from barnase integrated Bn#7 line or wild-type background were used in the experiment. The regeneration frequencies of homoplasmic secondary shoots from heteroplasmic leaf segments of Bn/Bs plants treated with estradiol tended to be higher than those of WT/Bs plants in which estradiol had or had not been treated (Figures 6, 7), demonstrating that the barnase–barstar system promoted the generation of homoplasmic-like plastid transformants to a greater extent than selection with spectinomycin alone. The efficiency of homoplasmic-like plastid transformants production in each line was relatively broad (Table 2). We expected that this is due to the slight differences in the heteroplasmic level of transformed plastids on each Bn/Bs line for the experiment, or slight differences in the absorbance of estradiol and we thought that there was no correlation of basic barnase expression level because the background of pLexBn integration, which is derived from Bn#7, is the same for each line.

The application of aadA as a selection marker in combination with a selection agent, spectinomycin or streptomycin, for the development of the plastid transformation system was key (Khan 2012; Svab and Maliga 1993). Although other selection markers such as kanamycin and chloramphenicol resistant genes have been used, their application was not as successful as that of aadA in plastid transformation model tobacco (Carrer et al. 1993; Li et al. 2011). In addition, herbicide-resistance genes such as bar, EPSPS, or mutated acetolactate synthase (Shimizu et al. 2008; Ye et al. 2001) were not sufficiently effective for primary selection but could be used in secondary selection procedures (Daniell et al. 1998; Lutz et al. 2001). We also failed to use the barnase–barstar system for primary selection by only adding estradiol in media instead of spectinomycin (data not shown). However, the barnase–barstar system is more suitable for application in secondary selection processes to promote homoplasmic production by excluding wild-type plastids from heteroplasmic cells.

Leaky barnase expression slightly promotes the production of homoplasmic plants

Barnase expression in Bn/Bs line tended to promote the regeneration frequency of homoplasmic-like shoots, however, there was not significant difference in regeneration frequency of homoplasmic-like shoots between Bn/Bs lines treated with or without estradiol (Figure 7). The regeneration frequency for homoplasmic-like plastid transformants of Bn/Bs lines not treated with estradiol was slightly higher than that for WT/Bs lines not treated with estradiol. Estradiol induction is reported to work more successfully than other chemical gene induction systems; however, slightly leaky expression without estradiol treatment has been found in some cases (Okuzaki et al. 2011; Zhang et al. 2012). Similarly, we detected slight expression of the barnase gene without estradiol treatment in the leaves of the pLexBn-integrated lines (Figure 5B). The growth rate of cultured leaf segments from the Bn/GFP and Bn/Bs lines not treated with estradiol seemed slightly suppressed by the leaky induction of barnase (Figure 4A). Additionally, the growth and flowering of the pLexBn-integrated line were slightly delayed compared with wild-type plants in the closed glasshouse but they could set seeds (data not shown). The leaky expression of plastid transit barnase in Bn/Bs lines seemed to slightly enhance homoplasmic-like shoot regeneration and was not lethal to reproductive organs. This slight level of leaky expression of barnase might be suitable for further growth steps after transplanting in the soil without spectinomycin selection in the glasshouse, if some wild-type plastids remain.
Future application of barnase–barstar system in plastids of other plant species

The generation of homoplasmic plastid transformants using the aadA marker is not always successful, even in nuclear transformation models such as Arabidopsis and rice (Khan and Maliga 1999; Lee et al. 2006). Recently, it was reported that a homoplasmic plastid transformant was regenerated by hygromycin selection after almost a year of selection in rice (Wang et al. 2018). Because regeneration frequency decreases with the lengthening period of tissue culture (Yang et al. 1999), selection systems must be modified to obtain healthy plants for setting seed. One strategy may be to use barnase/barstar for secondary selection in combination of hygromycin to promote the generation of homoplasmic plants. Other approaches may include the use of a mutant line as a donor to produce, for example, homoplasmic Arabidopsis plastid transformants (Yu et al. 2017). This method involves the use of acc2 mutants to improve the effects of spectinomycin selection in Arabidopsis. Several selection systems for plastid transformation have been developed to date, including our barnase–barstar system. These new approaches may enable the plastid transformation technique to be expanded to further plant species.

Producing vaccines or other useful products in plastid transformants has been studied in tobacco, tomato, and lettuce (Daniell et al. 2009; Davoodi-Semiromi et al. 2010; Zhou et al. 2008). Furthermore, field tests of plastid transformants have been undertaken with tobacco (Schmidt et al. 2019). Plastid DNA is inherited maternally and therefore the risk of gene flow from pollen in plastid transformants is extremely low compared with that for nuclear transformants (Ruf et al. 2007). Although the homoplasmic plastid transformants obtained in this study contained the pLexBn vector, this can be eliminated by segregation of the progeny. Moreover, aadA and barstar genes in plastid DNA can also be eliminated to leave the gene of interest by using additional techniques developed for eliminating genes from plastid DNA (Day and Goldschmidt-Clermont 2011; Khan 2012). In the near future, useful plastid transformants may be developed for practical use and cultivated in the field. We hope that the novel system developed in this study may be one of the tools used to produce useful plastid transformants.

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Compliance with ethical standards

1. Disclosure of potential conflicts of interest: No conflicts
2. Research involving Human Participants and/or Animals: Not applicable
3. Informed consent: Not applicable

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