Video Article

Floral-Dip Transformation of Flax (*Linum usitatissimum*) to Generate Transgenic Progenies with a High Transformation Rate

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Abstract

Agrobacterium-mediated plant transformation via floral-dip is a widely used technique in the field of plant transformation and has been reported to be successful for many plant species. However, flax (*Linum usitatissimum*) transformation by floral-dip has not been reported. The goal of this protocol is to establish that *Agrobacterium* and the floral-dip method can be used to generate transgenic flax. We show that this technique is simple, inexpensive, efficient, and more importantly, gives a higher transformation rate than the current available methods of flax transformation.

In summary, inflorescences of flax were dipped in a solution of *Agrobacterium* carrying a binary vector plasmid (T-DNA fragment plus the Linum Insertion Sequence, LIS-1) for 1 - 2 min. The plants were laid flat on their side for 24 hr. Then, plants were maintained under normal growth conditions until the next treatment. The process of dipping was repeated 2 - 3 times, with approximately 10 - 14 day intervals between dipping. The T1 seeds were collected and germinated on soil. After approximately two weeks, treated progenies were tested by direct PCR; 2 - 3 leaves were used per plant plus the appropriate T-DNA primers. Positive transformants were selected and grown to maturity. The transformation rate was unexpectedly high, with 50 - 60% of the seeds from treated plants being positive transformants. This is a higher transformation rate than those reported for *Arabidopsis thaliana* and other plant species, using floral-dip transformation. It is also the highest, which has been reported so far, for flax transformation using other methods for transformation.

Video Link

The video component of this article can be found at http://www.jove.com/video/52189/

Introduction

Flax (*Linum usitatissimum*) is an important crop grown widely for its fibers and oils. Transformation of the flax genome is possible with techniques such as wounding, *Agrobacterium* infection, and co-cultivation in tissue culture, applying biolistic particles or ultrasound sonication followed by regeneration. However, these techniques have many disadvantages, including proclivity to many mutational events and an extended time to obtain the transgenic lines. Some of these methods can also be expensive and require skilled and efficient manipulation of the instruments, resulting in low recovery seedlings. Most importantly, these technique often result in low transformation rates[2,6].

Agrobacterium-mediated plant transformation via floral-dip is a simple and efficient approach to generate transgenic plants. It has been routinely and successfully used for many plant species such as *Arabidopsis thaliana*[1,4], *Medicago truncatula*[11], tomato[12], wheat[13] and maize[10]. However, it has not been thought of as a viable technique for flax transformation due to several factors, such as the low numbers of flowers produced by flax, limited number of seeds obtained from each flower, the large seed size, and the thick coat, which could also be problematic for such genetic transformation process. Additionally, the selection segment of the floral-dip technique requires germinating transformed seeds on a plant media containing an antibiotic, with transformed progenies distinguished based on their ability to germinate and stay green, while non-transformed progenies either do not germinate or germinate but bleach out quickly and die. In the current literature, it has been noted that wild-type flax tends to escape high concentration of antibiotic selections, producing false positive results, and making the selection of T1 progenies based on antibiotic resistance more difficult[6,14]. Also, when a high concentration of antibiotic was added to the selection medium, the rate of observed transformation dropped dramatically[9].

In this protocol, we used *Agrobacterium* and the floral-dip method to transform a line of fiber flax, Stormont cirrus (responsive and plastic), which has been shown to respond to stresses in the environment by altering its genome[3,5]. To overcome the antibiotic escape problem, we have chosen to do direct PCR testing of DNA from T1 leaves, instead of selection by adding the antibiotic to the plant media. We took advantage of the simple anatomy of flax to track specific flowers at the time of treatment. This tracking system allowed selection of seeds from specific flowers and germination on soil without adding antibiotic. Positive transformants were simply identified by testing DNA obtained from leaves using the fast and efficient method of direct PCR. Our results demonstrate that the floral-dip method worked very well in this line of flax and surprisingly resulted in a very high transformation rate (50 - 60%), higher than those previously observed for *Arabidopsis thaliana*, which was reported to be

0.1 - 1%[1], and also higher than other plant species[10,12]. We also tested another variety of linseed (oil flax), Bethune (stable and non-responsive), and our preliminary data indicates that floral-dip also works for this variety of flax.

The goal of this protocol is to show that *Agrobacterium* and floral-dip can be used to generate transgenic flax. We show that this technique is simple, inexpensive, and quicker than other methods of flax transformation. More importantly it results in a much higher transformation rate than the other methods of flax transformation[2,6]. The anatomy of *Arabidopsis thaliana*, which has many branches and flowers, makes it difficult to distinguish dipped and non-dipped flowers on the same plant. Therefore, large numbers of seeds, approximately 20,000 seeds per plant, need to be screened to identify positive transformants[8]. Flax, on the other hand, has fewer branches (one main branch and a few side branches) and fewer flowers, producing approximately 100 seeds per plants, which makes it possible to track individual flowers and to select specific seeds during screening process.

We propose that floral-dip is an applicable method to transform any related species of flax, a genus of approximately 200 species. This method gives much higher transformation rate than other methods of flax transformation. We are also proposing that the direct PCR screening of T1 leaf DNA is an efficient way to overcome the problem of antibiotic resistance escape that often produces many false positives. Direct PCR screening can be applied to any other plant species and is not limited to flax. The simple seed tracking technique employed in this protocol can be applied to any other plant species with branching anatomy similar to flax.

Protocol

1. Growing the Plants

1. 6 weeks prior to dipping, fill 5 inch pots with soil and sow flax seeds ¼ inch deep in the soil (4 seeds per pot). Be sure to firm the soil over the seeds. Water the plants regularly and maintain them in long daylight (14 hr light and 10 hr dark).
2. Check plants regularly for primary inflorescences (clusters of flowers' organs). The plants are ready for transformation when the buds are visible and have just formed in the inflorescence (**Figure 1 and 2**). To see the buds, cut the leaves around it if necessary.
   NOTE: Using the best flower stage is critical and is detailed in the **Discussion**.
   1. Use the main branch of the plant for the experimental treatment as it produces more flowers than the side braches. Use the side branches either as controls (not to be dipped) or for other experimental treatments.
   2. Alternatively, use the main and side branches of the same plant for the experimental treatment and use another plant as a control or for other experimental treatments. Use labels to mark individual branches and individual flowers with the date and type of treatment.

2. Cloning and Transformation in to *Escherichia coli* (*E. coli*) Cells

1. Clone the fragment/gene of interest into the multiple cloning site of a plant binary vector harboring the T-DNA.
   1. Perform cloning in one step or two steps.
      1. Directly clone small inserts in this protocol (~500 bp) into the plant binary vector. In this protocol, use the plant binary vector (PRI909). Otherwise, use any other plant binary vectors in a similar strategy.
      2. Alternatively, clone large inserts (≥6.5 kb) in two steps: first using a general commercial cloning kit (not plant specific), then subclone into the plant binary vector.

2. Set up a PCR reaction to amplify the gene of interest. Design the primers with restriction sites at the 5' end (according to the multiple cloning site of the plant binary vector in use).
   1. Perform a standard PCR using genomic flax DNA with the following cycling conditions: an initial hold of 24 °C, then 2 min at 94 °C, followed by 30 cycles of 98 °C for 5 sec, 60 °C for 15 sec, and 72 °C for 2 min. Perform a final extension step at 72 °C for 5 min followed by an indefinite hold at 4 °C.
   2. Separate PCR products on 1% Tris/Borate/EDTA (TBE) agarose gel, run the gel at 100 V for 1 hr. Purify the products from the gel and quantify via Nanodrop.

3. Set up the ligation mix to clone the PCR product in the commercial cloning vector. Follow manufacturer's protocol.
4. Transform the ligation mix into chemically competent *E. coli* cells as follows.
   1. Add 2 µl of the ligation mix into one vial of chemically competent *E. coli* cells. Incubate on ice for 30 min.
   2. Heat shock the cells for 30 sec at 42 °C (Heat shock time and temperature depends on the type of cells used).
   3. Incubate on ice and add 250 µl of RT super optimal broth with catabolite repression (S.O.C) medium.
   4. Cap the tube and incubate in an orbital shaker at 200 rpm at 37 °C for one hr.
   5. Spread 10 - 50 µl from each transformation on a prewarmed LB plate (prewarmed at 37 °C) + appropriate selective antibiotic (determine the selective antibiotic based on the type of commercial cloning vector used). Incubate plates at 37 °C O/N.

5. Pick ~10 colonies for mini prep plasmid purification, using a commercial kit. Mini prep plasmid purification is generally performed as following.
   NOTE: Buffer names are specific to the commercial kit used but their general functions are similar.
   1. Inoculate the single colonies in 2 - 5 ml LB medium containing the appropriate selective antibiotic (determined based on the type of commercial vector used).
   2. Incubate for approximately 8 hr at 37 °C with vigorous shaking (~300 rpm).
   3. Harvest the cells by centrifugation at 6,000 x g for 10 min.
   4. Resuspend the pellet in the 250 µl resuspension buffer. Mix and vortex to completely disperse the pellet.
   5. Lyse the bacterial cells by adding 250 µl of the lysis buffer, mix thoroughly by inverting the tubes 4 - 6 times.
   6. Neutralize the lysate in the neutralization buffer and invert 4 - 6 times to mix. Centrifuge for 10 min at ~17,900 x g on a table-top microcentrifuge.

7. Transfer the supernatants from previous step to a spin column. Centrifuge again at ~17,900 x g for 30 - 60 sec.
8. Wash the spin column by adding 0.75 ml washing buffer and centrifuge for 30 - 60 sec. Discard the flow-through.
9. Centrifuge for an additional 1 min to remove residual wash buffer. Place the spin column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl water to the center of each column. Let stand for 1 min, and centrifuge for 1 min.

3. Analyze the Purified Plasmids for the Presence of Insert

1. Restriction Digest:
    1. Set up a restriction digest to determine the presence of the insert by digesting the plasmid with the restriction enzymes used to clone the insert. A typical double restriction digest reaction as follows: 1 µg of purified plasmid, 1 µl of each restriction enzymes, 2 µl 10x restriction digest buffer + 2 µl BSA (if applicable), X µl water (to a total of 20 µl)
    2. Mix gently and incubate at recommended temperature (varies from one enzyme to another).
    3. Analyze the restriction digest reaction on 1% TBE agarose gel, run at 100 V for 1 hr and look for drop-out of appropriate size.
2. PCR analysis:
    1. Set up a PCR with the purified plasmid, using primers from the commercial vector region to amplify inside the insert or the junction between the vector and the insert. Common primers are M13 forward and reverse and T3/T7 primers.
    2. In this protocol, use the following PCR cycling conditions: an initial hold of 24 °C, then 2 min at 94 °C, followed by 18 cycles at 98 °C for 5 sec, 60 °C for 15 sec, and 72 °C for 2 min. Perform a final extension step at 72 °C for 5 min followed by an indefinite hold at 4 °C.
    3. Load PCR products on a 1% TBE agarose gel and run for 100 - 120 V for 1 hr.
3. Sequencing:
    1. Send the purified plasmid to a commercial sequencing facility to analyze and confirm the presence of the insert.
    2. Once the correct construct is obtained, use it for the second step of cloning (into the plant binary vector).
    NOTE: **Steps 2.3 - 3.3** can be eliminated if the cloning is completed in one step.

4. Cloning into the Plant Binary Vector (PRI909) and *E. coli* Transformation

1. Set up a double restriction digest reactions to linearize the plant binary vector and isolate the previously cloned insert using the same restriction enzyme sites that were added to the primers (**step 2.2**).
2. Analyze the restriction digests using gel electrophoresis, run at 100 V for 1 - 2 hr. Cut the insert and the linearized plant binary vector from the gel.
3. Use a commercial kit to purify the gel products. Refer to manufacturer's manual.
4. Set up a ligation reaction to ligate the insert into the plant binary vector. The insert to vector ratio depends on the size of the insert and the plant binary vector. In this protocol the LIS1 insert was 6.5 kb and the plant binary vector was 9 kb. Hence, use a 1:2 insert to vector ratio.
5. Repeat **steps 2.4 - 3** for the bacterial transformation, plasmid purification and analysis. Once the correct construct (insert + plant binary vector) is obtained, proceed to the electroporation in **step 5**.

5. Electroporation into *Agrobacterium tumefaciens* Electrically Competent Cells

1. Thaw a vial of *Agrobacterium tumefaciens* electrically competent cells on ice.
2. Add 1 ng of binary vector plasmid DNA to 20 µl of competent cells, on ice, and mix gently.
3. Chill a 0.1 cm electroporation cuvette on ice.
4. Transfer the competent cell/DNA mix to the electroporation cuvettes, and tap to collect the mixture at the bottom. Put the cuvette in the electroporator machine and pulse (voltage and time conditions depend on the size of cuvette and the electroporator used).
5. Add 1 ml of SOC media and transfer cells to a 15 ml tube.
6. Incubate for 1 hr at 28 - 30 °C, shaking at 100 rpm. Plate 50 - 100 µl of cells on LB agar + appropriate selective antibiotic (depend on the plant binary plasmid and the strain of *Agrobacterium* used. In this protocol use 50 µg/ml kanamycin and 100 µg/ml streptomycin).
7. Incubate the plates up to 48 hr at 28 - 30 °C.
8. Repeat **step 2.5** for colony selection and plasmid purification.
9. Repeat **step 3** to analyze the plasmid and to check the integrity of the insert and T-DNA before using the construct for floral-dipping. Repeat **step 3.2**, using multiple PCR primers across the entire insert region and across the T-DNA regions and by sequencing as in **step 3.3**.
NOTE: In this protocol 4 different primers were designed across the T-DNA and 10 primers across the insert.
10. Once the presence of the plant binary plasmid in the selected *Agrobacterium* colony is confirmed, place cells in 50% glycerol and store at -80 °C. Store remaining colonies on the plate at 4 °C if they are to be used within one week[7].

6. Floral-Dipping

NOTE: 2 days prior to floral-dipping:

1. Grow *Agrobacterium* cells to the stationary phase (OD_{600} between 0.5 - 1 is acceptable) in LB + appropriate antibiotics in liquid media.
NOTE: These are the same antibiotic as in **step 5.6**, based on the plant binary vector and the type of *Agrobacterium* strain used.
2. Start the culture with 1:100 dilutions of a saturated (5 ml) O/N culture and grow for 24 - 48 hr at 28-30 °C while shaking at 150 rpm. The culture should have reached the midlogarithmic phase and more likely will be approaching or at stationary phase[1]. OD of approximately 0.8 (again OD between 0.5 to 1 are all acceptable)[8]. Collect the cells by centrifuging at 5,000 x g at RT.

3. Resuspend the cells in infiltration medium (5.0% sucrose + 0.05 - 0.003% Silwet L-77). For the first round of dipping, use 0.05% Silwet-77. For the second and third rounds, reduce the concentration to 0.03% (detailed in **Discussion**).
4. Proceed with the floral-dip step. Lay the plant on its side and dip only the visible buds in the infiltration medium for 1-2 min. Leave the plant on its side and cover it with plastic wrap to maintain high humidity in the dome (**Figure 3**).
5. On the next day, place the plant in an upright position and maintain normally.
6. When the bud grows larger (usually after about 10 - 14 days), repeat **steps 6.1 - 6.4**. Reduce the dipping time to 30 - 60 sec and Silwet-77 concentration to 0.003%.
7. Maintain the plants normally until their seeds are mature and ready to be collected (**Figure 4**).

7. Selection of Positive Transformants with Direct PCR

1. Sow the T1 seeds on soil as in **step 1.1**.
    1. Alternatively, make Murashige and Skoog basal salt medium (MS medium) by adding 2.2 g of MS medium + 4 g of agar in 500 ml water. Autoclave and pour into small plant pots. Keep in the 4 °C until used.
    2. Sow the seed by placing them on the solidified MS medium. Keep under long daylight (14 hr light and 10 hr of dark). Seeds will germinate in about 4 - 6 days (**Figure 5**).
    3. Use one seed from each flower as a starting point. If no positive transformant is obtained, repeat this step by picking another seed from the flower.
    NOTE: Test different seeds from the same flowers, because in some cases not all seeds from one flower will be transformed. Selecting additional seeds from experimental flowers is sometimes necessary.
2. Check on the seedlings regularly. In approximately 10 - 14 days after germination, when true leaves develop, test the plants with direct PCR.
3. Prepare the leaf DNA extract by cutting 2 - 3 leaves (5 - 10 mg in weight) from each seedling and place them in a microcentrifuge tube.
4. Add 180 µl of 50 mM NaOH to each tube, and incubate for 10 min at 95 °C.
5. Neutralize the extract by adding 20 µl of 1 M Tris-HCl (pH 8.0).
6. Use 1 µl of the extract in the direct PCR reaction, using primers designed across the T-DNA or the insert (**Figure 7**), to select for positive transformants.
    1. In this protocol, use direct PCR with an initial hold of 24 °C, then 2 min at 98 °C, followed by 40 cycles of (98 °C for 10 sec, annealing step for 15 sec, and an extension step at 68 °C). Perform a final extension step at 68 °C for 5 min followed by an indefinite hold at 4 °C. (Refer to **Table 1** for details on primers, annealing temperatures and extension times for the cycles).
7. Identify positive transformants and grow them to maturity. If the seeds were germinated in MS medium, transplant to soil in a bigger pot (**Figure 5**).

Representative Results

Figure 1 - 4 illustrate some of the steps within the protocol. In **Figures 1 and 2**, the leaves around the inflorescence buds are cut to expose them to the *Agrobacterium* cells and the different bud stages that were used to develop the protocol. **Figure 3** shows the process of flax floral-dip. **Figure 4**, shows an example of how the main and side branches can be labeled and how individual flowers can be tracked and identified. **Figure 5** shows how the T1 progenies can be germinated on the MS plant media and then transplanted to soil for maturity. **Figure 6** illustrates how wild-type flax can escape high concentrations of kanamycin, confirming previous findings in the literature[6,9,14].

Figure 7 shows an example of direct PCR amplification from positive T1 transformants. The T1 flowers were collected from the main and side shoots of a single T0 plant. As can be seen from the direct PCR, 8/12 T1 plants tested positive by PCR and have amplified the different regions across the T-DNA. Our primers were also designed between the LIS-1 insert and the multiple cloning sites (**Figure 7B and C**). We used additional primers from the plant binary vector to amplify different segments of the T-DNA, such as the left border and the NOS terminator (data not shown) or the right border and the multiple cloning site (**Figure 7D**). Primers specific to the LIS-1 insert were also used in this protocol (data not shown). A list of primers is provided in **Table 1**. However, the sequences of these primers depends on the sequence of the T-DNA plant binary vector and the insert used for the floral-dip. We also noted that there was no significant difference in the transformation rate between flowers collected from the main and side branches.

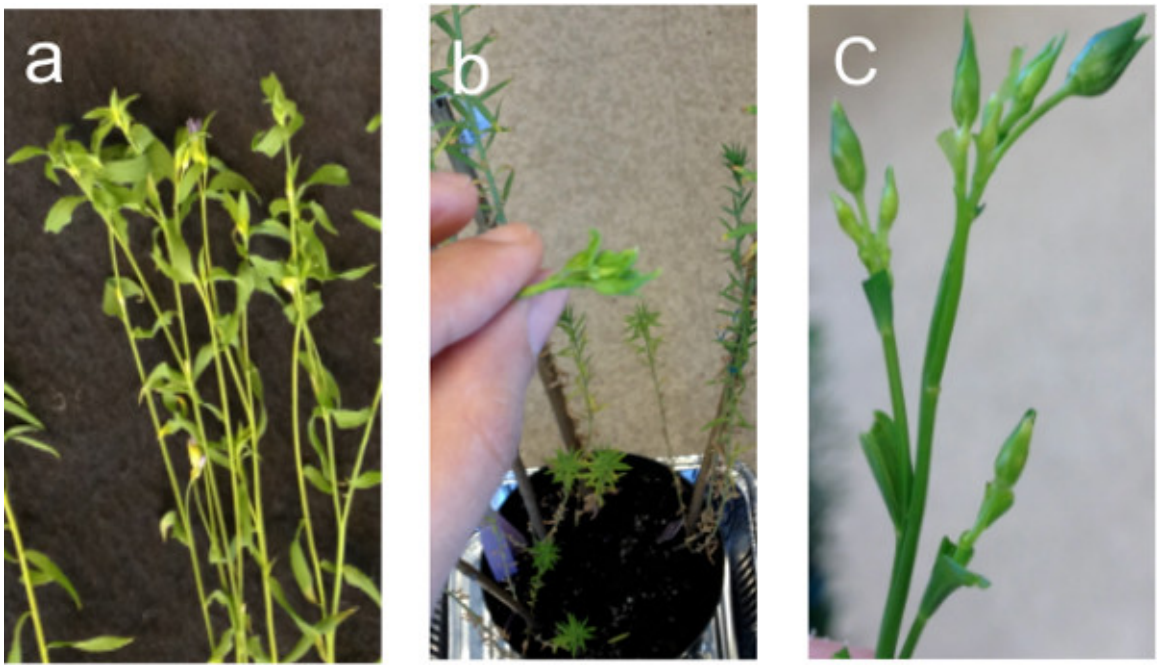

Figure 1. Cutting the leaves around the primary inflorescence buds to expose them to the *Agrobacterim* cells. **(A)** The buds are covered by leaves. **(B)** Leaves have been cut around the buds to expose them. **(C)** Magnified image from plant in **(A)** after cutting to expose buds. Please click here to view a larger version of this figure.

Figure 2. The different bud stages that were used in this protocol to determine the best stage to use for the floral dip. (A) The early stage bud is approximately 2 mm. **(B)** The middle stage bud is approximately 5 mm. **(C)** The Late stage bud is approximately about 1 cm. Please click here to view a larger version of this figure.

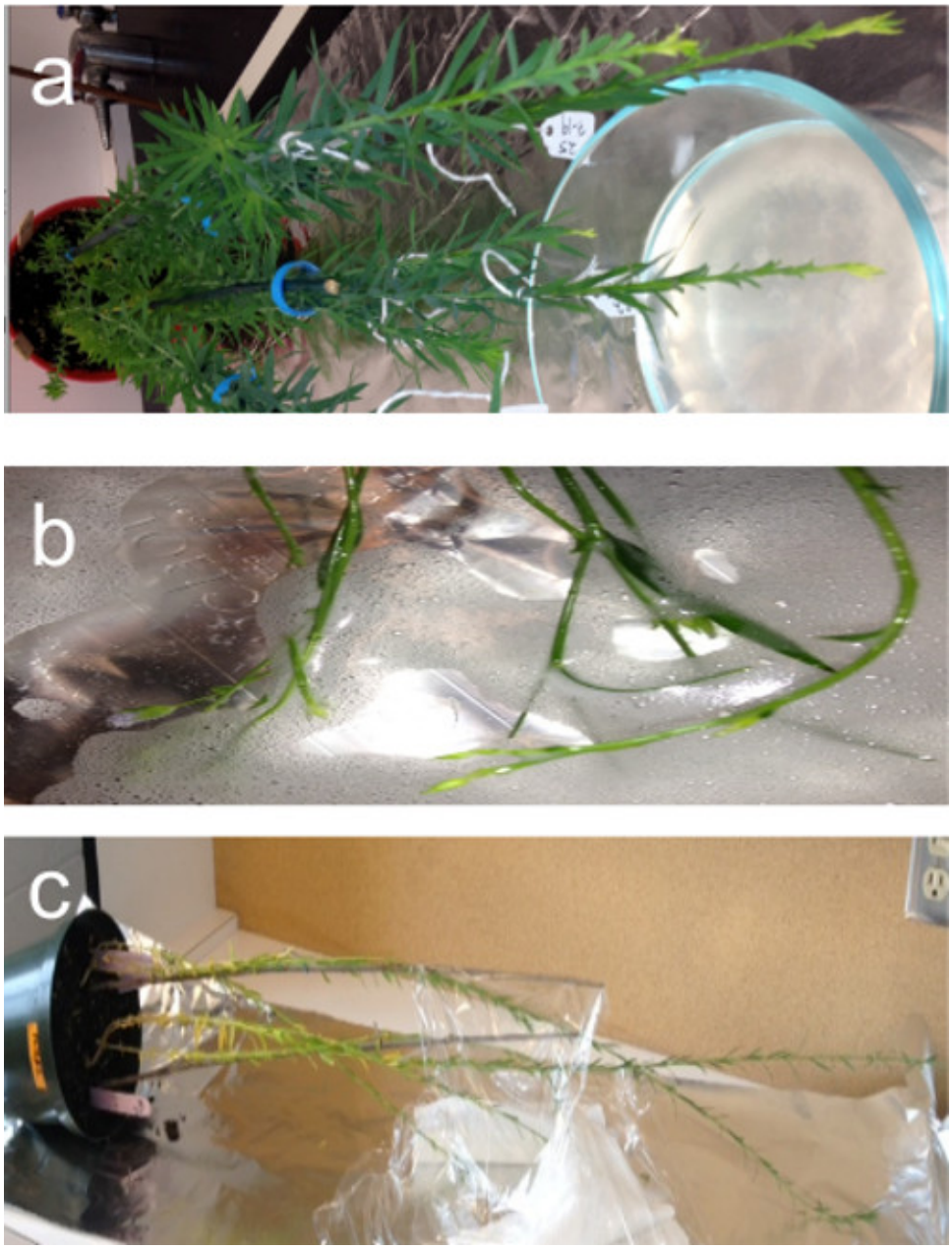

Figure 3. The process of flax floral-dipping. **(A)** The primary inflorescences are dipped in the infiltration media containing the *Agrobacterium* cells. **(B)** Magnified from **(A)**. **(C)** The dipped plants are laid flat until the next day, and the dipped branches are covered with plastic to maintain high humidity. Please click here to view a larger version of this figure.

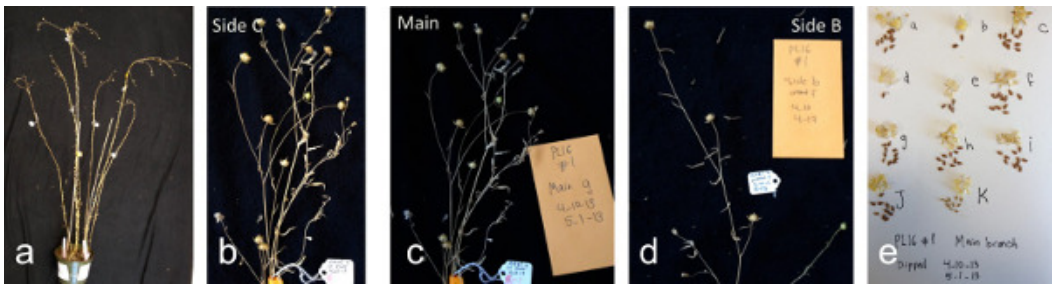

Figure 4. The process of flower tracking and seed collections, from the T0 treatedplants. (A) An example of the whole plant with the main branch (the tallest branch in the center) and the side branches. **(B - D)** An example of the flowers from the different branches. **(E)** An example of the seeds collected from individual flowers (labeled a - k) from the main branch. Please click here to view a larger version of this figure.

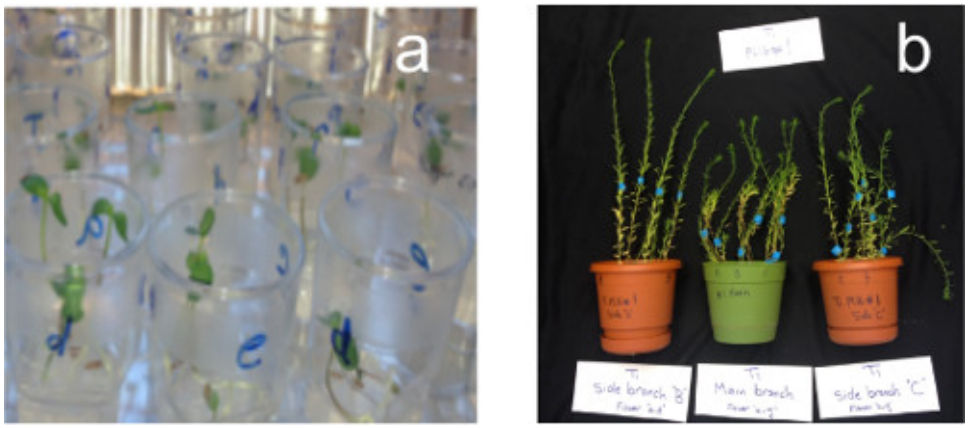

Figure 5. The T1 seedlings are grown without antibiotic selection. (A) T1 seeds are germinated on the MS plant media. **(B)** Positive transformants, as determined by direct PCR, are transplanted to soil and grown to maturity. Please click here to view a larger version of this figure.

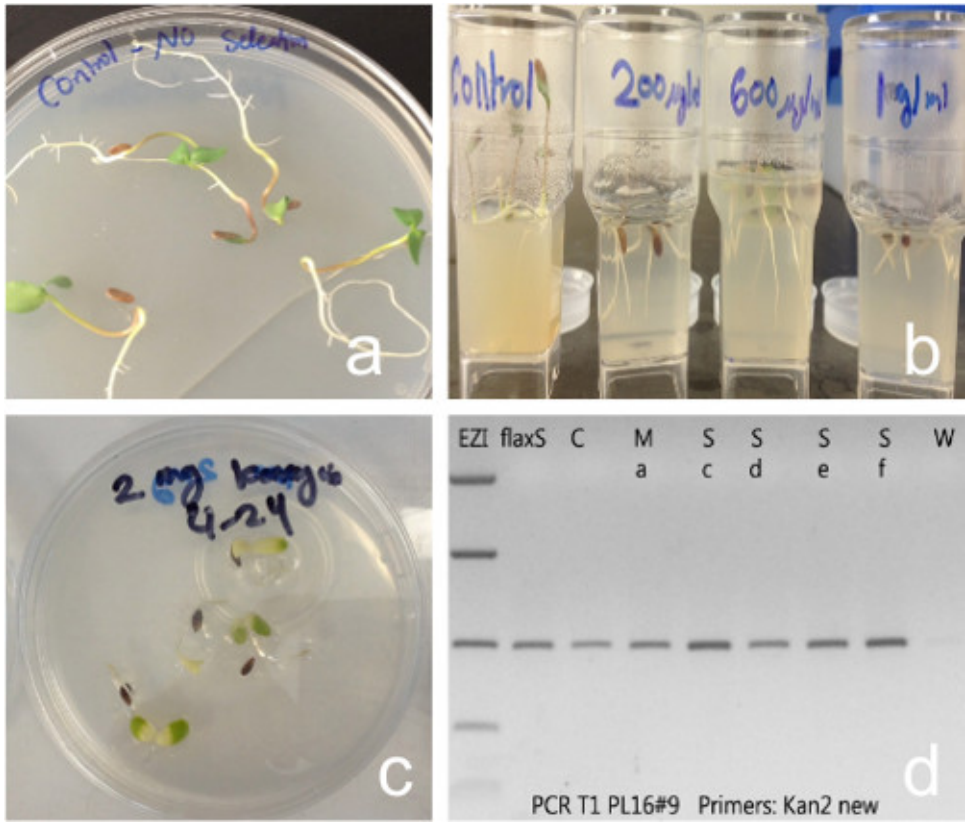

Figure 6. Antibiotic escape, a problem for T1 selection, is overcome by direct PCR screening. (A) Wild-type flax seeds germinated on MS plant media without antibiotic. **(B)** Wild-type flax seeds germinated on MS plant media+ increasing concentrations of kanamycin (200 µg/ml, 600 µg/ml, 1 mg/ml). **(C)** Wild-type flax seeds germinated on MS plant media with 2 mg/ml kanamycin. **(D)** PCR from wild-type flax and T1 seedling using kanamycin primers, all amplified the kanamycin gene (legends: EZ1: DNA marker, FlaxS, wild-type flaxS, C: control non-dipped branch, Ma: T1 progeny from flower “a” collected from the main branch, Sc,Sd,Se,Sf: T1 progenies of different flowers “c,d,e,f” collected from the side branch, W: no-DNA). Please click here to view a larger version of this figure.

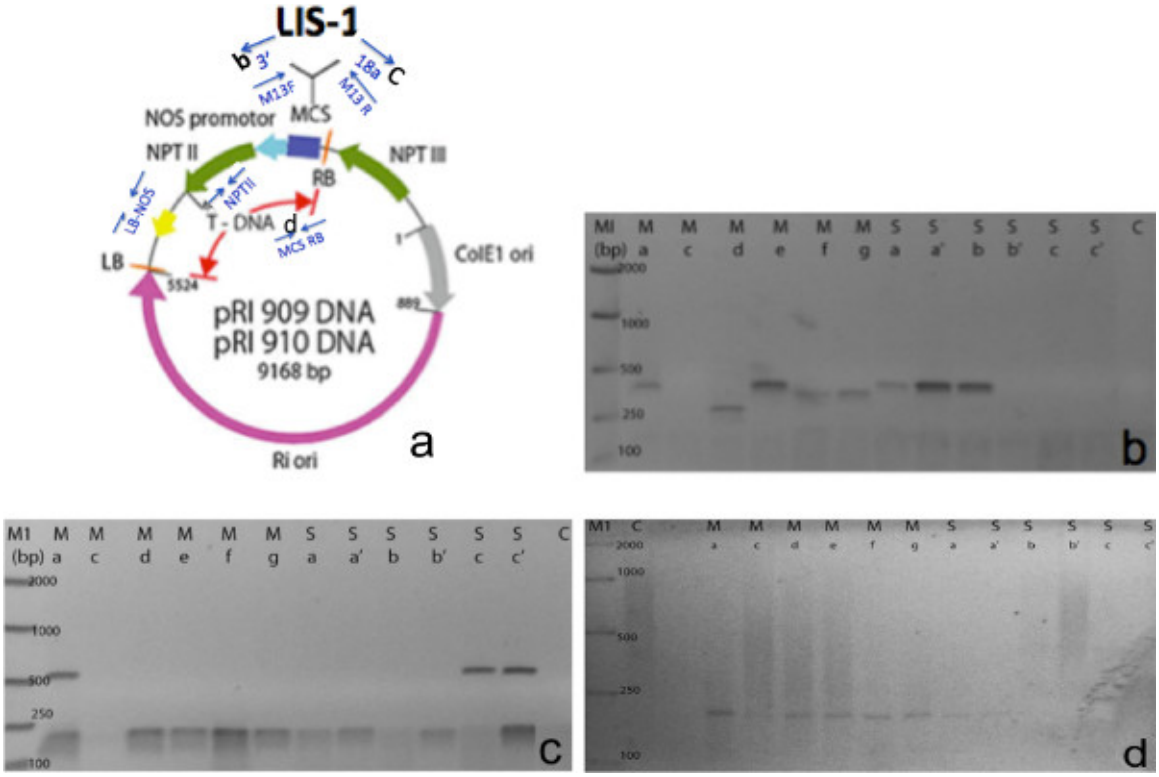

Figure 7. An example of successful PCR amplifications of T1 progenies using the direct PCR method. (A) Diagram of the Plant binary vector+ the cloned LIS-1 insert. Blue arrows indicate the position of PCR primers used in the direct PCR screening (modified from Takara). **(B)** PCR with primers M13F+3' **(C)** PCR with primers M13R+18a **(D)** PCR with primers right border (RB) and multiple cloning site (MCS) **Each lane represents T1 from individual flowers collected from C: control branch (non-dipped), M: main branch flower a-g, S: side branch flower b-c'. Please click here to view a larger version of this figure.

Forward primer sequence source	sequence 5'-3'	Reverse primer sequence source	Sequence 5'-3'	Annealing Temprature (°C)	Extension Time (Sec)	Expected size (bp)
M13F (T-DNA)	CTGCAAGGCG ATTAAGTTGG	3' (LIS-1 insert)	GAGGATGGAA GATGAAGAAGG	57	40	450
18a (LIS-1 insert)	TATTTTAACCC TATCTCCCAACAC	M13R (T-DNA)	ATTAGGCACC CCAGGCTTTA	57	40	520
MCS (T-DNA)	TGGTCATAGC TGTTTCCTGTG	RB (T-DNA)	TTTAAACTGA AGGCGGGAAA	60	20	200
LB (T-DNA)	TTTGATGGTG GTTCCGAAAT	NOS (T-DNA)	GAATCCTGTT GCCGGTCTT	60	30	380
NPTII (T-DNA)	GCGATACCGT AAAGCACGAG	NTPII (T-DNA)	GCTCGACGTT GTCACTGAAG	65	45	502

Table 1. Some of the primers used for the direct PCR testing.

Discussion

In some plant species, such as flax (*Linum usitatissimum*), successful plant transformation has been limited. Previously, transformation in flax has required an *Agrobacterium* infection by wounding and co-cultivation, applying biolistic particles or using ultrasound sonication, followed by regeneration; a process that is both long and prone to being accompanied by many mutational events. Moreover, the selection process of these techniques requires the use of antibiotic selectable markers such as kanamycin. However, it has been noted in the literature that this method of selection produces many false positives, as flax tends to escape high concentrations of antibiotics[6,9,14]. Another disadvantage of the previous techniques in flax transformation has been the low transformation rates[2,6].

In the protocol described here, *Agrobacterium*-mediated plant transformation via floral-dipping was shown to result in a high transformation rate for flax (50 - 60%). Transformants were obtained from flowers dipped and collected from main and side branches. Selection of positive transformants was simply done by growing T1 plants on soil and screening their leaves soon after they germinated, by-passing the use of antibiotic selection, a step previously used as a norm in floral-dip for other plant species. By performing direct PCR testing of leaves, and using the appropriate T-DNA primers, positive transformants can be rapidly selected. This technique is simple, inexpensive and easy to perform, yet

results in a much higher transformation rate than those previously reported for *Arabidopsis* and other plant species using this method[1,10,12]. It is also the highest reported transformation rate for flax.

However, there are critical steps in the procedures including the selection of the best flower stage and the best surfactant concentration, so that the *Agrobacterium* can penetrate into the plant cells without killing the flower organs. If an early bud stage is used (**Figure 2A**) with high Silwet-77 concentration of more than 0.05%, the flower will not develop nor set seeds. If late bud stage is used (**Figure 2C**), although the transformation might work, it will occur at a much lower rate. Similar results were obtained with *Arabidopsis* floral dip transformation[1,4]. For this protocol, all the floral stages were tested with different Silwet-77 concentrations and the best stage was determined to be the middle bud stage (**Figure 2C**) with Silwet-77 at 0.05% for the first dipping, followed by a second dipping at the late bud stage (**Figure 2C**) with a slightly reduced Silwet-77 concentration of 0.03%. The transformation also worked well using the early bud stage (**Figure 2A**) with a low silwet-77 concentration of 0.003%, followed by a second dipping with middle bud stage (**Figure 2B**) at higher Silwet-77 concentration of 0.05%.

In this protocol, some other parameters were attempted to optimize the transformation rate, but found to have no effects on the final outcome. Examples include extending the time after dipping that the plants lay on their side and covered in plastic from one day to two days; using an OD of more than 1 for *Agrobacterium* culture, instead of 0.5 - 1; increasing the dipping time to 5 - 15 min instead of 1 - 2 min. Again we have not noticed any effect on the transformation rate using these strategies. The most effective factors, however, were found to be using healthy plants at the correct flower stages, and using the best Silwet-77 concentration. We noticed that two dipping intervals, works somehow better than one time, even though one time dipping also works.

Modification to this protocol can be achieved by reducing the Silwet-77 concentration to as little as 0.003% in the second or third dipping. Since Silwet-77 is toxic, too high a concentration results in the flowers developing poorly, resulting in no seed yield. The dipping frequency can be reduced to one, with the second or third events eliminated if the plants are not looking healthy and the buds are not developing well.

A major limitation of this technique is the low number of flowers produced by the flax, the limited number of seeds obtained from each flower, and the long life cycle of flax. It takes 6 - 8 weeks from seed sowing to have the primary buds ready for the first dipping and an additional 8 - 10 weeks post-dipping to get to the T1 generation. In total, a range of 5 - 6 months is needed to obtain the T1 generation. Unlike other plant species, which flower anytime of the year, some flax varieties flower better at specific times the year. So thoughtful planning for this technique is important.

In summary, our results of floral dip with two different flax varieties: the fiber flax, Stormont Cirrus (responsive and plastic), and the oil flax, Bethune (stable and non-responsive), show that *Agrobacterium*-mediated plant transformation via floral-dip is an applicable and efficient method for flax transformation and can be used to replace the previously used techniques for flax transformation. The modifications of the floral-dip method in this protocol will be applicable for use with any other plant species and not limited to flax.

Disclosures

The authors have nothing to disclose.

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