Usage note:

FAST-R: Some transgenic lines with FAST-R gave red fluorescence in true leaves, while none of transgenic lines with FAST-G gave fluorescence in true leaves.

FAST-R07: The linker sequence between a gene of your interest and GFP is composed of 53 bp (see below), which is not a multiple number of 3. To avoid a frame shift, please add one base (G or C) to 3' side of the gene of your interest.
The linker sequence:
[a gene of your interest] G aagggtgggcgcgccgacccagctttcttgtacaaagtggtgatatcccgcgg [GFP]

Please let us know if you have any problems on the vectors.

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TECHNICAL ADVANCE

A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of *Arabidopsis thaliana*

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SUMMARY

The creation of transgenic plants has contributed extensively to the advancement of plant science. Establishing homozygous transgenic lines is time-consuming and laborious, and using antibiotics or herbicides to select transformed plants may adversely affect the growth of some transgenic plants. Here we describe a novel technology, which we have named FAST (fluorescence-accumulating seed technology), that overcomes these difficulties. Although this technology was designed for use in *Arabidopsis thaliana*, it may be adapted for use in other plants. The technology is based on the expression of a fluorescent co-dominant screenable marker FAST, under the control of a seed-specific promoter, on the oil body membrane. The FAST marker harbors a fusion gene encoding either GFP or RFP with an oil body membrane protein that is prominent in seeds. The marker protein was only expressed in a specific organ (i.e. in dry seeds) and at a specific time (i.e. during dormancy), which are desirable features of selectable and/or screenable markers. This technique provides an immediate and non-destructive method for identifying transformed dry seeds. It identified the heterozygous transformed seeds among the T_1 population and the homozygous seeds among the T_2 population with a false-discovery rate of <1%. The FAST marker reduces the length of time required to produce homozygous transgenic lines from 7.5 to 4 months. Furthermore, it does not require sterilization, clean-bench protocols or the handling of large numbers of plants. This technology should greatly facilitate the generation of transgenic Arabidopsis plants.

Keywords: ***Arabidopsis thaliana*, oleosin, seed, green fluorescent protein, transformation, screenable marker.**

INTRODUCTION

Many studies of plant physiology and molecular biology rely on the creation of transgenic plants, especially of model plants such as *Arabidopsis thaliana*. Two revolutionary methods for transformation of *A. thaliana*, namely vacuum infiltration (Bechtold and Pelletier, 1998) and floral dip (Clough and Bent, 1998), have been developed, and are widely used. These methods of transformation are most successful when applied to Arabidopsis plants, and are less efficient at generating transgenic plants in other species.

However, establishing transgenic plants is still a slow process that can take months, as only a small fraction of the seeds of the T_1 generation are typically transgenic. The transgenic plants are conventionally identified with a selectable marker that allows them to grow in the presence of an antibiotic or herbicide. However, this approach to generating transgenic plants has the following serious difficulties. First, transformed plants that have a defect in their growth are occasionally sensitive to antibiotics or herbicides, although they have an antibiotic- or herbicide-resistance gene (Yin *et al.*, 2008). Second, the conventional selectable markers require sterile work conditions, the handling of many T_2 plants and large spaces to grow the plants. Sowing many seeds onto agar plates to identify the plants that have been successfully transformed is time-consuming and laborious.

In this study, we established a novel technology of plant transformation, named FAST (fluorescence-accumulating seed technology), that overcomes these difficulties. This technology allows the identification of transformed dry seeds immediately after harvesting. It is thus not necessary to germinate plants on agar plates to identify the transformants. FAST markers are co-dominant markers that save time, eliminate the need for antibiotics and herbicides, and are cheap to produce and use. Much research focuses on applying the floral-dip method of transformation to other useful plants,

such as radish (*Raphanus sativus*; Curtis and Nam, 2001) and wheat (*Triticum* spp.; Agarwal *et al.*, 2009). The FAST marker may complement these studies by allowing the rapid identification of transgenic plants generated in these species.

RESULTS AND DISCUSSION

Seeds of transgenic plants expressing the OLE1-GFP fusion are fluorescent

We focused on using endogenous screenable markers to identify transgenic Arabidopsis seeds. Arabidopsis seeds accumulate a large quantity of oil in oil bodies, which are surrounded by phospholipid membranes with embedded proteins within the cell (Figure 1a) (Huang, 1992). Oleosins are abundant structural proteins embedded in oil body membranes (Abell *et al.*, 1997), have an important function in regulating the size of oil bodies (Siloto *et al.*, 2006), and confer freezing tolerance upon seeds (Shimada *et al.*, 2008). OLE1 is the most abundant oleosin in Arabidopsis seeds (Shimada *et al.*, 2008). We generated transgenic Arabidopsis plants that expressed the OLE1-GFP fusion protein, under the control of the *OLE1* promoter, in seeds. We investigated the subcellular localization of OLE1-GFP in the seed cells of transgenic plants by immunoelectron microscopy, using antibodies against either OLE1 or GFP. Gold particles revealing the localization of OLE1-GFP were specifically detected on the oil body membranes (Figure 1b,c). Confocal microscopy revealed that OLE1-GFP fluorescence was detected on the network-like formation that corresponds to the oil body membrane in the developing seeds of transgenic plants (Figure 1d). These results indicate that OLE1-GFP is correctly and specifically targeted to the oil bodies in the transgenic seeds. Surprisingly, the seeds of these transgenic plants emitted green fluorescence under a fluorescence stereomicroscope (see below). This result suggests that OLE1-GFP could be used as a screenable marker of transformation.

We constructed a plasmid vector that contained the *OLE1* promoter followed by *OLE1-GFP*, which we designated pFAST-G (plasmid of fluorescence-accumulating seed technology with OLE1-GFP). We introduced pFAST-G into Arabidopsis plants to obtain seeds of the first generation (T_1), and then inspected the seeds with a fluorescence stereomicroscope (Figure 2). A few of the T_1 seeds exhibited green fluorescence (Figure 2a, T_1 generation). The fluorescent T_1 seeds were then grown to obtain seeds of the second generation (T_2). Interestingly, the T_2 seed population contained three types of seeds: non-fluorescent, moderately fluorescent and strongly fluorescent seeds (Figure 2a, T_2 generation). We found that the number of fluorescent seeds in the T_2 generation was about three times greater than the number of non-fluorescent seeds (see below). We further generated T_3 seeds from the strongly fluorescent T_2 seeds. All of the T_3 seeds gave green fluorescence (Figure 2a, T_3 generation). These observations suggest that non-fluorescent seeds correspond to the non-transformed type, moderately fluorescent seeds corresponded to the heterozygous type and strongly fluorescent seeds corresponded to the homozygous type. If this is indeed the case, FAST-G may allow the instant identification of the heterozygous transformed seeds among the T_1 population, and then of the homozygous seeds among the T_2 population.

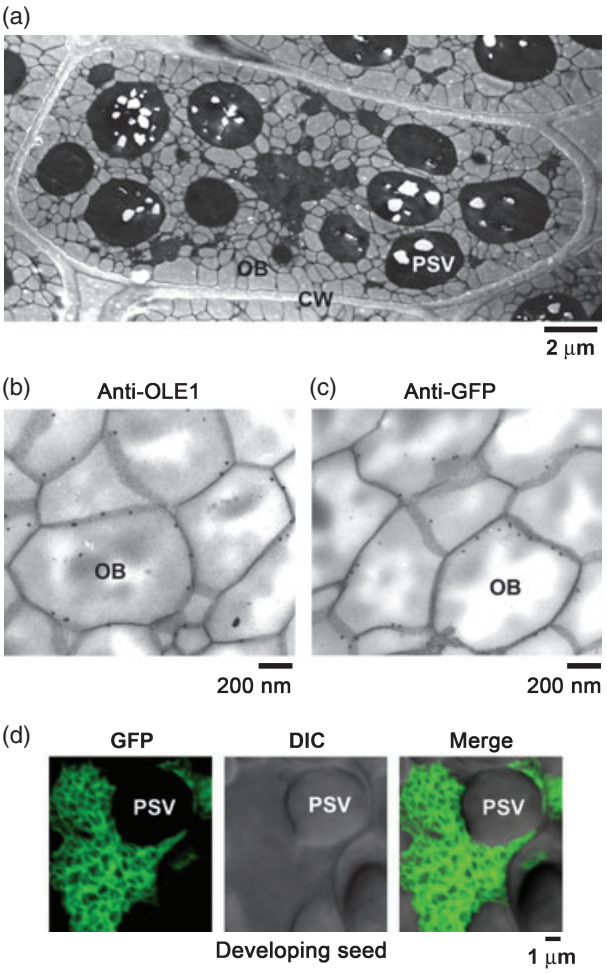


Figure 1. The OLE1-GFP fusion protein is localized to oil body membranes in the seed cells of transgenic Arabidopsis plants.
(a) Electron micrograph of dry seeds of Arabidopsis. CW, cell wall; OB, oil body; PSV, protein storage vacuole.
(b, c) Immunoelectron micrographs of oil bodies in transgenic Arabidopsis seeds expressing the OLE1-GFP fusion protein, showing the localization of OLE1 (b) and GFP (c) on the oil body membranes. OB, oil body.
(d) Confocal laser scanning microscopic image of oil body membranes in developing seeds of transgenic Arabidopsis expressing the OLE1-GFP fusion protein. DIC, differential interference contrast image; PSV, protein storage vacuole.

Homozygous seeds can be isolated from the T_2 seed population using OLE1-GFP

To evaluate the ability of the OLE1-GFP protein produced by FAST-G to function as a screenable maker, we measured the

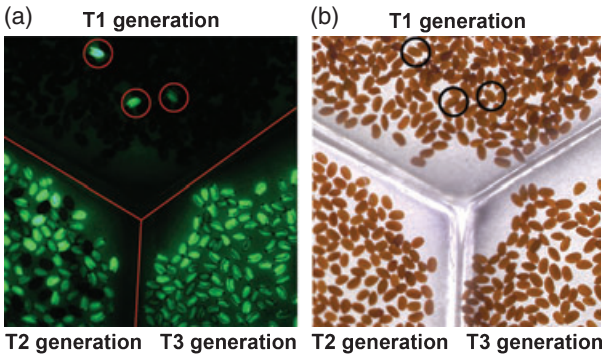
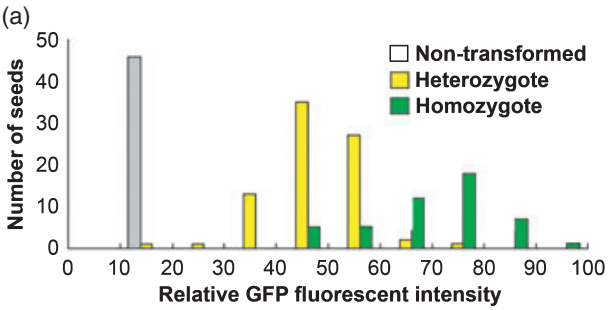


Figure 2. Seeds of Arabidopsis plants transformed with pFAST-G give green fluorescence.
(a) We introduced pFAST-G into Arabidopsis plants and then harvested seeds of the first (T_1) generation. The seeds were inspected with a fluorescence stereomicroscope. The fluorescent T_1 seeds (indicated by circles in the T_1 generation) were grown to obtain seeds of the second (T_2) generation. The T_2 seed population contained three types of seeds: non-fluorescent, moderately fluorescent and strongly fluorescent seeds. The strongly fluorescent seeds were grown to obtain seeds of the third (T_3) homozygous generation.
(b) The same field as in (a), obtained with a binocular microscope.

intensity of the GFP fluorescence emitted by each of the T_2 seeds (n = 174) of transgenic line 1, and then determined the genotype of the respective seedlings. We classified 46 seeds as being of the non-transformed type, 80 seeds as being heterozygous and 48 seeds as being homozygous. Frequency distributions of the fluorescence intensities of each genotype (Figure 3a) revealed that the fluorescence intensities correlated with the genotype of the seeds: the average fluorescence intensity and standard deviation were determined to be 14.76 ± 1.37% of the maximum fluorescent intensity for the non-transformed type, 46.90 ± 9.04% for the heterozygous type and 69.50 ± 14.10% for the homozygous type. As indicated in Figure 3a, seeds with fluorescence intensities of greater than 80% are likely to be homozygous.

The chi-squared (χ^2) test of the histogram presented in Figure 3a revealed that the frequency distribution was normal (P = 0.0911, for the heterozygous type; P = 0.619 for the homozygous type). Using a formula based on the assumption of a normal distribution (Figure 3b), we calculated the false discovery rate (FDR): the proportion of heterozygous seeds amongst seeds with fluorescence intensity above a certain value (X). We also calculated the proportion of seeds with intensities greater than X amongst all of the seeds. For example, fluorescent seeds with intensities in the top 11.6% or less might be homozygous, with an FDR of <1% (Figure 3c). We quantitatively analyzed two more transgenic lines with different fluorescence intensities (Figure S1). Although fluorescence intensity values vary amongst transgenic lines, seeds with fluorescence in the top 1% of all T_2 seeds of a transgenic plant are homozygous. These results suggest that homozygous seeds can be identified in the T_2 seed population using OLE1-GFP as a co-dominant screenable marker.



(b)

$$FDR(\%) = \frac{F(x; Avr^{Ht}, Sd^{Ht}, N^{Ht})}{F(x; Avr^{Ht}, Sd^{Ht}, N^{Ht}) + F(x; Avr^{Ho}, Sd^{Ho}, N^{Ho})} \times 100$$

$$F(x; \upsilon, \sigma, N) = \frac{N}{\sigma\sqrt{2\pi}} \int_{x}^{+\infty} e^{-\frac{(t-\upsilon)^2}{2\sigma^2}}\, dt$$

FDR, the proportion of heterozygous seeds in the seeds with fluorescence intensities higher than a certain value (X).

Avr^{Ht} and Avr^{Ho}, the averages of fluorescence intensities in the T2 heterozygous and homozygous populations, respectively.

Sd^{Ht} and Sd^{Ho}, the standard deviations.

N^{Ht} and N^{Ho}, the numbers of the T2 heterozygous and homozygous seeds.

$F(x; \upsilon, \sigma, N)$, a cumulative distribution function with an average (Avr) and a standard deviation (Sd).

(c)

FDR (false discoveru rate, %)	X	proportion (% of all)
1	72.3	11.6
5	65.6	17.9
10	62.0	22.0

Figure 3. Strongly fluorescent seeds that rank in the top approximately 10% of the T_2 seed population are homozygous, with a false discovery rate (FDR) of <1%.
(a) The T_2 seeds (n = 174) of transgenic line 1 were separated into three genotypes: non-transformed, heterozygous and homozygous. The fluorescence intensities of all seeds of each genotype were measured by setting the maximum value to 100.
(b) The formula used to calculate the FDR.
(c) The proportion of all seeds with intensities higher than X in the T_2 seed population is shown, when the FDR is 1, 5 or 10%.

OLE1-GFP is an efficient screenable marker of homozygous transformed T_2 seeds

We made use of the Gateway destination vector to generate a variant of pFAST-G that contains a multiple cloning site between the CaMV 35S promoter and 35S terminator, into which any gene can be incorporated (pFAST-G02). We incorporated the *caleosin-3* gene (*CLO3*) into the destination vector to produce pFAST-35S-CLO3 (Figure 4a), and then introduced it into Arabidopsis plants to determine whether pFAST-35S-CLO3 functions as an expression vector. It has been shown that *CLO3*, a member of the caleosin family (Chen *et al.*, 1999; Naested *et al.*, 2000; Frandsen *et al.*, 2001), is not expressed in young Arabidopsis leaves, and is

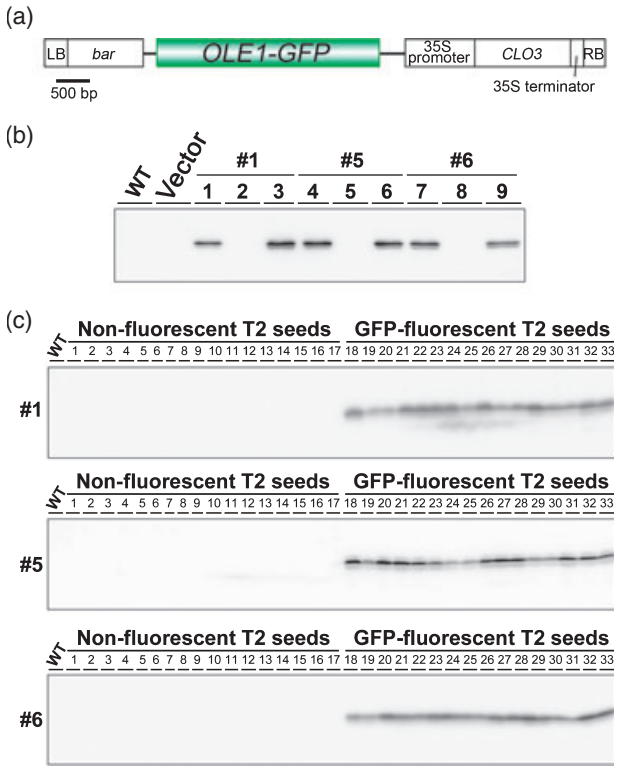


Figure 4. OLE1-GFP is an efficient marker for identifying transformed T_2 homozygous seeds.
(a) The expression vector pFAST-35S-CLO3, which was used to produce transgenic Arabidopsis plants that expressed the *caleosin 3* gene (*CLO3*) under the control of the 35S promoter. *bar*, Basta resistant gene.
(b) Immunoblot of T_2 seedlings from GFP-fluorescent (lanes 1, 4 and 7) and non-fluorescent seeds (lanes 2, 5 and 8), and T_3 seedlings (lanes 3, 6 and 9) of transgenic lines 1, 5 and 6, using anti-CLO3 antibody.
(c) Immunoblot of seedlings from GFP-fluorescent and non-fluorescent seeds of three T_2 lines, using anti-CLO3 antibody.

induced in leaves by desiccation, salt and abscisic acid (Takahashi *et al.*, 2000).

We selected three T_1 lines (1, 5 and 6) that gave GFP fluorescence in seeds and CLO3 expression in leaves. We separated the T_2 seeds from the three lines into a GFP-fluorescent seed population and a non-fluorescent seed population, and then grew the seeds to obtain 7-day-old seedlings. We performed an immunoblot analysis of each seedling using the anti-CLO3 antibody. Figure 4b shows that CLO3 was expressed in seedlings from fluorescent T_2 seeds of each line (lanes 1, 4 and 7) and in fluorescent T_3 seeds (lanes 3, 6 and 9), whereas CLO3 was not expressed in seedlings from non-fluorescent T_2 seeds (lanes 2, 5 and 8). Furthermore, CLO3 was not expressed in wild-type plants (WT, Col-0) or in plants transformed with pFAST-G that had no *CLO3* gene (vector). We further analyzed the fluorescent seeds (n = 16) and non-fluorescent seeds (n = 17) amongst the T_2 seeds of each line. Without exception, the transgene product CLO3 was expressed in the seedlings of all of the seeds that had been isolated using the fluorescent marker OLE1-GFP (Figure 4c). This suggests that OLE1-GFP can be used as a screenable marker of transgenic seeds.

We compared the fluorescent marker OLE1-GFP with a conventional antibiotic- or herbicide-resistance selectable marker, using Arabidopsis plants transformed with pFAST-35S-CLO3 that included the *bar* gene, which confers tolerance to the herbicide Basta (phosphinothricin, glufosinate) (Figure 4a). We separated the T_2 seeds (n = ~300) of each of four transgenic lines (i.e. lines 1, 5, 6 and 20) into a GFP-fluorescent seed population and a non-fluorescent seed population, and then sowed the seeds on agar plates in the presence of Basta to determine their resistance to Basta. We established that all of the fluorescent seeds of each line were resistant to Basta, whereas all of the non-fluorescent seeds were sensitive to Basta (Table 1), indicating that the fluorescent marker OLE1-GFP may be used in place of an antibiotic- or herbicide-resistance gene. The segregation rates of GFP +/Basta^r:GFP –/Basta^s in lines 1, 5 and 6 fitted the theoretical rate of 3:1 for a one-locus insertion of the transgene pFAST-35S-CLO3, and the rate in line 20 fitted the rate of 15:1 for two-locus insertions. As expected, all of the T_3 seeds of lines 1, 5 and 6 were fluorescent and resistant to Basta (Table 2). These results indicate that OLE1-GFP is an efficient marker for the identification of successfully transformed T_2 homozygous seeds.

Apparent segregation rates of GFP fluorescent seeds to non-fluorescent seeds were measured for 1937 T_2 lines that were transformed with pFAST-G (Figure S2). The first seed population (78.1%) with the rates of 3:1 was expected to have one locus of the transgene, whereas the second population (11.6%) with the rates of >15:1 was expected to have more than two loci of the transgene. These values are higher than the values in transgenic Arabidopsis lines generated by inoculation on the shoot apical meristem

Table 1 Ability of OLE1-GFP to detect Basta-resistant transgenic plants

		Number of seeds		
T_2 lines	GFP	Basta^r	Basta^s	Total
#1	+	227	0	301
	–	0	77	
#5	+	212	0	265
	–	0	53	
#6	+	215	0	283
	–	0	68	
#20	+	441	0	472
	–	0	31	

T_2 seeds (n ~ 300) of each of four lines transformed with pFAST-35S-CLO3 (i.e. lines 1, 5, 6 and 20) were separated into a GFP-fluorescent (GFP+) and non-fluorescent (GFP–) seed population, and were then grown in the presence of Basta to determine whether the line was resistant (Basta^r) or sensitive (Basta^s).

Table 2 All of the T_3 seeds identified by the FAST marker were resistant to Basta

	Number of seeds	
T_3 lines	Basta^r	Basta^s
#1	58	0
#5	59	0
#6	67	0

Basta^r, resistant to Basta; Basta^s, sensitive to Basta.

(SAM) (Chang *et al.*, 1994) or vacuum infiltration (Ye *et al.*, 1999). Both methods used conventional selectable markers. The third population (8.7%) produced only non-fluorescent seeds. This might be because of mis-selecting non-transformed T_1 seeds or gene silencing of the transgene. The fourth population (1.7%) exhibited unexpected segregation rates, such as 2:1, 1:1 and <1:1. This might be because of embryo/gametophyte lethal or gene silencing of the transgene. We did not detect gene inactivation in the T_3 generation from 20 independent T_2 fluorescent lines. These results suggest that the probability of gene inactivation is low, at least in the first three generations.

The screenable marker protein OLE1-GFP is only expressed in the seeds of transgenic plants

It has been shown that the *OLE1* promoter is active during seed maturation (Kim *et al.*, 2002). We investigated the expression profile of the fluorescent marker OLE1-GFP in various tissues of the T_3 plants, none of which had any defect of seed germination. The GFP fluorescence was reduced at around 3 days after germination, and disappeared by 5 days (Figure 5a). Fluorescence was observed in developing seeds at the full-sized embryo stage and in dry seeds, but not in the roots, leaves or stems of 7-week-old plants (Figure 5b). These results indicate that the screenable marker protein OLE1-GFP was detected only in the seeds of transgenic plants, and not in other tissues. One cannot exclude the possibility that OLE1-GFP is expressed in vegetative tissues because of the surrounding sequence of the T-DNA insertion site. A desirable feature of selectable and/or screenable markers is that they are expressed only in specific places and at specific times of development. Whereas OLE1-GFP is expressed in dormant seeds, the conventional antibiotic- and herbicide-resistance selectable markers are constitutively and widely expressed.

Purpose-designed variations of the FAST vectors

To expand the versatility, we designed and produced another type of FAST vector that contained the OLE1 promoter followed by *OLE1-TagRFP*, as *TagRFP*, which encodes RFP, produces much brighter fluorescence than mRFP (Merzlyak *et al.*, 2007). We designated this FAST vector pFAST-R (plasmid of fluorescence-accumulating seed technology

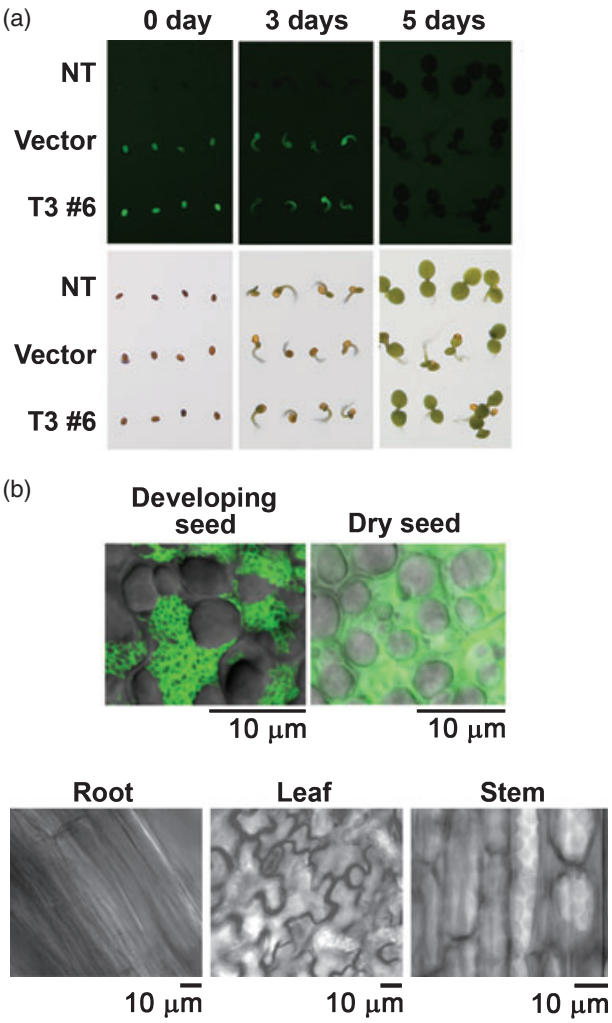


Figure 5. The OLE1-GFP screenable marker is only expressed in the seeds.
(a) The T_3 seeds of transgenic Arabidopsis plants expressing the OLE1-GFP fusion protein with pFAST-G (vector) and with pFAST-35S-CLO3 (T_3, line 6) were grown for 0, 3 and 5 days, and were then inspected with a fluorescence microscope (upper panels) or a stereomicroscope (lower panels). Non-transformed seeds (NT) were used as a control.
(b) Merged images of a confocal laser scanning microscopic image and a differential interference contrast image of each organ of the transgenic line (T_3, line 6). Green fluorescent images show OLE1-GFP on the oil body membrane.

with OLE1-TagRFP). The seeds of plants transformed with pFAST-R produced bright-red fluorescence under a fluorescence stereomicroscope (Figure 6). pFAST-R05, pFAST-R06 and pFAST-R07 (see below) are a valuable tool for determining the subcellular localization of the GFP-fused proteins.

We further generated four purpose-designed variations of the FAST-G vectors and six purpose-designed variations of the FAST-R vectors, as follows. pFAST-G01 and pFAST-R01 may be used to overexpress a gene driven by any promoter, and are useful for complementing a mutant phenotype

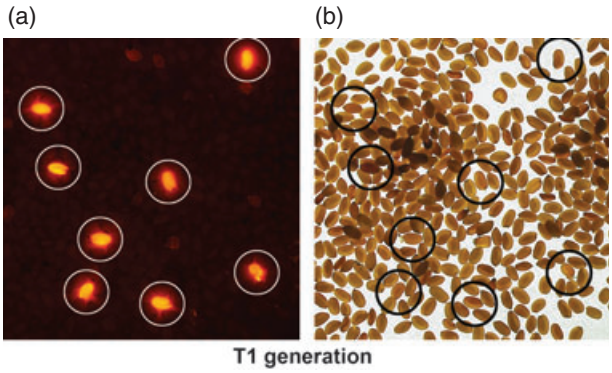


Figure 6. Seeds of Arabidopsis plants transformed with pFAST-R give red fluorescence.
(a) We introduced pFAST-R into Arabidopsis plants, and inspected the resulting seeds with a fluorescence stereomicroscope. A few seeds of the T_1 generation produced red fluorescence (indicated by circles).
(b) The same field of view of T_1 seeds as in (a), but obtained with a binocular microscope.

(Figure 7a,e). The pFAST-G02 and pFAST-R02 vectors may be used to overexpress a gene driven by the CaMV 35S promoter (Figure 7b,f). pFAST-G03 and pFAST-R03 are an RNA interference (RNAi) vector that may be used to knock down the expression of an interest gene (Figure 7c,g). pFAST-G04 may be used to study the temporal and spatial activity of a promoter that drives a *GFP-GUS*-fused gene (Figure 7d). pFAST-R05 and pFAST-R06 express a gene fused to *GFP*, driven by the CaMV 35S promoter, and is useful for studying the subcellular localization of the GFP fusion protein (Figure 7h,i). pFAST-R07 expresses a gene fused to *GFP*, driven by any promoter, and is useful for studying the temporal and spatial localization of the GFP fusion protein (Figure 7j).

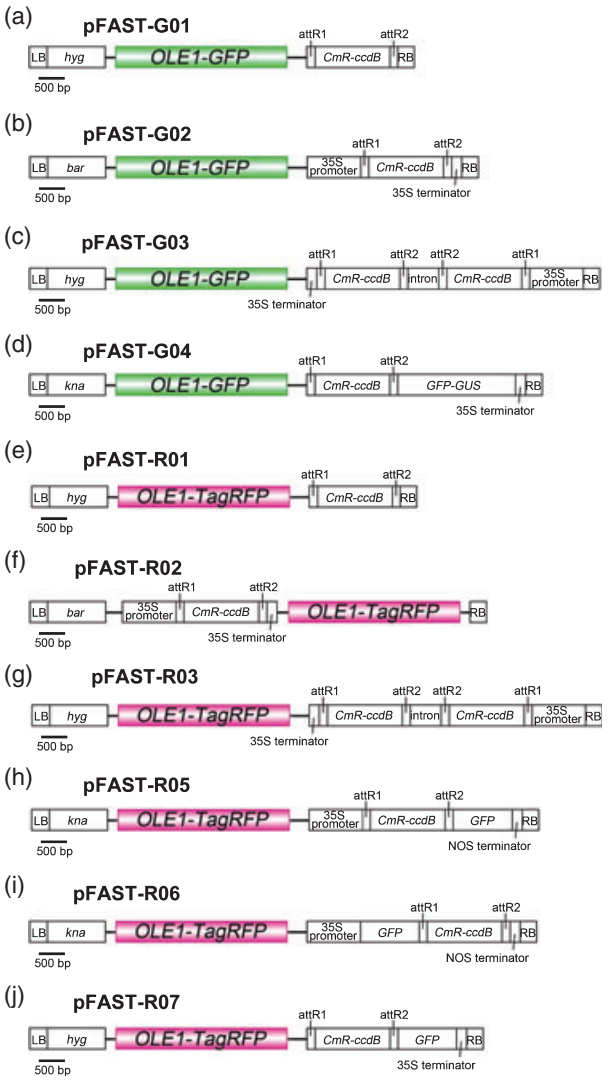


Figure 7. Schematic organization of four purpose-designed variations of the FAST-G vector and six variations of the FAST-R vector.
(a–j) Schematic diagrams of four FAST-G vectors and six FAST-R vectors. *bar*, Basta resistance gene; *ccdB*, negative selection marker used in bacteria; *CmR*, chloramphenicol resistance marker (chloramphenicol acetyl transferase); *GUS*, β-glucuronidase gene; *hyg*, hygromycin resistant gene; *kna*, kanamycin resistance gene; LB, left border; RB, right border; 35S promoter, CaMV 35S promoter; 35S terminator, CaMV 35S terminator.

The FAST method of selecting transgenic lines is rapid and non-destructive

The FAST marker has six advantages over conventional selectable markers, including antibiotic- and herbicide-resistance genes, as follows (Figure 8). (i) FAST enables us to efficiently isolate the heterozygous dry seeds at approximately 1 month after *Agrobacterium* infiltration. With the T_1 seeds, one can analyze any effect of a transgene that is expected in seeds. (ii) The time required for acquiring homozygous seeds with FAST (approximately 4 months) is shorter than that with the conventional method (approximately 7.5 months). (iii) FAST does not require the use of antibiotics or herbicides, which have been demonstrated to have adverse effects on the growth of transgenic plants (Yin *et al.*, 2008). (iv) Plants with a transgene that causes a defect in their growth are occasionally sensitive to antibiotics or herbicides, although they have an antibiotic- or herbicide-resistance gene (Yin *et al.*, 2008). FAST makes it possible to grow such plants. (v) FAST enables us to determine more accurate segregation rates of T_2 plants by inspecting all seeds (3000–5000 grains) of each T_2 line with a fluorescence microscope, which is important to isolate homozygous lines with a single transgene locus. (vi) FAST does not require sterilization procedures, clean-bench techniques, the handling of large quantities of T_2 plants or large space for plant growth.

On the other hand, there is a disadvantage of the FAST method. Previously, we reported that oleosin deficiency increases the size of oil bodies in seeds (Shimada *et al.*,

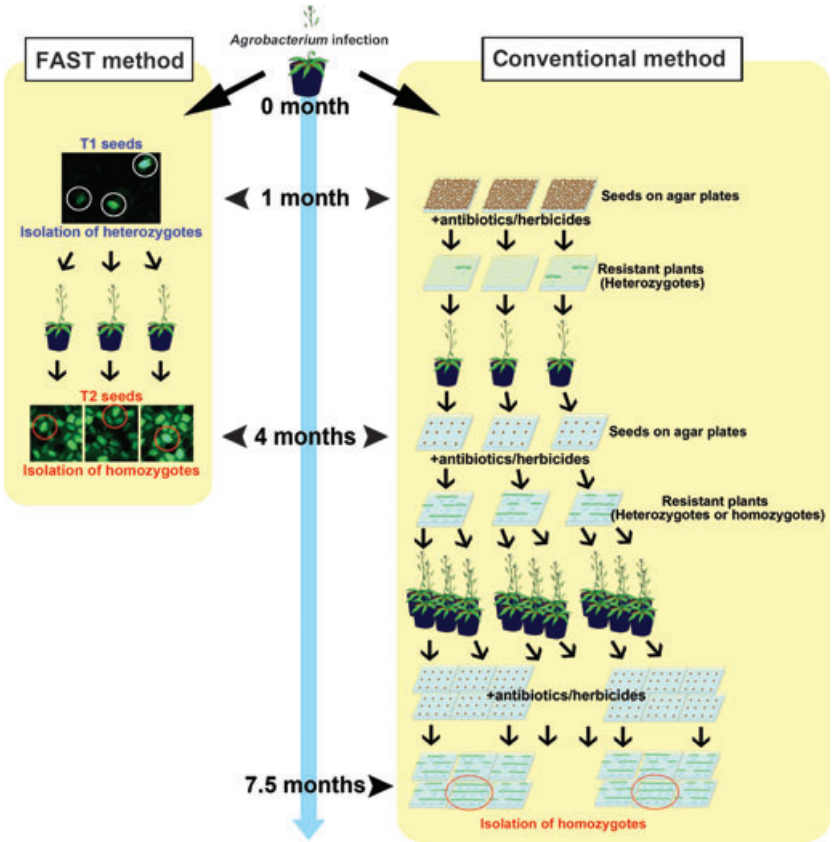


Figure 8. A comparison of the FAST method with the conventional method of transgenic line identification.
The fluorescent screenable marker OLE1-GFP makes the transformed seeds fluorescent, thereby allowing the identification of transformed seeds immediately after harvesting seeds. Homozygous seeds can be identified in the T_2 seed population using OLE1-GFP. The FAST method provides a time-saving, antibiotic- and herbicide-free, and labor-saving technique for detecting transformed plants. It is a simple method for determining the segregation rates of T_2 plants, based on the observation of seed populations with a fluorescence stereomicroscope.

2008), which implied that OLE1 overexpression decreases their size. We examined oil bodies in dry seeds of three independent T_2 lines by electron microscopy. We found that the size of oil bodies in fluorescent T_2 seeds were larger than those of non-fluorescent T_2 seeds (Figure S3), suggesting that the FAST marker causes an increase in the size of oil bodies. However, we have detected no defects in seed germination and subsequent seedling growth of the transgenic lines generated with the FAST method. Although the FAST method provided the variability of the GFP fluorescence among independent transgenic lines (Figure S4), the variability is not a major difficulty in screening with this method (Figures 3 and S1).

There are two potential difficulties of the FAST method. First, the silencing of the *OLE1* gene, the probability of which is not high (Figure S2), could affect seed germination. Second, as a result of unknown factors or conditions, the fluorescent markers OLE1-GFP and/or OLE1-RFP could unexpectedly express in vegetative tissues, in which the fluorescent proteins might be localized on oil bodies and endoplasmic reticulum in leaf cells (Wahlroos *et al.*, 2003).

Lu *et al.* screened for genes involved in the accumulation of hydroxy fatty acid in seed oils by using the selection marker DsRed driven by the cassava vein mosaic virus promoter pCVMV (Lu *et al.*, 2006; Lu and Kang, 2008). The fluorescent selection marker does not exhibit seed-specific expression, whereas the FAST marker is only expressed in dry seeds (Figure 5). Stuitje *et al.* reported an easy method for (co)transformation in Arabidopsis using fluorescent protein markers driven by a promoter of a storage protein napin (Stuitje *et al.*, 2003). Their fluorescent markers, which are distributed in the cytosol and nucleus of seeds, are hardly degraded during seed germination, whereas the FAST marker, which is localized on the oil body membrane in seeds, is degraded rapidly during germination (Figures 1 and 5). Additionally, Stuitje *et al.* focused on the T_1 seeds, but not on T_2 or T_3 generations. In contrast, the FAST marker makes it possible to instantly identify homozygous lines among T_2 seed populations (Figure 3).

The floral-dip technique was originally developed as a simplified method of infiltrating Agrobacteria bearing a transgene into Arabidopsis plants (Clough and Bent, 1998). Very recently, however, the floral-dip method was successfully applied to the transformation of starchy wheat seeds (Agarwal *et al.*, 2009). Considering that starchy seeds accumulate large quantities of oil in the embryo and in the aleurone layer, the FAST marker could be used not only for plants with oily seeds, but also for plants with starchy seeds, and thereby could potentially enable us to expand the versatility of the floral-dip method for use in other plant species.

EXPERIMENTAL PROCEDURES

Those who wish to use the FAST vectors are asked to contact us (ihnishi@gr.bot.kyoto-u.ac.jp).

Plant materials and growth conditions

Seeds of *A. thaliana*, ecotype Col-0, were surface sterilized with 70% ethanol, sown on MS medium (Wako, http://www.wako-chem.co.jp) with 1% (w/v) sucrose and 0.8% (w/v) agar, and incubated at 4°C for 3 days to break seed dormancy. The seeds were germinated and grown at 22°C under continuous light (100 mE s^{-1} m^{-2}) for 4 weeks, and then transferred to vermiculite for subsequent growth.

Electron microscopy and immunoelectron microscopy

Electron microscopy and immunoelectron microscopy of dry seeds of wild-type (Col-0) and *OLE1-GFP* transgenic Arabidopsis plants were performed as described previously (Shimada *et al.*, 2003, 2008). For immunoelectron microscopy, we used anti-OLE1 antibody (diluted 1:1000) (Shimada *et al.*, 2003) and anti-GFP antibody (diluted 1:500; Clontech, http://www.clontech.com). Ultrathin sections were examined with a transmission electron microscope (model JEM-1015B; JEOL, http://www.jeol.com).

Confocal laser scanning microscopy

The GFP images of transgenic plants were obtained with a confocal laser scanning microscope (LSM510 META; Carl Zeiss, http://www.zeiss.com) using the 488-nm line of a 40-mV Ar/Kr laser. Differential interference contrast (DIC) images were also obtained with this microscope. The procedures followed were essentially the same as those described previously (Tamura *et al.*, 2007).

Plasmid DNA construction for the FAST-G vectors

We produced the plasmid constructs using Gateway Technology (Invitrogen, http://www.invitrogen.com) with the destination vector pBGWFS7 (Plant System Biology, http://www.psb.ugent.be), which harbors a GFP gene and a β-glucuronidase gene. pBGWFS7 was digested with *Nru*1 and then electrophoresed to obtain an 11-kb fragment that lacks the β-glucuronidase gene. After purification, the fragment was self-ligated to produce a modified destination vector, pBGWF7. Position −1444 to 759 bp of the *OLE1* gene was amplified by PCR using Arabidopsis genomic DNA and the primer set 5′-CACCCTACTTAGATCAACACATAAA-3′ and 5′-GAGTAGTGTGCTGGCCACCACG-3′. The *OLE1* fragment was inserted into pENTER/D-TOPO (Invitrogen) by the TOPO reaction to produce an entry clone, pENTER/OLE1, and was then transferred into the destination vector, pBGWF7, by an LR recombination reaction, to create a vector, pBOLE1GFP, which we designated pFAST-G. The *Aat*2-digest of pFAST-G was treated with alkaline phosphatase. The fragment was ligated with the 3-kb fragment from the *Aat*2-digest of pH2GW7 (Plant System Biology) to produce the destination vector, pFAST-G02.

We generated three purpose-designed plasmid vectors based on pFAST-G, which harbors the *OLE1* promoter, *OLE1-GFP* and the CaMV 35S terminator. OLE1-GFP-*Apa*1 was generated by PCR using pFAST-G and the primer set 5′-CACCGGGCCCTACTTAGATCAACACATAAA-3′ and 5′-GGGCCCTCGCATGCCTGCAGGTCACTGGAT-3′. OLE1-GFP-*Spe*1 was generated by PCR using pFAST-G and the primer set 5′-CACCACTAGTTAGTAAGTGAAGAACCACAA-3′ and 5′-ACTAGTCGCATGCCTGCAGGTCACTGGAT-3′. OLE1-GFP-*Apa*1 and OLE1-GFP-*Spe*1 were inserted into pENTER/D-TOPO to produce entry clones: pENTER/OLE1GFP-*Apa*1 and pENTER/OLE1GFP-*Spe*1, respectively. After the DNA sequences were confirmed, pENTER/OLE1GFP-*Apa*1 and pENTER/OLE1GFP-*Spe*1 were digested with *Apa*1 and *Spe*1, and subjected to electrophoresis to yield 3.5-kb fragments: OLE1-GFP-*Apa*1 and OLE1-GFP-*Spe*1, respectively. OLE1-GFP-*Apa*1 was ligated into each of the destination vectors, pHGW and pH7GWIWG2(I) (Plant System Biology), which had been treated with *Apa*1 and alkaline phosphatase to produce pFAST-G01 and pFAST-G03, respectively. OLE1-GFP-*Spe*1 was ligated into the destination vector, pKGWFS7, which had been treated with *Spe*1 and alkaline phosphatase to produce pFAST-G04.

Plasmid DNA construction for the FAST-R vectors

We generated three purpose-designed plasmid vectors based on pFAST-R that were composed of the *OLE1* promoter, *OLE1-TagRFP* and the NOS terminator. A 2.2-kb fragment consisting of the *OLE1* promoter and *OLE1* was generated by PCR using pB-OLE1-GFP and the primer set 5′-CACCACTAGTGTATGTAGGTATAGTAACAT-3′ and 5′-CAGCTCGCTCATAGTAGTGTGCTGGCCACC-3′. The 0.7-kb *TagRFP* fragment was generated by PCR using a *TagRFP* fragment (Evrogen JSC, http://www.evrogen.com) (Merzlyak *et al.*, 2007) and the primer set 5′-CAGCACACTACTATGAGCGAGCTGATTAAG-3′ and 5′-TGTTTGAACGATTCACTTGTGCCCCAGTTT-3′. The 0.2-kb NOS terminator fragment was generated by PCR using an NOS terminator fragment (Evrogen JSC) and the primer set 5′-GGGCACAAGTGAATCGTTCAAACATTTGGC-3′ and 5′-ACTAGTGATCTAGTAACATAGATGACACC-3′. OLE1-TagRFP-*Spe*1 was generated by PCR using the three fragments (i.e. the *OLE1* promoter and *OLE1*, *TagRFP*, and the NOS terminator), and the primer set 5′-CACCACTAGTGTATGTAGGTATAGTAACAT-3′ and 5′-ACTAGTGATCTAGTAACATAGATGACACC-3′. OLE1-TagRFP-*Spe*1 was inserted into pENTER/D-TOPO to produce the entry clone, pENTER/OLE1-TagRFP-*Spe*1. After confirming the DNA sequences, OLE1-TagRFP-*Hind*3 was generated by PCR using pENTER/OLE1-TagRFP-*Spe*1 and the primer set 5′-CACCAAGCTTCAAGTGTATGTAGGTATAGT-3′ and 5′-AAGCTTGATCTAGTAACATAGATGACACC-3′. OLE1-TagRFP-*Hind*3 was inserted into pENTER/D-TOPO to produce an entry clone, pENTER/OLE1-TagRFP-*Hind*3. OLE1-TagRFP-*Apa*1 was generated by PCR using pENTER/OLE1-TagRFP-*Spe*1 and the primer set 5′-CACCGGGCCCTTCAAGTGTATGTAGGTATA-3′ and 5′-GGGCCCATCTAGTAACATAGATGACACC-3′. OLE1-TagRFP-*Apa*1 was inserted into pENTER/D-TOPO to produce an entry clone, pENTER/OLE1-TagRFP-*Apa*1. The pENTER/OLE1TagRFP-*Spe*1, pENTER/OLE1-TagRFP-*Hind*3 and pENTER/OLE1-TagRFP-*Apa*1 entry clones were digested with *Spe*1, *Hind*3 and *Apa*1, respectively, and were then subjected to electrophoresis to yield the 3.5-kb fragments: OLE1-TagRFP-*Spe*1, OLE1-TagRFP-*Hind*3 and OLE1TagRFP-*Apa*1, respectively.

pHGWFS7 (Plant System Biology) was digested with *Nru*1 and then electrophoresed to obtain an 11-kb fragment that did not contain the β-glucuronidase gene. After purification, the fragment was self-ligated to produce a modified destination vector, pHGWF7. To produce pFAST-R07, OLE1-TagRFP-*Spe*1 was ligated into the destination vector, pHGWF7, which had been treated with *Spe*1 and alkaline phosphatase. To produce pFAST-R06, OLE1-TagRFP-*Hind*3 was ligated into the destination vector, pGWB406 (Nakagawa *et al.*, 2007), which had been treated with *Hind*3 and alkaline phosphatase. To produce pFAST-R02, OLE1-TagRFP-*Apa*1 was ligated into the destination vectors, pB2GW7 (Plant System Biology), which had been treated with *Apa*1 and alkaline phosphatase.

Transformation of Arabidopsis plants

A DNA fragment of *CLO3* was amplified by PCR using Arabidopsis genomic DNA and the primer set 5′-CACCATGGCAGGAGAGGCAGAGGCTT-3′ and 5′-TTAGTCTTGTTTGCGAGAATTGGCCC-3′. The *CLO3* fragment was inserted into pENTER/D-TOPO to produce an entry clone, pENTER/CLO3, and was then transferred into the

pFAST-G02 and pFAST-R06 destination vectors to create the pFAST-35S-CLO3 and pFAST-GFP-CLO3 expression vectors, respectively. pFAST-G, pFAST-35S-CLO3 and pFAST-GFP-CLO3 were each introduced into Arabidopsis (Col-0) plants via *Agrobacterium tumefaciens* (strain GV3101), by means of the floral-dip method (Bechtold and Pelletier, 1998). Plants transformed with pFAST-G expressed the fusion gene *OLE1-GFP* driven by the *OLE1* promoter, whereas plants transformed with pFAST-35S-CLO3 expressed both *CLO3*, driven by the 35S promoter, and the fusion gene, *OLE1-GFP*, driven by the *OLE1* promoter.

Segregation test of transgenic plants

Seeds emitting green fluorescence (GFP+) were picked up with a wet wooden toothpick from the T_1, T_2 and T_3 seed populations, while being viewed with a fluorescence stereomicroscope (MVX10; Olympus, http://www.olympus-global.com). To examine the correlation between seed fluorescence (GFP+) and herbicide resistance (Basta^r), the T_2 and T_3 seeds were sown on MS medium that contained Basta (phosphinothricin, 10 mg l^{-1}). The fluorescence intensities of the T_2 seeds were measured from photographs using PHOTOSHOP ELEMENTS 5.0 (Adobe, http://www.adobe.com).

For genotyping each T_2 seed, we sowed the seed and then examined its T_3 seeds with the fluorescence stereomicroscope. We determined a genotype of each T_2 seed based on the concept as follows: T_2 homozygous seed should give fluorescent T_3 seeds, T_2 non-transgenic seed should give non-fluorescent T_3 seeds and T_2 heterozygous seed should give the 3:1 segregation rate of fluorescence seeds (GFP+) to non-fluorescent seeds (GFP−).

Specific antibodies

A peptide derived from CLO3 (CVTSQRKVRNDLEETL) was chemically synthesized with a peptide synthesizer (model 431 A; Applied Biosystems, http://www.appliedbiosystems.com). The peptide was cross-linked to BSA with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Sigma-Aldrich, http://www.sigmaaldrich.com). The peptide-BSA conjugate was subcutaneously injected into a rabbit with complete Freund's adjuvant. After 3 weeks, four booster injections with incomplete adjuvant were given at 1-week intervals. Blood was drawn 1 week after the booster injections, and the antibodies were prepared.

Immunoblot analysis

One 7-day-old seedling of each line was homogenized in 50 μl SDS sample buffer that contained 100 mM Tris/HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethnol. The homogenates (15 μl) were subjected to SDS–PAGE on a 15% acrylamide gel, and immunoblot analysis was performed as described previously (Shimada *et al.*, 2003, 2008). Anti-CLO3 antibody (diluted 1:5000) and horseradish peroxidase-conjugated goat antibody against rabbit IgG (diluted 1:5000; Pierce, http://www.piercenet.com) were used. Immunodetection was performed with an ECL Kit (Amersham, now part of GE Healthcare, http://www.gelifesciences.com).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Homozygous seeds can be efficiently isolated from various T_2 seed populations using OLE1-GFP.

Figure S2. Almost 80% of T_2 lines with pFAST-G had a single locus of the transgene.

Figure S3. OLE1-GFP fluorescent seeds had smaller oil bodies than non-fluorescent seeds.

Figure S4. Variability of fluorescence intensities of T_1 seeds expressing OLE1-GFP.

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Accession numbers: Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *OLE1* (At4G25140) and *CLO3* (At2G33380).

