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A NAPIN PROMOTER ACTIVATES GENE EXPRESSION IN DEVELOPING SEEDS OF *LESQUERELLA FENDLERI*

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ABSTRACT

Lesquerella fendleri produces industrial useful Hydroxy Fatty Acids (HFA) in seed oil. To improve oil and HFA of *L. fendleri*, it is desirable to use of seed-specific promoters to control the expression of target genes by genetic engineering. A seed-specific promoter fragment, -397 to -1 of a napin gene (*PnapA*) from *Brassic napus* was isolated by PCR and constructed to a small promoter-testing vector named pGPro4. A *nopaline synthase* (*nos*) promoter was used to control the expression of the selectable marker of pGPro4. pGPro4 also contains a bifunctional *β-glucuronidase-enhanced Green Fluorescent Protein* (*gusA-eGFP*) reporter gene that provides visual detection of reporter gene expression using either fluorescence in live cells or histochemical detection of β-glucuronidase activity. To demonstrate the usefulness of *PnapA*, *L. fendleri* was transformed with the pGPro4-PnapA vector. Primary transgenic shoots were generated from explants at an expected frequency of 13 to 23%, indicating that the nos promoter drove sufficient *hptII* expression to generate hygromycin resistant plants. Five independent transgenic *L. fendleri* lines were grown to maturity and generated T_1 seeds. Segregation analysis of T_1 seeds indicated that the transgenic *L. fendleri* lines contain one, two or more integration sites. The *gusA-eGFP* reporter gene activity was examined in various organs of all these transgenic lines by standard GUS assay. Only seeds showed positive GUS stain, confirming that *PnapA* confers seed-specific expression in transgenic *L. fendleri*.

Keywords: *Lesquerella Fendleri*, Napin Promoter, Hygromycin Phosphotransferase II, B-Glucuronidase, *Agrobacterium*-Mediated Transformation

1. INTRODUCTION

Lesquerella fendleri (A. Gray) S. Wats. (Brassicaceae), under development as a new industrial oilseed crop in the southwestern region of the U.S., is valued for its unusual Hydroxy Fatty Acid (HFA). HFAs and derivatives are used as raw materials for numerous industrial products, such as lubricants, plasticizers and surfactants (Caupin, 1997). *L. fendleri* is amenable to *Agrobacterium*-mediated transformation (Chen, 2011; Skarjinskaia *et al*., 2003; Wang *et al*., 2008), thus genetic transformation provides an alternative means to improve this crop. To engineer oils or fatty acid contents in *L. fendleri* seed, it is preferable to utilize seed specific promoters, to limit the changes of oils and fatty acids to the seed and to avoid the changes in membrane lipids in other parts of the plant. It is known that napins, a group of storage proteins, are tightly regulated by seed developmental process, their synthesis is induced by abscisic acid and restricted to the developing seeds (Crouch and Sussex, 1981; Crouch *et al*., 1983). The physiological role of napins is to provide the growing seedling with essential nutrients prior to the establishment of the photosynthetic capacity.

Napins are encoded by a multigene family in rapeseed and other Brassica species. One of napins, napA, has been cloned and sequenced (Josefsson *et al*., 1987) (Genbank accession: J02798). NapA has been demonstrated to be highly expressed in developing seeds (Stalberg *et al*., 1993) and its promoter could also drive

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the expression of foreign genes efficiently in the embryos of transgenic plants, including modification of the lipid profile of oilseeds (Broun *et al*., 1998; Eccleston and Ohlrogge, 1998; Topfer *et al*., 1995). Based on reports, *napA* promoter (-309 to +45) directs strong reporter gene activity in transgenic tobacco seeds (Stalberg *et al*., 1993). In the -309 *napA* promoter, a highly conserved region between positions -152 and -120 is crucial for promoter function

in transgenic rapeseed (Ellerstrom *et al*., 1996; Stalberg *et al*., 1996) and tobacco (Ellerstrom *et al*., 1996). Two *cis* elements were identified in this region: an ABRE (abscisic acid-responsive element)-like element situated between -148 and -139 and a CA-rich element between -130 and -124 (Ellerstrom *et al*., 1996). Other *cis* elements include two RY-repeat motifs bordering a G-box at -78 to -50 and the B-box situated between -152 and -120 (Ezcurra *et al*., 1999). These seed-specific *cis* elements are highly conserved seed promoters (Baumlein *et al*., 1992) and are crucial for seed-specific expression directed by napA, leguminin and glycinin promoters (Baumlein *et al*., 1992; Ezcurra *et al*., 1999; Lelievre *et al*., 1992).

We have cloned and sequenced the upstream regulatory region of *napA* gene to test the tissue specific expression pattern of this promoter in *L. fendleri*. We constructed the promoter sequence from -397 to -1 (corresponding to Genbank accession J02798, 708 to 1145) in pGPro4 vector (Chen *et al*., 2011) (GenBank accessions JN593324). This vector contains a promoterless *gusA-eGFP* (*β-glurcuronidase-enhanced Green Fluorescent Protein*) reporter gene, a small size plasmid backbone and many unique restriction sites. More importantly, pGPro4 was designed for precise testing an organ-specific promoter. Because in many popular vectors such as pCAMBIA (www.cambia.org) and pGreen (Hellens *et al*., 2000), one overlooked problem is the use of the CaMV35S promoter with one or more enhancer sequences to drive selectable marker gene expression. When an organ-specific promoter-target gene cassette is placed within a such vector, the enhancer can bidirectionally interfere with the transcription of the target gene as well as nearby genes, affecting the fidelity of the organ-specific gene expression and/or causing unintended misexpression of nearby genes (Yoo *et al*., 2005; Xie *et al*., 2001). To avoid this problem, a *nopaline sythase* promoter (nosP) is used to drive a selectable marker gene, hptII (*hygromycin phosphotransferase II*), in pGPro4. We report here the construction of the *napA* promoter to upstream of *gusA* gene and analysis the tissue specific pattern of this promoter in transgenic *L. fendleri* carrying this construct.

2. MATERIALS AND METHODS

2.1. Construction of the pGPro4-napa Promoter (pGPro4-PnapA) Binary Vector

The vector map of the pGPro4 vector was described (Chen *et al*., 2011) and the annotated sequence is available from Genbank (accession JN593324). The promoter sequence of *napA* from -397 to -1 was synthesized by using PCR and the genomic DNA of Brassic napus, cultivar svaloefs karab (USDA-ARS germplams accession number PI 535869) as template. The PCR primers, forward 5' GAGCTCCCAATTTATATTCCCAACGGC 3', reverse 5' GAATTCGTGTATGTTTTTAATCTTGTTTGTATTG ATG 3' were designed based on the reported *napA* promoter sequence (GenBank accession J02798) and restriction sites, *Sac*I and *Eco*RI were added to facilitate the cloning of the PCR product in pGPro4 vector. The accuracy of *pGPro4-PnapA* clone was confirmed by sequencing analysis.

2.2. Plant Material, Bacterial Strain

The *L. fendleri* seeds, WCL-LY2 (Dierig *et al*., 2001), were kindly provided by Dr. Dave Dierig (USDA-ARS, Arid-Land Agricultural Research Center, Maricopa, AZ). Plants were grown in a greenhouse at temperatures between 28°C (day) and 18°C (night), with supplemental metal halide lighting to provide a 15-hr-day length (1000 to 1250 μmol m^{-2} sec^{-1}). Mature female flowers were hand-pollinated and the seeds were harvested at about 49 days after pollination. An *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*., 1991) carrying binary vector pGPro4-PnapA was used for plant transformation. An AGL1/pGPro4-PnapA culture was started with the inoculation of one clone in 1 mL Luria Broth (LB, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} sodium chloride, 1 g L^{-1} glucose, pH 7) supplemented with 50 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin shaken at 200 rpm overnight at 28°C, followed by sub-culturing of 0.1 mL of the overnight culture in 50 mL fresh LB for overnight.

2.3. Tissue Culture, Transformation and Regeneration

The previously described protocol (Chen, 2011) was utilized for *L. fendleri* transformation. In brief, leaves harvested from plants in sterile condition were wounded by scratching slightly on the underside of a leaf and then cultured on Callus and Shoot Induction (CSI) medium. After two days, the leaves were dipped in the half strength MS medium containing AGL1/pGPro4-PnapA

for five minutes. Following the inoculation, leaves were blotted on sterilized filter paper and transferred to Callus and Shoot Induction (CSI) medium composed of basal medium (BM, half strength MS medium plus 30 g L^{-1} sucrose and 6 g L^{-1} agar, pH 5.7) supplemented with 1 mg L^{-1} 6-BENZYLAMINOPURINe (BA) and 0.1 mg L^{-1} 1-Naphthaleneacetic Acid (NAA). After incubating the infected leaves in the growth chamber for 2 days, the leaves were cut into 5 mm segments and cultured on CSI media plus 25 mg L^{-1} hygromycin (hyg) for transgenic selection and 100 mg L^{-1} timentin for inhibiting the *Agrobaterium* growth. In 6-8 weeks, yellow-greenish hygromycin resistant calli started to appear on the leaf segments. The calli were then transferred to a timentin-free subculture medium CSI+ (CSI with hygromycin increased to 50 mg L^{-1}). Green shoots were developed from the calli on CSI+ medium in about 2-4 weeks after the transfer. To eliminate chimeras, each shoot was cut into small pieces (about 1×1 mm) and placed on the CSI+ medium for shoot-regeneration. After 4 rounds of successive regenerations, shoots were sub-cultured on BM plus 1 mg L^{-1} BA, 1 mg L^{-1} Indole-3-Butyric Acid (IBA) and 50 mg L^{-1} hyg for multiplication. Shoots 10-15 mm in length were transferred to rooting medium (BM plus 1 mg L^{-1} IBA and 50 mg L^{-1} hyg). When a shoot developed 2-3 roots (usually in 3-5 weeks), it was then transferred to a Magenta box (Sigma, St. Louis, Mo) containing sterilized peat-vermiculite growth mixture (Sunshine mix #4, Planet Natural, Bozeman, MT) pre-soaked with 1 mg L^{-1} IBA water solution. After 8-10 weeks in the growth mixture, well-developed primary plants showing 8-12 normal leaves and 2-3 inch height were transferred to a 6-inch pot and placed under a transparent plastic cover for the first 2 weeks for acclimation in the greenhouse.

2.4. Detection of β-Glucuronidase Gene Activity

Developing seeds at different stages were dissected out and the Histochemical GUS assays (*gusA* gene activity) were performed using the Jefferson method (Jefferson *et al*., 1987). The assay solution contained 0.5 mM potassium ferrocyanide, 0.3 % (v/v) triton X-100 and 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-gluc) in 50 mM phosphate buffer, pH7.0. Samples were incubated at 37°C overnight in the assay solution and then transferred to 70% ethanol. GUS staining images were documented using a Leica dissecting microscope (Leica micro-system Ltd., CH-9435 Heerbrugg, Switzerland) equipped with a digital camera. Mature seeds harvested from primary transgenic plants or Wild type (Wt) seeds were surface-sterilized in a 0.25% (v/v) sidium hypochlorite solution for 15 min followed by five rises in sterile deionized water. Wt seeds were germinated on Germination Medium (GM) containing half strength of MS salts and 0.6% agar. Transgenic seeds were germinated on GM supplemented with 0.5% sucrose, 50 mg L^{-1} hyg and 0.6% agar. Two-week old seedlings were tested for GUS activity as described above.

3. RESULTS AND DISCUSSION

3.1. *L. Fendleri* Transformation with the pGPro4-PnapA Vector

To examine the functionality of the *PnapA*, we constructed the pGPro4-PnapA plasmid by ligation of a 406 bp promoter PCR fragment (material and method) upstream of the *gusA-eGFP* reporter gene and tested it in *L. fendleri* by *Agrobacterium*-mediated transformation using a previously described protocol (Chen, 2011). In three separate experiments, the percentage of primary hygromycin resistant shoots generated from the explants ranged from 13-23% (**Table 1**). The frequency of primary shoot regeneration is lower than that of 22-60% when a pCAMBIA plasmid was used (Chen, 2011), but still gave rise a sufficient number of transformants. Five independent primary transgenic *L. fendleri* plants were grown to maturity in the greenhouse to produce next generation (T_1) seeds. The number of unlinked integration sites was estimated from the segregation of hyg-resistant (hyg^R) T_1 seedlings. The analysis showed that transformation with *pGPro4-PnapA* construct gave rise to collection of plants containing different numbers of integration sites, from one to more than two (**Table 2**). The result is similar to what we observed when a pCAMBIA plasmid was used (Chen, 2011). The presence of *PnapA* was verified by PCRs with *PnapA* specific primers. All lines tested showed presence of the *PnapA* (data not shown).

Table 1. Number of calli and shoots produced in leaf segments

Expt. No.	No. of segments	No. of calli[a]	No. of shoots[b]	Shoot (%)
1	30	7	6	20
2	30	5	4	13
3	30	9	7	23

[a]; The number was scored at the 8th week after placing the explants on CSI medium plus 25 mg L^{-1} hygromycin and 100 mg L^{-1} timentin [b]; The number was scored at the 4th week of subculture on CSI medium plus 50 mg L^{-1} hygromycin

Table 2. T1 progeny numbers and segregation ratios of hygromycin-resistant (hyg^R) and hygromycin-sensitive (hyg^S) phenotype

T_1 line	Total No of seeds	No of germinated	hyg^R : hyg^S seedlings	No of *hptII* gene insertion
1	42	39	30:9	1
2	48	45	33:12	1
3	53	50	47:3	2
4	38	37	35:2	≥2
5	31	29	29:0	>2

Seeds were sterilized and placed on GM plus 50 mg L^{-1} hyg, hyg^R or hyg^S seedlings were documented after 2 weeks of culture in a growth chamber. A typical hyg^R seedling had normal cotyledons, true leave and roots (**Fig. 2A**), while a hyg^S seedling was arrested at early stage of germination and showed bleached cotyledons and radicle (**Fig. 2D**)

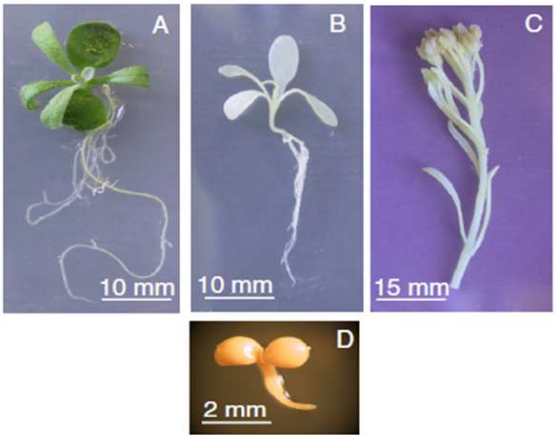

Fig. 1. Phenotype of 2-week old T1 seedlings. A, a hygR seedling; B, a hygR seedling after GUS staining assay; C, a shoot branch from a hygR T1 plant after GUS staining assay; D, a hygs seedling killed by hygromycin

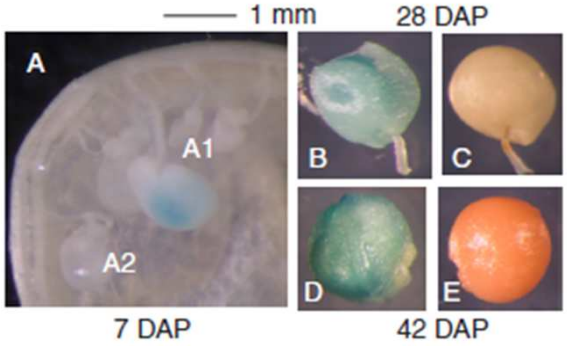

Fig. 2. GUS staining assay of developing T1 seeds. DAP stands for days after pollination. GUS+ seeds (A1, B and D) showing blue stain in embryos, while GUS- seeds (A2, C and D) showing no stain

3.2. Tissue Specificity of PnapA

To investigate the tissue specific activity of *PnapA*, we analyzed GUS activity in the shoot branches of primary (data not shown) and T1 plants (including leaves, stem and flower buds) and two-week old hyg^R T1 seedlings (including root, cotyledon, hypocotyls and leaves). None of these five lines showed any activity in these tissues (**Fig. 1**). When GUS activity was examined in developing T1 seeds of these five lines, we found that most seeds showed positive GUS staining and few seeds failed to take up the stain, consistent with the expectation of observing null segregants lacking the *gusA* transgene. The expression of GUS was detected as early as 7 Days After Pollination (DAP) and the expression persisted throughout the rest of seed development (mid-stages 21-35 DAP and late stage 42-49) (**Fig. 2**). This clearly indicated that the cloned *PnapA* had a seed specific expression pattern in *L. fendleri*.

4. CONCLUSION

We have constructed a binary vector, *pGPro4-PnapA-gusA-eGFP* to test *PnapA* activity in transgenic *L. fendleri*. By using histochemical analysis of the *gusA* gene activity, we confirmed that *PnapA* was active only in seeds. The *PnapA* can be used to express a target gene and to facilitate future research on genetic engineering of seed oil in *L. fendleri*.

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