Ectopic expression of *Atleafy* in *Brassica juncea* cv. Geeta for early flowering

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Abstract High temperature stress during pod filling severely affects the yield of *Brassica juncea*. Early flowering can evade the terminal heat stress and result in early maturity of the crop. In this study, a regeneration and transformation protocol has been standardized for *B. juncea* cv. Geeta. Hypocotyl from 5-day-old seedlings were used as explants. Of the various combinations of auxins and cytokinins tried along with Murashige and Skoog's (Physiol Plant 15:473–497, 1962) medium, MS + IAA (0.2 mg/l) + BA (3 mg/l) proved best for shoot regeneration with 89.9 % regeneration efficiency. To induce early flowering *Leafy* gene from *Arabidopsis thaliana* was transformed using *Agrobacterium* mediated transformation method. After 12 weeks transgenic plants showed flowering in vitro whereas their untransformed counterpart did not flower even after 16 weeks. The maximum transformation frequency was 4 %.

Keywords *Brassica juncea* · *Atleafy* · Early flowering · Transformation · Micropropagation

Introduction

Brassica juncea (Indian mustard) is second most important source of edible oil next to groundnut and contributes 19.6 % to the total oilseeds production in India (Anonymous 2009). Abiotic stresses specifically high temperature adversely affects its growth and productivity by triggering a series of morphological, physiological, biochemical and molecular changes. The Intergovernmental Panel for Climate Change (IPCC) has projected an average temperature increase of 0.5–1.2 °C by 2020 with higher increase in rabi season (Boomiraj et al. 2010). High temperature stress causes flower abortion (Hall 1992; Rao et al. 1992), reduced siliques per plant, decreased seeds per silique, smaller seeds and low oil content hence decreased yield. Opportunity to recover from the stress damage decreases with the advancement of developmental stages (Angadi et al. 2000); pod development and oil filling stages are most affected by high temperature (Gan et al. 2004). *B. juncea* generally has life cycle of 140–160 days, and varieties with shorter life cycle can evade the temperature stress during terminal stages. Flowering is an important step in life cycle of a plant and mark the transition from vegetative to reproductive phase. The complex mechanism of flowering has been elucidated in *Arabidopsis* and four different pathways were identified that promote this transition viz. long-day photoperiod, gibberellin dependent, vernalization and autonomous promotion. These pathways converge on a set of floral pathway integrator that activates flower meristem identity genes such as *LEAFY* (*LFY*) and *APETALA1* (*API*).

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Fig. 1 Regeneration from hypocotyl of 5-day-old seedlings of *B. juncea*. **a** Shoot regeneration after 3 weeks of inoculation, **b** Individual plant after 5 weeks, **c** Regenerated plant after 8 weeks

LFY is the first gene to be expressed amongst them. Besides regulating the transition from vegetative to reproductive phase, *LFY* also has role in floral organ identity. Overexpression of *LFY* and its homologues have been found to accelerate flowering in many species such as aspen (*Populus tremuloides* Michx.) and tobacco (*Nicotiana tabacum* L.) (Weigel and Nilsson 1995), rice (*Oryza sativa* L.) (He et al. 2000), poplar (*Populus trichocarpa* Torr. & A. Gray ex Hook.) (Rottmann et al. 2000), and *Citrus* (Pena et al. 2001). This ability of *LFY* to accelerate flowering is tapped in this study to develop early flowering transgenics of *Brassica* by overexpression of *AtLFY*. *B. juncea* cv. Geeta selected for the study though has high oil content and suitable for rainfed conditions of Delhi and Haryana, matures in 148–160 days (Agricoop 2012).

Material and methods

Seeds of *B. juncea* cv. Geeta were procured from Bawal Regional Research Station, Rewari, Haryana, India. Geeta was recommended for Haryana and Delhi region for its high oil content and tolerance to rain-fed conditions. *Agrobacterium tumefaciens* strain GV3101 transformed with pROK II *LFY* GUSint harbouring *LFY* gene driven by CaMV 35S promoter was a kind

gift from Prof J P Khurana (South Campus, University of Delhi, New Delhi, India).

Seeds were surface sterilized using 5 % Savlon (Johnson & Johnson) for 10 min, followed by 1 % Bavistin (BASF) for 15 min and 0.1 % mercuric chloride for 10 min and subsequent washing with sterile distilled water thrice. Sterilized seeds were stratified overnight at 4 °C in water for better seed germination and inoculated on MS basal semisolid medium. Hypocotyl from 5-day-old seedlings served as explants and inoculated on different combination of IAA (0.01–0.05 mg/L) and BA (1–5 mg/L) for determination of best shoot regeneration medium. For all the regeneration experiments, 2 % sucrose and 0.6 % Agar (Duchefa) was used. The pH was adjusted to 5.8. The cultures were maintained in a culture room illuminated by cool, white fluorescent tubes of 36 W (Philips, India) at 25±2 °C with 55±5 % relative humidity for 16 h photoperiod.

Transformation of *B. juncea* cv. Geeta was performed according to the protocol of Pental et al. (1993) with modifications. *A. tumefaciens* strain GV3101 containing the binary plasmid was grown overnight in Luria Bertini (LB) broth without antibiotics. 1 mL of the overnight culture (OD₆₀₀ 1) was inoculated in 50 mL of LB medium supplemented with kanamycin (50 mg/L) and rifampicin (50 mg/L). The cells obtained after centrifugation were resuspended in liquid MS basal medium (OD₆₀₀ 0.5). Approximately 200 hypocotyl segments

Table 1 Effect of different concentrations of IAA and BA on shoot regeneration efficiency of 5-day-old hypocotyl segments of *B. juncea* cv. Geeta. (Mean ± SD)

IAA BAP (in mg/l)	0	0.01	0.02	0.03	0.04	0.05
0	0±0	0±0	0±0	0±0	0±0	0±0
1	16.2±1.8	18.8±1.3	33.7±3.1	21.3±1.8	17.4±1.3	11±1.7
2	25.8±2.8	31.2±1.3	49.5±4.6	35.2±3.8	27.9±5.6	20.3±2.0
3	50.0±3.1	75.3±4.7	89.9±5.9	73.3±3.9	57.7±3.0	41.2±4.2
4	23.5±3.7	26.3±4.1	45.63±5.9	26.9±2.8	24.6±3.4	19.2±5.7
5	12±1.7	14.52±4.1	18.33±3.8	13.26±2.2	0±0	0±0

Value represent the mean of three repeated experiments

Table 2 Determination of Minimum inhibitory concentration (MIC) of kanamycin in wild explants for transgenic selection

Kanamycin concentration (in mg/L)	Regeneration %
0	86.1
10	77.8
20	63.9
30	47.2
40	22.2

(0.7–0.9 cm) from 5-day-old seedlings in four batches were precultured for 12–24 h before infecting them for variable time period with *A. tumefaciens* harbouring *Atleafy* and then co-cultivated on regeneration medium for 36–48 h. Co-cultivated explants were washed with MS liquid medium containing cefotaxime 250 mg/l, blot dried and inoculated on regeneration medium containing 30 mg/l kanamycin for selection. The hypocotyl segments were subcultured after every 2 weeks till shoots regenerated. Shoots were then transferred to MS + IBA (1 mg/l) for rooting.

Screening of putative transformants

Preliminary screening of kanamycin selected transformed explants was done through determination of GUS expression as described by Jefferson (1987). Integration of *Atleafy* into putative transgenic plants was verified by PCR using *Atleafy*, *uidA* (GUS) and *nptII* (kanamycin) specific primers. Primers used for amplification are:

AtLfy–Forward- 5'CTT GTG GGT ATG AAG GAC GAG G 3'

Reverse- 5'AAA TAC CGC CAA CTA AAG CCG 3'

uidA–Forward- 5' CGG GTG AAG GTT ATC TCT ATG A 3'

Reverse- 5'AGC CAT GCA CAC TGATAC TCT T 3'

nptII–Forward- 5' GAG GCT ATT CGG CTA TGA CTG 3'

Reverse- 5'ATC GGG AGC GGC GAT ACC GTA3'

Fig. 2 Putative transgenic leaves showing GUS expression. **a** Untransformed regenerated leaf without GUS expression **b** Regenerated plant along with callus **c** Transgenic leaf showing GUS staining

Results and discussion

The sterilants, their concentration and duration of treatment proved satisfactory in sterilization of seeds.

Regeneration

The regeneration medium for hypocotyl of *B. juncea* cv. Geeta was optimized using different concentrations of IAA (0.01–0.05 mg/l) and BA (1–5 mg/l) in MS medium. IAA (0.02 mg/l) + BA (3 mg/L) proved to be the best medium for shoot regeneration with 89.9 % regeneration efficiency (Fig. 1; Table 1). There was increase in percentage shoot regeneration with increase in concentration of BA till 3 mg/l. Further increase in concentration of BA affected the regeneration efficiency. Bhuiyan et al. (2011) used 0.1 mg/l NAA and 1 mg/l BA for efficient regeneration of *B. juncea* cv. Rai-5, whereas Bano et al. (2010) demonstrated MS + NAA 0.3 mg/l and BA 3 mg/L as the best regeneration medium for three *B. juncea* cultivars viz. UCD-635, RL-18 and NIFA-RAYE. The regeneration efficiency, however, differ on account of genetic difference among the cultivars. When cotyledonary petiole was used as explant MS medium supplemented with IAA 0.2 mg/l and BA 3 mg/l proved to be the best regeneration medium for *B. juncea* cv. Pusa bold (Singh et al. 2009). Our results and earlier reports clearly demonstrate that different combination of auxins and cytokinins are the best regeneration medium for different genotypes and that also depends on the type of explant used. According to the survey of literature, this appears to be the first report of establishment of regeneration protocol for *B. juncea* cv. Geeta.

Transformation

For transformation, the first step was to optimize the concentration of selection agent. Minimum inhibitory concentration (MIC) of kanamycin was determined by inoculating hypocotyl segments from 5-day-old seedling on shoot regeneration medium supplemented with varying concentration of kanamycin (Table 2). 47.2 % hypocotyl segment

Fig. 3 **a** Vector diagram of pROKII harbouring *Atleafy*. **b–d** PCR amplification of 11 transgenic lines with *Atleafy*, *nptII* and *uidA* specific primers. M- 100 bp ladder, C- Nontransformed *B. juncea* as negative control P- Plasmid harbouring CaMV 35S driven *Atleafy* gene as positive control and T1-T11 are transgenic lines (**b**) 8 out of 11 transgenic lines showed amplification of 975 bp *Atleafy* gene, (**c**) 8 lines showed 800 bp band with Kan specific primers and (**d**) 8 out of 11 lines showed 700 bp amplified product through GUS specific primers

showed regeneration at 30 mg/l of kanamycin and was taken as MIC. The concentration of kanamycin was increased to 40 mg/l on subsequent subculturing. The selected transformants were subsequently screened for GUS assay and the putative transgenics whose representative leaves showed blue colouration (positive GUS results) were selected for further screening through PCR (Fig. 2).

Confirmation through PCR

Genomic DNA of the GUS positive lines was isolated and was PCR amplified using *Atleafy*, *uidA* and *nptII* specific primers. 11 putative transgenic lines were PCR screened and 8 showed positive amplified bands on gel

Table 3 Transformation frequency of *B. juncea* cv. Geeta when transformed with *Atleafy*

Batch no.	No. of explants co-cultivated	No. of green shoots	Transformation frequency %
1	65	1	1.5
2	72	2	2.5
3	64	3	4.0
4	55	2	3.2

electrophoresis for *Atleafy* genes for Kan and GUS specific markers. 8 of them were considered stable transgenic for *Atleafy* (Fig. 3). Transformation frequency was calculated from four batches of transformation and maximum was found 4.0 (Table 3).

Phenotypic analysis

After 12 weeks the transgenic lines showed flowering in-vitro whereas their untransformed counterparts did not even

Fig. 4 Transgenic plants showing flower buds after 12 weeks of transformation

Fig. 5 Morphological changes in vegetative parts of transgenic plants. **a** fasciated petiole ↓ **b** fasciation of leaf blade ↓

after 16 weeks (Fig. 4). Early onset of flowering in transgenic plants is in concordance with the earlier reports of *Leafy* overexpression in different species tobacco (Weigel and Nilsson 1995), aspen (Wiegel and Nilsson 1995), rice (He et al. 2000), poplar (Rottmann et al. 2000), citrus (Pena et al. 2001) and *B. juncea* (Roy et al. 2009). The PCR positive lines also showed different morphological defects. Fasciation of petioles and leaves was observed (Fig. 5). This result is in confirmation with the earlier reports in tomato, petunia and other legumes where there is role of leafy homologues in compound leaf development Hofer et al. (1997) but is in contrast with the findings of Roy et al. (2009) in *B. juncea* cv. Varuna.

Early flowering is a desirable trait that makes the *B. juncea* to fit in multiple cropping system and its expansion in non-traditional areas like north-east. The study also demonstrates the exploitation of *LFY* in evading the terminal heat stress and hence in preventing the yield loss. Study is in progress on finding the effect of overexpression of *Atleafy* on yield.

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