Lipoxygenase-2-free Indian soybean (*Glycine max* L.) genotypes

Numerous nutraceutical components present in soybean are reported to check the onset of killer diseases like diabetes, estrogen deficiency-induced cancer, atherosclerosis, etc. Also, its richness in basic nutrients which can fight the diseases originating from mal and/or under nutrition have made soybean the 'functional food of the century'. Despite these virtues, barely 5–7% of the soybean seeds produced in India is processed for soyfood and snacks[1]. The off-flavour associated with soy products is one of the major deterrents in the widespread acceptance of soyfood. This is due to the hexanal compounds released by the catalytic oxidation of polyunsaturated fatty acids in the oil fraction by seed lipoxygenase enzyme. When the seeds are subjected to grinding for making any soy product, lipoxygenase comes in contact with its substrate (polyunsaturated fatty acids) and thereby triggers the catalytic oxidation in the presence of oxygen. In fact, lipoxygenase in soybean seed exists in three isozymic forms[2] – lipoxygenase-1, lipoxygenase-2 and lipoxygenase-3. Presence of each of the isozymes is controlled by single dominant genes, i.e. *Lox1*, *Lox2* and *Lox3* and their absence is ascribed to the corresponding null alleles (*lox1*, *lox2* and *lox3*). Soy preparations made from seeds of lipoxygenase-free genotypes are better accepted due to production of low levels of hexanal compounds[3]. Though lipoxygenases are heat labile, heat inactivation employed at industrial level not only incurs extra cost, but also affects the solubility and functionality of proteins[4]. In India, none of the soybean varieties released so far is free from any of the three lipoxygenases. Lipoxygenase-2 is mainly responsible for generation of off-flavour imparting *n*-hexanal in soybean[5]. Genetic removal of lipoxygenase-2 from the seeds has been shown to significantly improve the flavour of soy products[6]. Therefore, bringing lipoxygenase-2-free soybean under cultivation and making the seeds available for soy processors can boost utilization of soybean in food.

In view of this pressing need of the soy processing industry, a breeding programme aimed at the development of lipoxygenase-2-free soybean genotypes was initiated at the Directorate of Soybean Research, Indore. Here we report the successful development of lipoxygenase-2-free genotypes NRC109 and NRC110 by crossing varieties Samrat and PI086023. Samrat is a farmers' variety of soybean cultivated widely in Central India. PI086023 is a source of *lox2* allele which was procured from the United States Department of Agriculture. Its plant type is agronomically poor and not adaptable to Indian conditions. The crossing programme was carried out up to F7 generation. The selection for null lipoxygenase-2 plants in each generation was made by rapid assay in the seeds, according to Suda *et al.*[7]. Briefly, 2.5 mg soybean seed flour was mixed with 0.5 ml of distilled water by mild stirring and kept for 3–10 min and mixed with 2 ml dye substrate. The dye substrate stock was prepared by mixing 154 mg dithiothreitol, 25 ml 200 mM sodium phosphate buffer (pH 6.0), 5 ml 100 μM methylene blue, 5 ml 10 mM sodium linoleate substrate and 5 ml acetone. Change in colour after 3 min was recorded visually. In the test tubes containing lipoxygenase-2-positive seed flour, the blue colour of the dye bleached because of the quenching of free radicals generated due to the action of lipoxygenase-2 on polyunsaturated fatty acids, whereas in the lipoxygenase-2-free soy flour the blue colour of the dye persisted. Validation of null lipoxygenase-2 plants in the advanced generations of Samrat × PI086023 was performed using null allele-specific marker recently designed by Shin *et al.*[8] from the sequence analysis of *lox2* gene (null allele of lipoxygenase-2) of Korean cultivar Jinpumkong 2 (Table 1). DNA amplification using this gene-specific marker was confirmed by deploying it on null allele of lipoxygenase-2 from genotype PI086023 in the present study. For this purpose, genomic DNA extracted from the young leaves of NRC109, NRC110, Samrat (the recipient) and PI086023 (the donor of null allele of lipoxygenase-2) using the CTAB method of Doyle and Doyle[4] was utilized

Table 1. Forward and reverse sequences of primer specific to *lox2*

Primer	Sequence 5′ → 3′
Forward	AAACCAGTAAGATAACAGCAGATG
Reverse	AATGGCTCAATCACCGCT

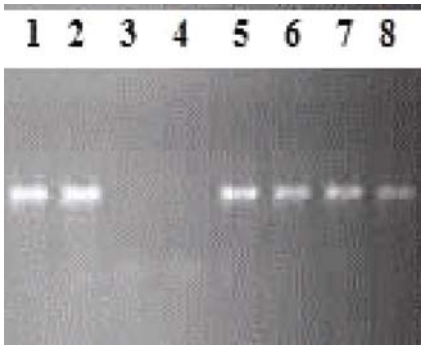

Figure 1. PCR amplicons generated using null allele-specific marker for lipoxygenase-2. Lanes 1, 2, NRC109; lanes 3, 4, Samrat; lanes, 5, 6, PI086023 and lanes 7, 8, NRC110.

Figure 2. Freshly harvested mature seeds of NRC109 (left) and NRC110 (right).

as template for amplification using the primer specific for the null allele of lipoxygenase-2. The oligonucleotide sequence of this null allele-specific marker was obtained from Sigma Aldrich. PCR reactions were performed in a thermocycler (model PTC100) and the reaction mixture (10 µl) contained 2 µl DNA (20 ng/µl), 1 µl PCR (10×) buffer, 1.1 µl $MgCl_2$ (25 mM), 0.1 µl dNTPs (25 mM), 0.4 µl each of forward and reverse SSR primers (30 ng/µl), 0.068 µl *Taq* DNA polymerase (3 U/µl) and 4.932 µl distilled water. Initially, DNA was denatured at 94°C for 1 min followed by 30 cycles, each cycle comprising denaturation at 94°C for 2 min, primer annealing at 68°C for 2 min and primer elongation at 72°C for 3 min. Finally, elongation was carried out at 72°C for 10 min. The PCR products were resolved on 3% metaphore gel. Amplicons of 769 bp size were observed in NRC109 and NRC110, similar to the donor parent PI086023 (Figure 1) and no amplicon was seen in Samrat, the lipoxygenase-2-positive parent. This confirmed the transfer of null allele of lipoxygenase-2 in NRC109 and NRC110. There was minor difference in plant height and flowering time between NRC109 and NRC110 and both the varieties bore purple flowers. NRC109 yielded seeds with yellow seed coat and black hilum, whereas the seeds of NRC110 were greenish-yellow with brown hilum (Figure 2). Furthermore, both the genotypes are bold-seeded and the weight of 100 seeds for NRC109 and NRC110 was 16.5 and 16.3 g respectively. NRC109 and NRC110 are early-maturing, a character desired by the farmers in Central India, with yield potential of 2.6 and 2.8 tonnes/ha respectively. NRC109 and NRC110 attain maturity in 85 and 93 days respectively. In brief, NRC109 and NRC110 are the first two Indian soybean genotypes which are free from lipoxygenase-2 isozyme, and can contribute immensely in boosting the soy food industry for health and nutritional security of the masses of the country.

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Ground feeding observations on corn (*Zea mays)* by eastern hoolock gibbon (*Hoolock leuconedys*)

Hoolock gibbons (genus *Hoolock)* inhabit primary tropical evergreen and subtropical semi-evergreen rainforests and also semi-deciduous forests of Southeast Asia[1], extending from Brahmaputra River east to the Salween River[2]. Globally, they are widely distributed in different forest types from the foothills to the mountains in Northeast (NE) India, South China, Bangladesh and Myanmar between lat. 20–28°N and long. 98–99°E. Two distinct species of hoolock gibbons are recorded from India, viz. western hoolock gibbon (*Hoolock hoolock*) and eastern hoolock gibbon (*Hoolock leuconedys*). In India, *H. hoolock* is distributed throughout the seven NE states except Sikkim[3], while *H. leuconedys* is only reported in protected and unprotected lowland tropical evergreen and semi-evergreen forest areas of Lohit and Lower Dibang Valley districts in Arunachal Pradesh and the eastern most part of Assam as well as south bank of Brahmaputra–Dibang river system[4,5].

Generally, gibbon species are highly selective feeders that are largely dependent on small, scattered fruit patches[6,7] and mostly feed on sugar-rich, juicy ripe fruits and figs[8]. Gibbons inhabiting tropical forests like the warm lowland forests in Bangladesh have been found to consume more fruit (70%) and less leaves (15%)[9,10]. Feeding ecology of the genus *Hoolock* is mostly documented for western hoolock gibbon. Only a few studies have been conducted on eastern hoolock gibbon in India, with particular emphasis on distribution and population status[4,5]. However, no study with regard to diet and behaviour has been made for *H. leuconedys* in India. Recently, a study in the northern montane forest of China has documented the seasonal variation of diet and time budget of the species[11].

During our long-term study on behavioural observation of *H. leuconedys*, we recorded uncommon ground feeding behaviour on agricultural crops (corn, *Zea mays*) by one of our selected study gibbon groups, which was mainly surviving in the highly fragmented, unclassed forest areas in the southeast boundary of Mehao Wildlife Sanctuary, Lower Dibang Valley district, Arunachal Pradesh. Geographically the area lies between 27°58′30″–28°03′38″N lat. and 95°50′30″–95°58′18″E long., and altitude ranges from 145 to 390 m amsl. The selected group was composed of one adult male