DOI : 10.5958/0974-0112.2019.00012.4

Indian J. Hort. 76(1), March 2019: 80-85

Development of molecular marker for nitrate reductase (NR) gene to improve nitrogen use efficiency in potato

Jagesh K. Tiwari*, Rasna Zinta, Vinod Kumar, Devendra Kumar, Tanuja Buckseth, RK Singh, Shashi Rawat and SK Chakrabarti

ICAR-Central Potato Research Institute, Shimla 171001, Himachal Pradesh

ABSTRACT

Two diverse potato varieties Kufri Jyoti (N inefficient) and Kufri Gaurav (N efficient) were used to amplify, clone and analyze the sequence of nitrate reductase (NR) gene, involved in N metabolism in plants. Only single, distinct and un-fractioned two fragments amplified by the NR gene-based primers were cloned and sequence analyzed. Following sequence analysis, non-redundant sequences with uninterrupted open reading frames of NR homologues were identified to the known N metabolism. A cleaved amplified polymorphic sequence (CAPS) marker of the NR gene was developed for marker-assisted selection (MAS) in potato. The CAPS marker was validated in a tetraploid mapping population of 123 individuals and 48 potato varieties for its polymorphism. Thus, the development of new CAPS marker would be useful for MAS with better nitrogen use efficiency (NUE) in potato.

Key words: *Solanum tuberosum*, *Cloning, CAPS marker, marker-assisted selection, nitrogen use efficiency.*

INTRODUCTION

Potato is the third most important food crop in the world after rice and wheat in terms of human consumption (Tiwari, 10). Potato is an input intensive crop and requires high N fertilizer for high tuber yield (Vos, 14). In India, a mature crop of potato yielding 25-30 t/ha tubers with recommended N fertilizer dose varies between 150-240 kg N/ha (Trehan, 12). However, potato plants utilize nearly 40-50 per cent of total applied N for their growth and development and excess N lost in the environment (Fageria *et al.*, 3). This is more aggravated in shallow-rooted potato and irrigated cultivation on sandy-loam soil, where nitrate leaching and contamination to the ground water and deteriorating soil health are predominant problems (Ospina *et al.*, 5). Hence, improving nitrogen use efficiency (NUE) of plant is essential to save cost with low-input N supply while maintaining the yield for sustainable potato production.

It is a known fact that plant N uptake and its regulation is a complex system, researchers have attempted to delineate the underlying mechanism of NUE. However, success in releasing nitrogen efficient genotypes has been limited may be due to its complex genetics and interactions with environmental variables (Gutiérrez, 4; Tiwari *et al.*, 10). Potato NUE involves complex interactions among various physiological processes such as N uptake by roots/ stolons, N assimilation/ utilization by shoots and roots/stolon/tubers, and N remobilization from source tissues. In-season good N management is critical for successful potato production (Zebarth and Rosen, 15). Substantial genetic variation for NUE in potatoes has been observed in commercial cultivars (Ospina *et al.*, 5) and wild species (Zebarth *et al.*, 17). A few studied on gene expression has been conducted in traits involved in N metabolism (Zebarth *et al.*, 16; Tiwari *et al.*, 11).

Previous studies at our institute have shown that Kufri Gaurav is N efficient potato variety, whereas cv. Kufri Jyoti is N inefficient (Trehan 12; Trehan and Singh, 13). However, knowledge about the genes involvement in the N metabolism and their sequence variation remains elusive. Therefore, objectives of this study were to isolate, clone and analyze sequence variations in the NR candidate key gene involved in the N metabolism; and develop molecular markers for marker-assisted selection in potato breeding.

MATERIALS AND METHODS

Two contrasting Indian potato varieties Kufri Gaurav (KG: N efficient) and Kufri Jyoti (KJ: N inefficient) were grown in the earthen pots (in duplicates) and leaf tissues were used for DNA analysis. Leaf samples of a mapping population of 123 progenies (Kufri Jyoti x Kufri Gaurav) and potato varieties were collected from field at Indian Council of Agricultural Research (ICAR)-CPRI, Regional Station, Modipuram, Meerut, UP, India and ICAR-CPRI, RS, Kufri, Shimla for the study.

*Corresponding author's Email: jageshtiwari@gmail.com

Total genomic DNA was isolated from the leaf tissues using DNeasy plant mini kit (Qiagen, Venlo, Limburg, Netherlands). Quality and quantity of the DNA was confirmed by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA), and 1% (w/v) agarose gel. Two primer-pairs were designed and tested for the presence of NR gene associated with N metabolism (Table 1). The PCR reaction prepared using Taq PCR core kit in a total volume of 25 µL consisted of 50 ng of DNA template in 1× PCR buffer (contained 15 mM $MgCl_2$), 200 µM dNTP, 0.5 µM of each primer (forward and reverse) and 1 U of Taq polymerase (Qiagen, Venlo, Limburg, Netherlands). The PCR was performed in a Veriti Thermal Cycler (Life Technologies, Carlsbad, California, USA) following 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 45 sec at the 57 °C, and 1 min at 72 °C; and final extension of 10 min at 72 °C. The amplified DNA products were separated on a 1.6 % agarose gel, documented and analyzed as described by Tiwari *et al.* (9).

Single and distinct PCR products were gel-eluted using QIAquick gel extraction kit (Qiagen, Venlo, Limburg, Netherlands) for cloning and sequencing. Cloning and sequencing procedures were followed as described earlier by Tiwari *et al.* (8). The nucleotide sequences of the isolated genes (KJ: KU965589 and KG: KU965590) in this study were submitted to the National Centre for Biotechnology Information Centre (NCBI) database. The resulting contigs were analyzed for homology search in the NCBI GenBank and the Potato Genome database for the available N metabolism genes. The phylogenetic analysis of the nucleotide sequences of the isolated two *N*-homologues (KJ: KU965589 and KG: KU965590) and the known N metabolism genes (PGSC0003DMT400077648 and NM_001288022.1) from the potato genome was carried out using the software program Molecular Evolutionary Genetics Analysis 6 (MEGA6) (Tamura *et al.*, 6).

Cleaved amplified polymorphic sequence **(**CAPS) markers were designed through the CAPS designer tool of Sol Genomics Network (https://solgenomics.net/tools/caps designer/caps _input.pl) using the isolated NR gene sequences (KJ: KU965589 and KG: KU965590). The PCR amplification, restriction digestion using respective enzymes and gel electrophoresis was followed as above. CAPS marker was validated in a mapping population (123 progenies) and varieties (48).

Table 1. Details of the primers used in PCR amplification and sequencing of the NR gene (PGSC0003DMT400077648) homologues isolated from potato varieties Kufri Jyoti and Kufri gaurav.

Particular	Primers	Sequence (5'→3')
PCR amplification	NR_Forward	AGAGACGAAGGTACCGCTGA
	NR_Reverse	TCCATGTCTCTCCTCCATCC
Internal sequencing	1S	CAGGCAGTTCACTCACCTTG
	2S	CGAAGGTACCGCTGATAATTG
	3S	AGAAGGAATTCGCCATGGAT
	4S	TGAGGTGCTCGACTTGCTTA
	5S	TGGAGGACTTTAGGATTGGTG
	6S	CACATTGAATATCAAGGCAAGG

RESULTS AND DISCUSSION

Leaf tissues of two potato cv. Kufri Jyoti (N inefficient) and Kufri Gaurav (N efficient) were PCR amplified using the NR gene-specific primers. We obtained nearly 2000 bp band size in both samples (Fig. 1). Single and distinct PCR products (~ 2000 bp) from each sample were sequenced and two N homologous genes were identified. The nucleotide sequences of isolated genes were deposited to the NCBI GenBank (KJ: KU965589 and KG: KU965590 (Table 2). Multiple sequence alignment of the nucleotide sequences of two N homolgues (KU965589 and KU965590) and known potato genes (PGSC0003DMT400077648) of N metabolism is shown in Fig. 2. Further, a phylogenetic analysis was carried out using the MEGA6 program to understand their relationships (Fig. 3).

In a process to design cleaved amplified polymorphic sequence (CAPS) marker, several restriction enzymes were analyzed in N metabolism genes (NRT, AMT,

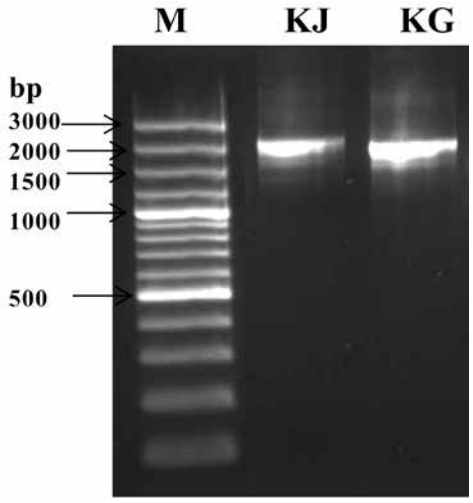

Fig. 1. Selected PCR amplification products in two contrasting potato varieties Kufri Jyoti (N inefficient) and Kufri Gaurav (N efficient) using NR gene specific primers. KJ: Kufri Jyoti; KG: Kufri Gaurav.

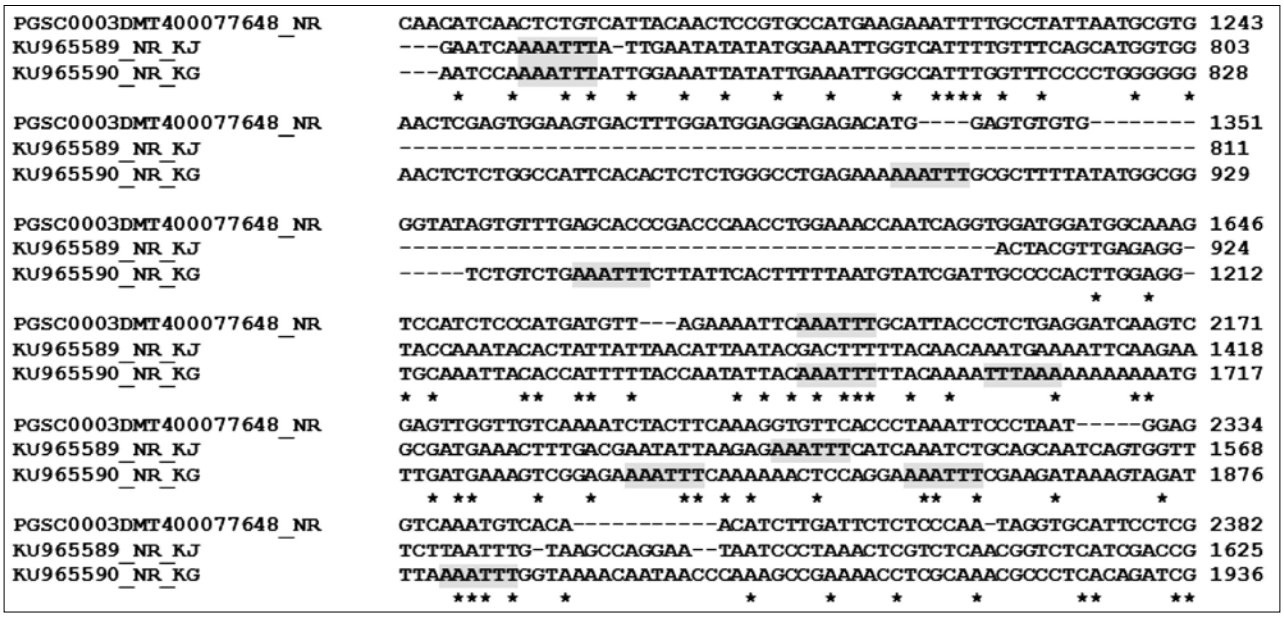

Fig. 2. Sequence alignment of the isolated nitrate reductase (NR) gene fragments from the potato varieties Kufri Jyoti (KU965589_NR_KJ) and Kufri Gaurav (KU965590_NR_KG) with the NR gene (PGSC0003DMT 400077648) and highlighted *Dra*I restriction site in the sequences (5'-TTT^AAA-3' and 3'-AAA^TTT-5').

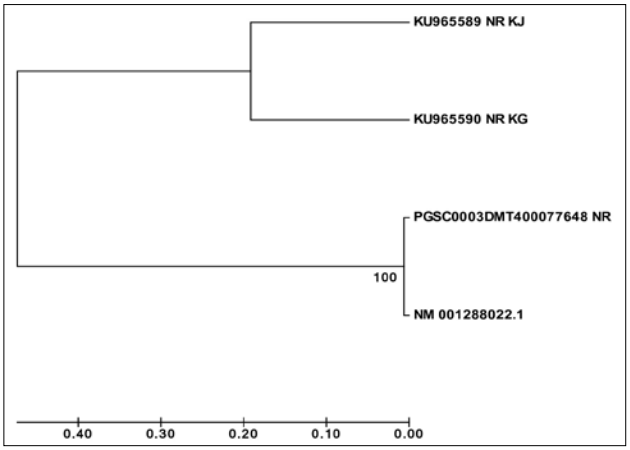

Fig. 3. A cluster analysis based on the UPGMA method showing relationship between the nucleotide sequences of the two N-homologues and two known N metabolism genes from the NCBI/Potato Genome database. The values at the nodes represent bootstrap support at 1000 replications.

NIR, NR and AS). However, only one CAPS marker of the NR gene (PGSC0003DMT400077648) with our designed primers (F: AGAGACGAAGGTACCGCTGA; and R: TCCATGTCTCTCCTCCATCC) was found effective. The PCR procedures (Ta = 57°C) followed by restriction digestion with *Dra*I enzyme resulted into distinct polymorphism in both varieties. A few sections of the sequence alignment of the NR gene with the isolated genes is shown in Fig. 2 and restriction sites (forward and reverse) of *Dra*I enzyme are highlighted. The PCR yielded 2000 bp band size products in both varieties and upon restriction with *Dra*I enzyme it produced variable bands (Kufri Jyoti: 250, 500, 1200 bp; Kufri Gaurav: 250, 500, 900, 1000 and 1200 bp of approximate sizes). This CAPS marker was found to be effective for selection of N use efficient genotype for breeding through marker-assisted selection.

CAPS marker was validated for its polymorphism in 49 varieties and segregating progenies (F_1 mapping population) of 123 individuals developed

Table 2. Sequence analyses and similarity search details of the two *N*-homologous genes isolated from the potato varieties.

S. No.	Variety	Primer	Sequence length	ORF	Accession No.	NCBI BLASTn homology search			Potato Genome BLAST search		
						Maximum score	Identity (%)	Accession No.	Maximum score	Identity (%)	PGSC transcript ID
1.	Kufri Jyoti	PgNR7648	1724	1681	KU965589	1275	99	NM_001288022.1	668	98.60	PGSC0003DMT400077648
2.	Kufri Gaurav	PgNR7648	2044	1990	KU965590	1024	92	NM_001288022.1	491	98.47	PGSC0003DMT400077648

NCBI Accession No. of the BLASTn search: NM_001288022.1 (*Solanum tuberosum* NADH nitrate reductase (NR3), mRNA).
Potato Genome BLAST search was performed to the Potato Genome Sequence database (http://solanaceae.plantbiology.msu.edu/blast.shtml). *E*-value is 0.0 for all accessions.

by crossing these both varieties (Kufri Jyoti x Kufri Gaurav) (Figs. 4 and 5). Variable amplification patterns of PCR products (275, 500, 1250 and 1500 bp) were observed in 99 out of 123 individuals (Fig. 4), perhaps due to recombination during crossing over in both varieties. Further, CPAS marker was also validated in 48 Indian potato varieties, as Fig. 5 shows different banding patterns (275, 500, 900, 1000 and 1250 bp). In the most N efficient potato variety Kufri Gaurav three distinct bands (275, 500 and 1250 bp), where as in the N inefficient variety Kufri Jyoti five bands (275, 500, 900, 1000 and 1250 bp) were observed. Similarly, in another moderate N efficient variety Kufri Pukhraj (Fig. 5, gel image SN 3) showed similar amplification patter like N efficient Kufri Gaurav. However, validation of other varieties under different N regimes needs to be confirmed either in field/pot or controlled experiments.

Identification of key genes regulating N metabolism has been considered an important approach to improve NUE in plants, as reviewed by Tiwari *et al.*, (10). A multitude of research has been attempted to uncover the genetic basis of NUE in many crops, but the utility of the results is limited because of complex genetics of NUE. We focussed to understand NUE in two contrasting potato varieties Kufri Jyoti and Kufri Gaurav selected based on the previous field-based studies (Trehan, 12; Trehan and Singh, 13). With the availability of potato genome sequence database, using homology-based gene cloning approach, we isolated N metabolism-associated candidate genes, sequenced, analysed their sequence variations and developed a CAPS molecular marker of NR gene for marker assisted selection. Previous researchers have shown that the significant genetic variations are available for NUE, particularly agronomic variables in potato germplasm (Zebarth *et al.*, 17) and commercial cultivars (Ospina *et al.*, 5).

Exploiting the sequence homology and PCR-based cloning approaches, late blight resistance genes have been isolated from wild potato species (Tiwari *et al.*, 8). Using similar approach, efforts were made to isolate genes involved in N metabolic pathways from the contrasting potato varieties using genes-specific primers. As a result of sequence variations in the NR gene, we developed CAPS marker using NR gene homologues of two contrasting varieties for molecular breeding purpose and to characterize mapping population and potato germplasm. Though, while validation of CAPS marker in a tetraploid mapping population, 24 individuals could not amplify the CAPS marker; this might be due to the loss of restriction site in the population. Since, NUE work in potato is very limited except a few gene expression work, our

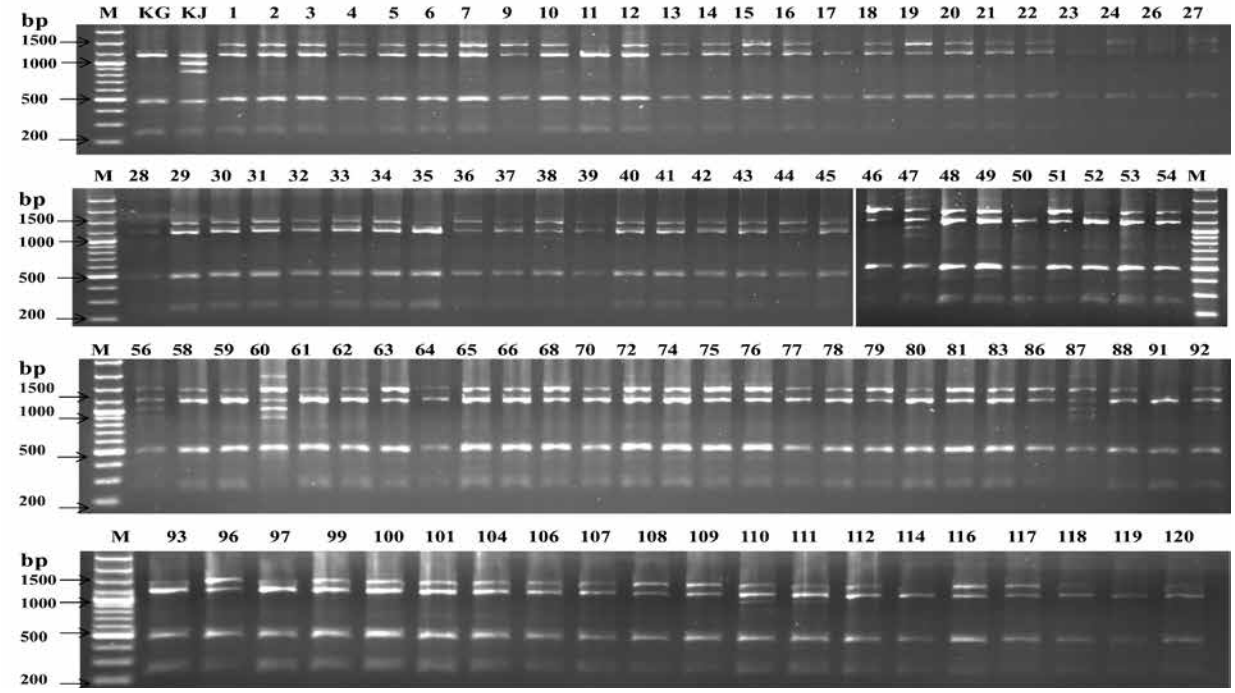

Fig. 4. Development of CAPS marker for the NR gene and validation in a mapping population of 123 individuals developed by crossing between Kufri Jyoti (KJ) and Kufri Gaurav (KG). Gel SN 1-120 shows amplification of the CAPS marker (restricted PCR products: 500, 900, 1000 and 1200 bp) by *Dra*I enzyme in the mapping population. Missing individual nos. has no amplification, so gel image is not shown.

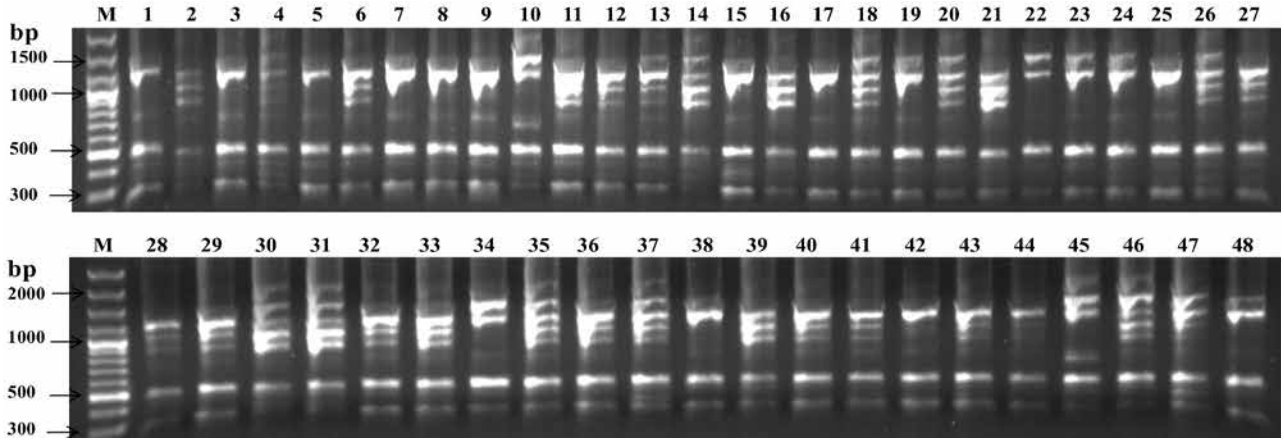

Fig. 5. Validation of CAPS marker of NR gene in 48 Indian potato varieties showing variable amplification pattern. Gel SN 1. Kufri (K) Gaurav, 2. K. Jyoti, 3. K. Pukhraj, 4. K. Garima, 5. K. Kanchan, 6. K. Alankar, 7. K. Anand, 8. K. Arun, 9. K. Ashoka, 10. K. Badshah, 11. K. Bahar, 12. K. Chamatkar, 13. K. Chandramukhi, 14. K. Chipsona-1, 15. K. Chipsona-2, 16. K. Chipsona-3, 17. K. Dewa, 18. K. Frysona, 19. K. Girdhari, 20. K. Giriraj, 21. K. Himalini, 22. K. Himsona, 23. K. Jawahar, 24. K. Jeevan, 25. K. Khasigaro, 26. K. Khyati, 27. K. Kuber, 28. K. Kumar, 29. K. Kundan, 30. K. Lalima, 31. K. Lalit, 32. K. Lauvkar, 33. K. Megha, 34. K. Muthu, 35. K. Neela, 36. K. Pushkar, 37. K. Red, 38. K. Sadabahar, 39. K. Safed , 40. K. Shailja, 41. K. Sheetman, 42. K. Sherpa, 43. K. Sinduri, 44. K. Surya, 45. K. Sutlej, 46. K. Swarna, 47. K. Chipsona 4, and 48. K. Mohan.

findings corroborate with previous results in other crops. Boisson *et al.* (2) presented partial sequence genes controlling N metabolism in wheat species in order to find fragments sequence variation of NR, NIR, GDH and GOGAT genes for mapping study. In another study, allele re-sequencing of *RTCL*, *RTH3*, *RUM1* and *RUL1* genes related to rot architecture and grain yield under N stress conditions by Abdel-Ghani *et al.* (1) showed considerable allelic diversity within the candidate genes that can be utilized to develop functional markers for identification of maize improvement. A few gene expression studies have been reported in potato using candidate genes involved in N metabolism (Zebarth *et al.*, 16; Tiwari *et al.*, 11).

To conclude, the present study shows identification of two *N*-homologous genes in two contrasting potato varieties Kufri Jyoti (N inefficient) and Kufri Gaurav (N efficient) for NUE based on the sequence homology of the N metabolism genes. The development of CAPS marker of the NR gene will be useful for characterization of N-use efficient potato genotypes for marker-assisted selection.

ACKNOWLEDGEMENT

The authors are grateful to the ICAR-CPRI, Shimla for necessary supports under the biotechnology program (Project No. HORTCPRICIL201500300131) and CAB in Scheme (IASRI, New Delhi). Sincere help of Ms. Sapna Devi, Ms. Nilofer Ali and Mr. CM Bist is gratefully acknowledged.

REFERENCES

1. Abdel-Ghani, A.H., Kumar, B., Pace, J., Jansen, C., Gonzalez-Portilla, P.J., Reyes-Matamoros, J., Martin, J.P.S., Lee, M. and Lübberstedt, T. 2015. Association analysis of genes involved in maize (*Zea mays* L.) root development with seedling and agronomic traits under contrasting nitrogen levels. *Plant Mol. Biol.* **88**: 133-47.

2. Boisson, M., Mondon, K., Torney, V., Nicot, N., Laine, A.-L., Bahrman, N., Gouy, A., Daniel-Vedele, F., Hirel, B., Sourdille, P., Dardevet, M., Ravel, C. and Le Gouis, J. 2005. Partial sequences of nitrogen metabolism genes in hexaploid wheat. *Theor. Appl. Genet.* **110**: 932-40.

3. Fageria, N.K., Baligar, V.C. and Li, Y.C. 2008. The Role of nutrient efficient plants in improving crop yields in the twenty first century. *J. Plant Nutr*. **31**: 1121-57.

4. Gutiérrez, R.A. 2012. Systems biology for enhanced plant nitrogen nutrition. *Science*, **336**: 1673-75.

5. Ospina, C.A., van Bueren, E.T.L., Allefs, J.J.H.M., Engel, B., van der Putten, P.E.L., van der Linden, C.G. and Struik, P.C. 2014. Diversity of crop development traits and nitrogen use efficiency among potato cultivars grown under

contrasting nitrogen regimes. *Euphytica*, **199**: 13-29.

6. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6:molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**: 2725-29.

7. Tiwari, J.K., Devi, S., Ali, N., Dua, V.K., Singh, R.K. and Chakrabarti, S.K. 2018. Cloning and sequence variation analysis of candidate genes involved in nitrogen metabolism in potato (*Solanum tuberosum*). *Indian J. Agric. Sci*. **88**: 751-56.

8. Tiwari, J.K., Devi, S., Sharma, S., Chandel, P., Rawat, S. and Singh, B.P. 2015a. Allele mining in *Solanum* germplasm: cloning and characterization of RB-homologous gene fragments from late blight resistant wild potato species. *Plant Mol. Biol. Rep*. **33**: 1584-98.

9. Tiwari, J.K., Devi, S., Sundaresha, S., Chandel, P., Ali, N., Singh, B., Bhardwaj, V. and Singh, B.P. 2015b. Microarray analysis of gene expression patterns in the leaf during potato tuberization in the potato somatic hybrid *Solanum tuberosum* and *Solanum etuberosum*. *Genome*, **58**: 305-13.

10. Tiwari, J.K., Plett, D., Garnett, T., Chakrabarti, S.K. and Singh, R.K. 2018. Integrated genomics, physiology and breeding approaches for improving nitrogen use efficiency in potato: translating knowledge from other crops. *Funct. Plant Biol*. **45**: 587-605.

11. Tiwari, J.K., Trehan, S.P., Sundaresha, S., Poonam, Singh, B.P., Dua, V.K. and Bhardwaj, V. 2014. Gene expression analysis: indicators of nitrogen use efficiency in potato cultivars. *Potato J.* **41**: 175-79.

12. Trehan, S.P. 2009. Improving nutrient use efficiency by exploiting genetic diversity of potato. *Potato J.* **36**: 121-35.

13. Trehan, S.P. and Singh, B.P. 2013. Nutrient efficiency of different crop species and potato varieties – in retrospect and prospect. *Potato J.* **40**: 1-21.

14. Vos, J. 2009. Nitrogen responses and nitrogen management in potato. *Potato Res* **52**: 305-17.

15. Zebarth, B.J. and Rosen, C.J. 2007. Research perspective on nitrogen BMP development for potato. *American J. Potato Res*. **84**: 3-18.

16. Zebarth, B.J., Tai, H., Luo, S., Millard, P., De Koeyer, D., Li, X.-Q. and Xiong, X. 2012. Effect of nitrogen form on gene expression in leaf tissue of greenhouse grown potatoes during three stages of growth. *American J. Potato Res.* **89**: 315-27.

17. Zebarth, B.J., Tarn, T.R., de Jong, H. and Murphy, A. 2008. Nitrogen use efficiency characteristics of andigena and diploid potato selections. *American J. Potato Res.* **85:** 210-18.

Received : May, 2018; Revised : February, 2019;
Accepted : February, 2019