Indian Journal of Agricultural Sciences **88** (5): 751–6, May 2018/Article

Cloning and sequence variation analysis of candidate genes involved in nitrogen metabolism in potato (*Solanum tuberosum*)

JAGESH KUMAR TIWARI[1], SAPNA DEVI[2], NILOFER ALI[3], VIJAY K DUA[4], RAJESH K SINGH[5] and SWARUP K CHAKRABARTI[6]

ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh

Received: 26 August 2017; Accepted: 22 March 2018

ABSTRACT

Objective of this study was to isolate, sequence and analyze variations in candidate genes involved in nitrogen (N) metabolism in potato (*Solanum tuberosum* L.) for future breeding application. Two contrasting potato varieties, viz. Kufri Gaurav (N efficient) and Kufri Jyoti (N inefficient) were used. Of the total 17 PCR primers tested for N metabolism genes in the both varieties and only 12 distinct bands were cloned and sequenced, which were amplified by six primers of five genes (nitrate transporter-NRT, ammonium transporter-AMT, nitrate reductase-NR, nitrite reductase-NIR and asparagines synthetase-AS). Following sequence analysis, non-redundant sequences with uninterrupted open reading frames of 12 '*N*-homologous genes' were identified to the known N metabolic pathway genes. Thus, the identified 12 N-homologous genes may serve as an important genomic resource for novel gene/marker discovery and would be useful for marker-assisted selection (MAS) in potato with better nitrogen use efficiency (NUE).

Key words: Cloning, Nitrogen metabolism genes, Nitrogen use efficiency, Potato, Sequencing

Nitrogen (N) is the most important nutrient for plant growth and development. High N requirement, shallow root system and irrigated cultivation of potato (*Solanum tuberosum* L.) increase the chance of nitrate leaching and contamination to the groundwater and thus degrade soil health (Ospina *et al.* 2014). Therefore, enhancing nitrogen use efficiency (NUE) of potato is essential for its sustainable cultivation. Potato NUE involves complex interactions among various physiological processes such as N uptake and its assimilation/utilization by potato plant. Substantial genetic variation for NUE has been observed in potato (Zebarth *et al.* 2004, Ospina *et al.* 2014). With the advancement of genomics and NUE research in other plants like *Arabidopsis*, rice, wheat and maize, very recently a holistic view for improving NUE in potato has been presented by Tiwari *et al.* (2018) and also discussed genomics approaches (Tiwari *et al.* 2017). However, nitrogen metabolism has been meagrely studied in potato, and it was mostly focussed on agronomic management. A few reports on measurement of N sufficiency by gene expression analyses of N metabolism genes are available (Tiwari *et al.* 2014, Zebarth *et al.* 2012, 2011; Li *et al.* 2010).

[1]Scientist (Senior Scale) (e mail: jageshtiwari@gmail.com), [2]JRF (e mail: sapnadogra609@gmail.com), [3]JRF (e mail: sweetnilofer786@gmail.com), [4]Head and Principal Scientist (e mail: vkdua65@yahoo.com.in), [5]Head and Principal Scientist (e mail: rjan_1971@yahoo.com.in), [6]Director (e mail: Chakrabarti.SK@icar.gov.in.).

Previous studies at our institute have shown that Kufri Gaurav is N efficient potato variety, whereas cv. Kufri Jyoti is N inefficient one. Higher N use efficiency in Kufri Gaurav is probably due to better utilization capacity of absorbed N than in Kufri Jyoti (Trehan 2009, Trehan and Singh 2013). However, knowledge about genes involved in the N metabolism and their sequence variation remains elusive. Therefore, objective of this study was to isolate, clone and analyze sequence variations in the candidate genes involved in the N metabolism.

MATERIALS AND METHODS

Two Indian potato varieties, viz. Kufri Gaurav (N efficient) and Kufri Jyoti (N inefficient) were grown in the earthen pots (in duplicates) and leaf tissues were used for this study. Total genomic DNA was isolated from the leaf tissues using DNeasy plant mini kit. DNA was quantified using NanoDrop 2000 spectrophotometer, and quality was checked on 1% (w/v) agarose gel. Seventeen primer-pairs were tested for the N metabolism-associated genes (NRT, AMT, NR, NIR, AS, glutamate dehydrogenase-GDH, and glutamine synthetase-GS) to amplify the genomic DNA of samples with annealing temperature (Ta) as shown in Table 1. The PCR reaction was 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. The PCR was performed in a Veriti Thermal Cycler following procedure of an initial

Table 1 Summary of nitrogen metabolism-associated genes and primers used for PCR amplification in potato varieties

Crop	Primer	Sequence (5'→3')	Annealing temp (Ta)	N metabolism pathways gene	Gene ID
Potato	PgNRT1304	F: GGATCACCGGGGAGTTCTAT R: TTGGTGTCAAATTTGGTGGA	57	Nitrate transporter (NRT)	PGSC0003DMT400031304
Potato	PgAMT9775	F: TTTGCAGCAGTCCTTGTTTG R: GCCAATACACCTGCCAGAAT	57	Ammonium transporter (AMT)	PGSC0003DMT400049775
Tomato	LeAMT1-3	F: GCTGGTTCTGTTAGGGCAAA R: CGTAGGCATAACCTCCGTGA	57	Ammonium transporter (AMT1-3)	NM_001247287.1
Potato	PgNR7648	F: AGAGACGAAGGTACCGCTGA R: TCCATGTCTCTCCTCCATCC	57	Nitrate reductase (NR)	PGSC0003DMT400077648
Potato	PgNIR1310	F: CCATCGGAGAAAACACCACT R: CAGACGGAGCTCTCCTGAAC	57	Nitrite reductase (NIR)	PGSC0003DMT400021310
Potato	PgAS0685	F: TGACATGCTGGATGGAGTGT R: TTTGGAAGGTACGGTTGCTC	57	Asparagine synthetase (AS)	PGSC0003DMT400010685
Tobacco	NtNRT1.1	F: GCAGTGGAGAGGCTAACGAC R: TCAATCCCACACTCAGCAAG	50	Nitrate transporter (NtNRT1.1-t)	AB102806
Tomato	LeAMT1-2	F: CTCAGCTGCCGAGCTCTT R: TGCAAATCCTCCATGTCTTG	50	Ammonium transporter (AMT1-2)	NM_001247324
Tomato	Lenii2	F: GCGGAGATAGCTGCTGAAAG R: TCTCTTGGAACAGCACCAAA	50	Nitrite reductase (nii2)	XM_004248688.1
Potato	StNR5	F: AATCGTCGGAAAGAGCAGAA R: GCCAATGCCAATGTTGTATG	50	Nitrate reductase (NR)	AB062142.1
Tomato	LeAS1	F: ATTTTGGCTTTGTTGGGTTG R: GCAACACCGGATAGATGGTT	53	Asparagine synthetase (AS1)	XM_004240358.1
Potato	StGS1	F: TCAGGAGGGGCAACAATATC R: CGCGTACATAGAGGTCACGA	50	Glutamine synthetase GS1 (gln)	AF302115.1
Potato	StGS2	F: CCTTTCCGTGGTGGTAACAA R: GAGATCTTTTGGGCAGCAAG	50	Glutamine synthetase GS2 (gln)	AF302113.1
Tobacco	NpGDH	F: CAGCCACCAACCGTAACTTT R: ACGCCTCCCAACCTCTTAAT	53	Glutamate dehydrogenase (GDH)	AB062142.1
Tomato	LeARG1	F: GAGGAGTGCTGGAAGAATGG R: AACCACATCAGCACCAACAA	53	Arginase 1 (ARG1)	AY656837.1
Potato	PgGDH7579f1	F: ACCTTGTCGGCTCTCTTTCA R: TTGCTGAAAACTAAGCCCATC	53	Glutamate dehydrogenase	PGSC0003DMT400017579
Potato	PgGDH7579f2	F: GATAGGGGTGAGACGCATGT R: GCAGCCATAGAAGGAGCAAC	53	Glutamate dehydrogenase	PGSC0003DMT400017579

denaturation of 5 min at 94°C; followed by 35 cycles of 1 min at 94°C, 45 sec at the T_a, and 1 min at 72°C; and a 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.* (2015).

Single and distinct PCR products were gel-eluted using QIAquick gel extraction kit for cloning. Cloning, sequencing, processing and analysis procedures were followed as described earlier by Tiwari *et al.* (2015). Sequencing was performed using the universal primer M13 on '3500 Genetic Analyzer'. The nucleotide sequences of the isolated genes (KU965581- KU965592) (hereafter referred to '12 N-homologues') in this study were submitted to the National Centre for Biotechnology Information Centre (NCBI) database. The sequences were aligned by multiple sequence alignment using default parameters of the Clustal Omega tool of EMBL-EBI program. The phylogenetics analysis of the isolated 12 N-homologues and the known N metabolism genes was carried out using the software 'Molecular Evolutionary Genetics Analysis 6 (MEGA6)' (Tamura *et al.* 2013).

RESULTS AND DISCUSSION

PCR amplification

Out of total 17 primers tested (Table 1), 15 primer pairs were amplified in both the varieties, and amplifications products are summarized in Table 2. Primers of the genes involved in N uptake/transport (AMT and NRT) and utilization/assimilation (NR, NIR, GDH and AS) produced

Table 2 Details of PCR products (approximate size in bp) amplified and cloned from potato varieties

Primer	Variety		Total fragments	No of fragments cloned
	Kufri Jyoti	Kufri Gaurav		
PgNRT1304	2000	2000	2	2
PgAMT9775	2200	2200	2	2
LeAMT1-3	1200	1200	2	2
PgNR7648	2000	2000	2	2
PgNIR1310	1500	1500	2	2
PgAS0685	2000, 1000, 400	2000, 400	5	2
NtNRT1.1	1200	1200	2	0
LeAMT1-2	800, 1000	800, 1000	4	0
Lenii2	500, 2000	500, 2000	4	0
StNR5	300, 500	300, 3000	4	0
LeAS1	500, 600, 700	500, 600, 700	6	0
StGS1	1200	1200	2	0
StGS2	2800, 700, 600, 500	2800, 700, 600, 500	8	0
NpGDH	0	0	0	0
LeARG1	3000	1500	2	0
PgGDH7579f1	0	0	0	0
PgGDH7579f2	400, 500, 600, 800	400, 500, 600, 800	8	0
Total			55	12

Underlined 12 PCR products (single and distinct band) were gel-eluted, cloned, sequenced and analyzed. Isolated products were homologues to the six known N metabolic pathways genes. Of which five are potato genes: PGSC0003DMT400031304 (Nitrate transporter, NRT); PGSC0003DMT400049775 (Ammonium transporter, AMT); PGSC0003DMT400021310 (Nitrite reductase, NIR); PGSC0003DMT400077648 (Nitrate reductase, NR); PGSC0003DMT400010685 (Asparagine synthetase, AS); and one known tomato gene NM_001247287.1 (Ammonium transporter, AMT1-3). Faint bands were excluded from the study.

desired fragments for further use. Only single and distinct 12 fragments were amplified in both varieties with following gene/primer combinations: (i) NRT: PgNRT1304, (ii) AMT: PgAMT9775 and LeAMT1-3, (iii) NR: PgNR7648, (iv) NIR: PgNIR1310, and v) AS: PgAS0685 (Fig. 1).

Cloning, sequencing and phylogenetics analysis

Details of the PCR products gel-purified, cloned and sequence submission/analysis of 12 amplified single and distinct PCR products are summarized in Table 3. CAP3 analysis resulted into 12 contigs of homologous genes involved in N metabolic pathways. The subsequent BLASTn analysis of the 12 N-homologues showed 86-97% sequence identity (E-value 0.0) to known N metabolism genes in the NCBI database (Table 3). In addition, these isolated new homologues showed 96-99% identity with the known potato genes in the BLAST search to the Potato Genome Sequence database. Amino acid translations of the DNA sequence of N-homologues using EMBOSS Transeq tool produced 12 sequences with uninterrupted ORFs in at least one of the reading frames. The length of the identified 12 N-homologues varied from 1173 to 2164 bp. The UPGMA tree consists of six groups (I-VI), where each group includes three sequences of one known gene and two 'N-homologues'. Thus, the phylogenetic tree indicated the existence of relationship of N-homologues and known genes involved in N metabolism.

Previous researchers have shown that the significant genetic variations are available for NUE, particularly for both NUpE (plant N content/soil N supply) and NUtE (plant dry matter/plant N content) among potato germplasm (Zebarth *et al.* 2004, 2008; Trehan 2009, Ospina *et al.* 2014). We focussed to understand NUE in two contrasting potato varieties Kufri Gaurav (N efficient) and Kufri Jyoti (N inefficient) selected based on the previous field-based studies (Trehan 2009, Trehan and Singh 2013). We were successful in amplifying single and distinct PCR products of homologues of N metabolism genes. The identified potential 12 N-homologues had 86-99% sequence identity to known N genes, indicating that these genes are well conserved in

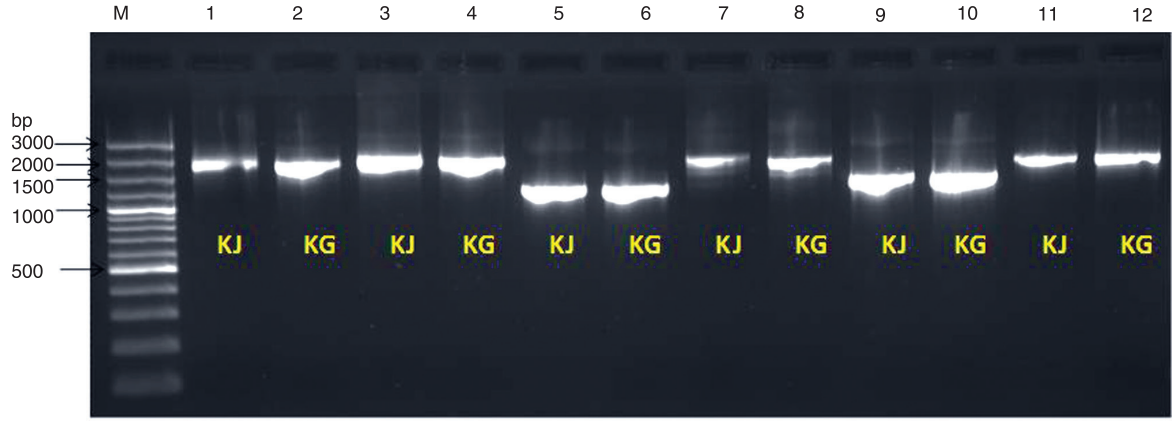

Fig 1 Selected PCR amplification products in two contrasting potato varieties, viz. Kufri Jyoti (KJ: N inefficient) and Kufri Gaurav (KG: N efficient) by the primers of the genes involved in the N metabolism pathways. SN 1-12 denotes varieties (KJ/KG) amplified by primers: Lane #1-2 (PgNRT1304), #3-4 (PgAMT9775), #5-6 (LeAMT1-3), #7-8 (PgNR7648), #9-10 (PgNIR1310), and #11-12 (PgAS0685).

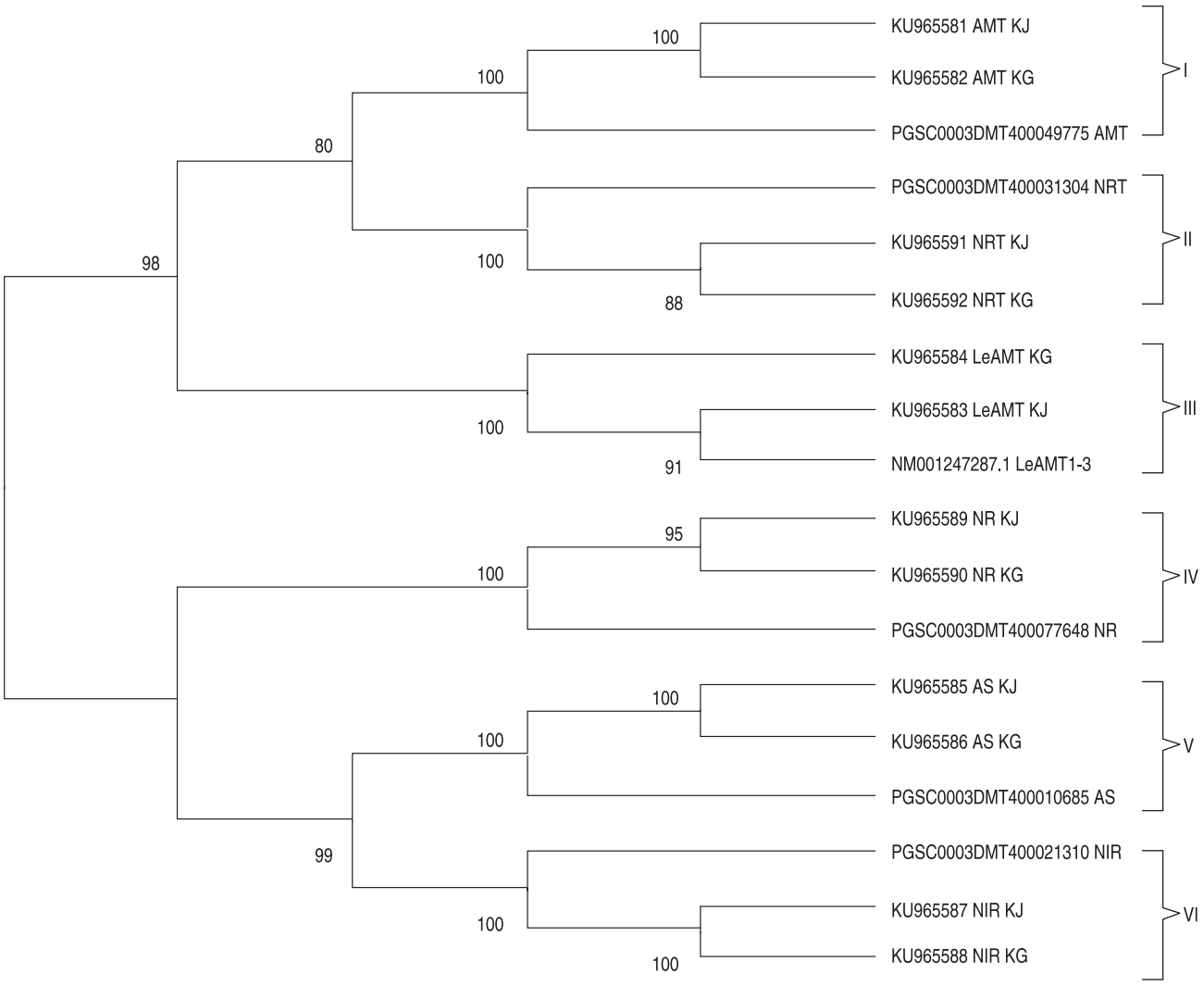

Fig 2 A cluster analysis based on the UPGMA method showing relationship among the nucleotide sequences of 12 N-homologues and known N metabolism genes from the NCBI/Potato Genome database.

potato. Their presence in two varieties suggested that they might be functional N genes that may provide improved NUE to crops. Further, the UPGMA cluster analysis showed six groups (I-VI) that N-homologous genes cluster in together in one group with known respective N gene source but in separate clade due to underlying sequence variations. Our study indicates that the potato species may contains multitudes of sequence variations in N genes isolated from these two contrasting varieties. Thus, the process of evolution of homologous genes may involve conserved common features and subsequence accumulation of mutations within the motif regions. A few gene expression studies have been reported in potato using candidate genes involved in N metabolism pathways (Tiwari *et al*. 2014, Zebarth *et al.* 2011, 2012).

Conclusion

The present study shows identification of '12 N-homologous genes' in two contrasting potato varieties Kufri Gaurav (N efficient) and Kufri Jyoti (N inefficient) for NUE based on the sequence homology of the N metabolism genes. In future, it would be worthwhile to develop molecular marker like SNP and/or CPAS based on the sequence variations for screening of germplasm and breeding purpose, analyse gene expression pattern of these '12 N-homologues' in cDNA pool and regulatory small/ microRNAs involved in NUE in potato under different N regimes under controlled conditions.

ACKNOWLEDGEMENTS

The authors are grateful to the ICAR-CPRI, Shimla for necessary support under the biotechnology program (Project No. HORTCPRICIL201500300131). Sincere help of Mr. CM Bist is gratefully acknowledged for sequencing. The authors thank anonymous reviewer for valuable inputs to improve the manuscript.

REFERENCES

Li X-Q, Sveshnikov D, Zebarth B J, Tai H, Koeyer D D, Millard P, Haroon M and Singh M. 2010. Detection of nitrogen

Table 3 Sequence analyses and similarity search details of the 12 'N-homologous genes' isolated from the potato varieties

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
Kufri Jyoti	PgNRT1304	1679	1536	KU965591	1871	89	HG975445.1	655	96.94	PGSC0003DMT400031304
Kufri Gaurav	PgNRT1304	1740	1665	KU965592	2263	90	HG975445.1	651	96.26	PGSC0003DMT400031304
Kufri Jyoti	PgAMT9775	1919	1917	KU965581	2054	86	HG975448.1	444	95.69	PGSC0003DMT400049775
Kufri Gaurav	PgAMT9775	1959	1959	KU965582	937	89	HG975448.1	529	99.81	PGSC0003DMT400049775
Kufri Jyoti	LeAMT1-3	1568	1512	KU965583	2017	97	XM_006346793.2	1035	96.94	PGSC0003DMT400048284
Kufri Gaurav	LeAMT1-3	1173	1140	KU965584	1179	95	XM_006346793.2	580	95.65	PGSC0003DMT400048284
Kufri Jyoti	PgNIR1310	1456	1418	KU965587	1439	86	HG975522.1	316	97.19	PGSC0003DMT400021310
Kufri Gaurav	PgNIR1310	1717	1665	KU965588	1408	87	HG975522.1	324	97.75	PGSC0003DMT400021310
Kufri Jyoti	PgNR7648	1724	1681	KU965589	1275	99	NM_001288022.1	668	98.60	PGSC0003DMT400077648
Kufri Gaurav	PgNR7648	2044	1990	KU965590	1024	92	NM_001288022.1	491	98.47	PGSC0003DMT400077648
Kufri Jyoti	PgAS0685	2085	2058	KU965585	743	93	HG975518.1	205	97.78	PGSC0003DMT400010685
Kufri Gaurav	PgAS0685	2164	2128	KU965586	592	90	HG975518.1	158	92.92	PGSC0003DMT400010685

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 search except 1e-112 and 6e-84 for KU965585 and KU965586, respectively.

sufficiency in potato plants using gene expression markers. *American Journal of Potato Research* **87**: 50–59.

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.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–9.

Tiwari J K, Devi S, Ali N, Buckseth T, Moudgil V, Singh R K, Chakrabarti S K, Dua V K, Kumar D and Kumar M. 2017. Genomics approaches for improving nitrogen use efficiency in potato. (*In*) *The Potato Genome. Compendium of Plant Genomes*. Chakrabarti S K, Conghua X and Tiwari J K (Eds) Springer, Cham.

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

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. *Functional Plant Biology*, https://doi.org/10.1071/FP17303.

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 Journal* **41**: 175–9.

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

Trehan S P. 2009. Improving nutrient use efficiency by exploiting genetic diversity of potato. *Potato Journal* **36**: 121–35.

Zebarth B J, Tai G, Tarn R, de Jong H and Milburn P H. 2004. Nitrogen use efficiency characteristics of commercial potato cultivars. *Canadian Journal of Soil Science* **84**: 589–98.

Zebarth B J, Tai H, Luo S, Millard P, De Koeyer D, Li X-Q and Xiong X. 2011. Differential gene expression as an indicator of nitrogen sufficiency in field-grown potato plants. *Plant and Soil* **345**: 387–400.

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 Journal of Potato Research* **89**: 315–27.

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