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Effect of nitrogen-starvation on growth pattern and expression of nitrogen assimilation genes

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ABSTRACT

Studying plant response and adaptation under low nitrogen stress condition is pre-requisite to enhance nitrogen use efficiency in crops. The present study investigated the physiological and molecular responses of maize (*Zea mays* L.) to nitrogen stress during early vegetative stage. Maize seedlings were grown hydroponically under controlled environmental conditions in phytotron. One set of plants were nutritionally stressed by eliminating nitrogen source in hydroponic culture while the other set was provided with nitrogen (2 mM KNO_3). Under nitrogen-starvation condition, plant growth and physiological parameters changed dramatically. Significant reduction in chlorophyll content, total soluble proteins and nitrate reductase activity was observed. Further, nitrogen-starvation resulted into differential expression of genes related to nitrogen-assimilation and metabolism. The present study might be useful to improve our understanding towards plants adaptive response under nitrogen-starvation conditions.

Key words: Gene expression, Maize, Metabolism, Nitrogen starvation, Physiological parameters

Nitrogen (N) is a major limiting factor in most agricultural systems. Because the N availability strongly influences crop productivity, a vast amount of N fertilizers is applied to maximize yields. However, the nitrogen use efficiency (NUE) for cereals is as low as 33%, which means that more than 60% of the applied fertilizer is lost to the environment (Raun and Johnson 1999). NUE involves efficiency in N uptake, assimilation, remobilization and utilization. The improvement of NUE in cereal crops is essential not only to reduce the cost of cultivation but also to reduce environmental pollution, save energy which is consumed during the production of chemical fertilizers, improve soil health, and ultimately help in mitigating climate change.

The N uptake, assimilation and metabolism pathways in higher plants have been well documented. N is absorbed as nitrate (NO_3^-) and NH_4^+ by roots through high- and low affinity nitrate transporters (NRT1 and NRT2) and ammonium transporters (AMTs). The absorbed nitrate is first reduced into nitrite by cytosolic nitrate reductase (NR) enzyme and finally into ammonium by nitrite reductase (NiR) enzyme in chloroplast. Subsequently, ammonium is incorporated into the organic form via assimilation by glutamine synthetase (GS) and glutamate synthase/glutamine-2-oxoglutarate aminotransferase (GOGAT), also known as GS/GOGAT cycle (Krapp 2015). Subsequent to GS/GOGAT pathway, two crucial enzymes are involved in N re-assimilation/ remobilization and metabolism, and biosynthesis of other amino acids, *viz*. cytosolic asparagine synthetase (AS), and mitochondrial NADH-glutamate dehydrogenase (GDH). The AS enzyme catalyses the conversion of glutamine into asparagine, which is a transport form of N in phloem and source of N for biosynthesis of other amino acids in sink tissue (Gaufichon *et al.* 2013).

In order to develop N use efficient genotypes, a better understanding of plants response towards N-deficiency, adaptive changes to minimize the negative effects of such stress condition and changes in expression of key genes playing pivotal role in N-assimilation and metabolism is needed. In this context, the present study has investigated the effect of N-starvation and expression profile of selected N-assimilation and metabolism related genes in maize.

MATERIAL AND METHODS

Plant material and growth conditions: Maize hybrid, HQPM-1 seeds were surface sterilized and allowed to germinate at 25°C in wet germination paper in the dark. The germinated seedlings were grown hydroponically in

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plastic trays. The hydroponic experiment was performed at National phytotron facility, IARI, New Delhi (2016) under controlled conditions, i.e. 29/21(± 2)°C day/night temperatures with 32–53% humidity. For initial 3 days, seedlings were grown hydroponically in modified Hoagland solution containing 0.1 mM $CaCl_2$, 0.75 mM K_2SO_4, 0.25 mM KH_2PO_4, 0.65 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM Fe-EDTA, 0.01 mM H_3BO_3, 1 µM $MnSO_4 \cdot H_2O$, 1 µM $ZnSO_4 \cdot 7H_2O$, 0.5 µM $CuSO_4 \cdot 5H_2O$, 0.005 µM $(NH_4)_6MO_7O_{24} \cdot 4H_2O$ and pH of the nutrient solutions was adjusted to 6.0. Nitrogen was given in the form of 2 mM KNO_3. After 3 days, one set of experiment was grown in modified Hoagland solution with 2mM KNO_3 (represented as sufficient N or +N condition) while other set was supplied with modified Hoagland solution without KNO_3 (represented as N-deprived or –N condition). The nutrient solutions were continuously aerated by an electric pump and replaced every 3 days. Both the sets were allowed to grow in respective solutions for 21 days. After 21 days, plants were harvested and half of them were used immediately for measuring growth parameters while remaining were frozen in liquid nitrogen and stored at –80°C for further use.

Measurement of growth related parameters: A total of 3 plants were randomly taken from both the sets (+N and –N) after 21 days, washed with running tap water and various physiological parameters such as stem girth, shoot fresh weight, root length, root fresh weight, and root volume were measured. Subsequently, shoot and root from these plants were dried in oven at 65°C for 72 h and followed by measuring root and shoot dry weights.

Estimation of pigments, amino acid and soluble protein content: The Chlorophyll (Chl) and carotenoid estimation was performed by slight modification in dimethyl sulphoxide (DMSO) Chl extraction method described by Hiscox and Israelstam (1979). For determining total amino acids content, samples were processed as described by Naidu (1998). Total amino acids were measured by slight modification in ninhydrin method described by Moore and Stein (1954). The amino acid concentration in the extract was calculated from the standard curve of alanine and then converted to tissue amino acid content expressed as micromoles per gram of fresh weight (Chen *et al.* 2007). The total soluble proteins were estimated as per Bradford (1976) method.

Nitrate reductase (NR) activity estimation: Estimation of *in-vivo* nitrate reductase activity was done as previously described by Klepper *et al.* (1971). The nitrite produced in the reaction was estimated by the method of Evans and Nason (1953). The assay involved nitrate reduction in the presence of NADH solution which subsequently reacted with sulphanilamide and NEDD (1-Naptyl ethylene diamine dihydrochloride) solution.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis: Total RNA was isolated from frozen leaf and root samples using AmbionPureLink™ Plant RNA kit (Invitrogen) according to the manufacturer's protocol. The quantity of the RNA was assessed using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). The first strand cDNA was synthesized using Superscript II reverse transcriptase kit (Invitrogen) as per manufacturer protocol. Maize N assimilation and metabolism related gene sequences were obtained from NCBI and gene specific primers for quantitative real time polymerase chain reaction (qRT-PCR) were designed using Primer 3.0 software (Table 1). The qRT-PCR was performed in triplicates using the Brilliant II SYBR Green QPCR Master mix (Agilent) in real time PCR (Agilent Technologies, USA) detection system. Maize α *tubulin* gene was used as an internal control to normalize gene expression values. The relative expression levels of genes were calculated as per Livak *et al.* (2001). Standard errors and standard deviation were calculated from replicates and significance was measured at the level of $P \leq 0.05$.

Statistical analysis: Analysis of variance (ANOVA) was performed using OPSTAT analysis software (http://14.139.232.166/opstat/default.asp). One-way analysis of variance was used to evaluate whether significant difference existed at $P \leq 0.05$ between the +N and the –N treatments; among leaves and roots. The means in all these analyses were separated using the least significant difference test at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of N starvation on plant growth: Maize plants grown under N-deprived conditions exhibited visual symptoms of N deficiency such as stunted growth, pinkish

Table 1 List of nitrogen assimilation and metabolism related key genes and their primers used for qRT-PCR analysis

Accession number	Annotation	Gene name	Primer pairs (5′-3′)
NM_001291791.1	*Asparagine synthetase*1	*Zm AS1*	F-TCCCAAGCCTGCAGCAAGGTC R-AAGCAGCACGGCCAGAAGGA
NM_001112223.1	Ferredoxin-dependent *glutamate synthase*1	*ZmFd-GOGAT1*	F- TTGTCTGAACGTGGAGCTTG R-GGCCCAGTCATCAAACAAGT
X65926.1	*Glutamine synthetase*1	*Zm GS1.1*	F-TTTCTTCTGCACAACGCATC R-GAAGCACAGCCAAACGTACA
X64446.1	*Nitrate reductase*	*Zm NR1*	F-GGTGAACGGCAAGCAGCGTC R-CAGCCAAGCGCGGTCGATCT
AJ420856.1	Alpha *tubulin*4	*Zm TUB*	F- CCAACTCCACCAGTGTTGTG R-ACCGACCTCCTCGTAGTCCT

red coloration in shoots, yellow coloration in older leaves, upright leaves with light green/yellow color and burnt leaf margin etc. Previous studies have reported accelerated yellowing and senescence of old leaves as one of the typical symptoms of N deficiency in plants (Soltabayeva *et al.* 2018). Further, under N-deprived condition, significant reduction in various morphological parameters was observed, such as shoot fresh and dry weight which decreased by 77.32% and 69.56%, respectively, while root fresh and dry weight reduced by 20.52% and 35%, respectively (Table 2). The shoot height and stem girth was reduced by 25.22% and 57.65%, respectively. However, there was a pronounced increase in root length (59.07%) and root volume (60.63%) under N-deprived condition as compared to N-sufficient condition (Table 2). It is important to note that mostly growth parameters responded negatively to N-deficiency stress except the root length and root volume. Drastic reduction in stem girth, shoot fresh and dry weight indicated severe stress-related growth retardation and hence suggested the importance of N for biomass accumulation. The increase in root length and volume indicated the adaptive response of plant under N-deprivation to maximize N uptake from rhizosphere by increasing the surface area for acquisition. Similar pattern of results has been reported in N-limiting conditions after 30 days treatment in temperate maize (Schluter *et al*. 2012) and after 15 days treatment in wheat (Sinha *et al.* 2015).

Effects of nitrogen availability on photosynthetic pigments, total soluble proteins, total amino acids and nitrate reductase activity: In order to further characterize the physiological status of the plants under N-starvation, the levels of photosynthetic pigments (chlorophyll and carotenoid), amino acids content, total proteins and NR enzyme activity were evaluated (Fig 1). The N-deprivation resulted in significant reduction in photosynthetic pigments in leaf tissue. The chlorophyll A and total chlorophyll (Chl A+ Chl B) level decreased upto 59.2% and 62.7%, respectively, while reduction in chlorophyll B and carotenoids was very less (Fig 1A). This indicated that chlorophyll A is more sensitive to N-starvation stress than chlorophyll B and carotenoids. Our results suggested that prolonged N-starvation led to reduction in chlorophyll content which in turn induces early senescence in older leaves. N deficiency-induced lower chlorophyll content in plants is considered as one of the most common indicators of leaf senescence (Yadava *et al*. 2019). The chlorophyll content of plants positively correlates with their photosynthetic activity and adverse environmental conditions induces reduction in chlorophyll level and hence inhibition of photosynthesis. In photosynthetically active leaves, about 80% of N is bound in chloroplast proteins (Kant *et al.* 2011). Soltabayeva *et al.* (2018) has shown that N deficiency-induced chloroplastic protein degradation leads to early senescence in older leaves. Further, reversal of senescence in N-starved plants by N resupply has also been seen (Balazadeh *et al.* 2014). These previous reports and our results suggest that proper N nutrition is important in preventing premature senescence.

Table 2 Physiological parameters of maize plants under N-deprived (-N) and sufficient nitrate (+N) conditions. All the values shown represent the mean of three plants ±SD

Parameter	Sufficient-N condition	N-deprived condition
Shoot fresh weight (g)	12.72 ± 0.68	2.88 ± 0.25
Shoot dry weight (g)	0.92 ± 0.38	0.28 ± 0.03
Stem girth (cm)	3.07 ± 0.08	1.30 ± 0.06
Root length (cm)	32.53 ± 1.62	51.74 ± 3.81
Root fresh weight (g)	1.51 ± 0.04	1.22 ± 0.11
Root dry weight (g)	0.2 ± 0.01	0.13 ± 0.02
Root volume (cm^3)	3.73 ± 0.32	9.44 ± 0.11

In the roots and leaves of N-starved plants, total soluble proteins and amino acids level decreased significantly compared to N-sufficient condition (Fig 1B-C). The total soluble proteins and amino acids content were lower in root tissue compared to leaf. It is well known that N transported from root to leaf which act as major sink for N during the vegetative stage and consequently utilized for synthesizing bio-molecules in leaf via assimilation (Kant *et al*. 2011, Gaufichon *et al.* 2013, Krapp 2015). However, N assimilation occurs in root and shoot both, but usually a larger proportion of NO_3^- reduction and assimilation occurs in shoot than root (in tropical and subtropical species including maize) (Andrews 1986, Scheurwater *et al.* 2002). Therefore, it is obvious that leaf tissue will have more amino acids and total soluble proteins (due to higher N assimilation) than root under N-sufficient growth environment. Moreover, similar pattern was observed in N-starvation condition also. It has been reported that under N-stress condition, proteolytic degradation of chloroplastic proteins accelerated (Krapp 2015), which in turn might resulted in high level of free amino acids and soluble proteins inside leaf compared to root. Further, 21 days of culturing under N-deprived solution, caused 38% and 28.2% reduction in total soluble proteins as compared to N-sufficient condition in leaves and roots, respectively (Fig 1B). Similar responses were observed for free amino acids contents in leaf and root (Fig 1C). N absorbed by roots is assimilated into amino acids inside plant (glutamine, glutamate, asparagine and later into other amino acids). Therefore, under N-starvation condition, availability of N for amino acids and hence soluble protein biosynthesis decreases drastically, as reflected by reduction in their content. Previous studies also reported the reduction in amino acids and proteins under N-limitation condition in hydroponic and field experiments (Prinsi *et al.* 2009, Schluter *et al.* 2012).

Nitrate reductase (NR) is the first enzyme which reduces the absorbed nitrate into nitrite. We observed that maize plant had higher NR activity in leaf than root in +N and –N conditions. Under sufficient N condition, the enzyme activity in leaf was observed nearly 2.53 fold compared to activity in root (Fig 1D). Further, drastic reduction in NR

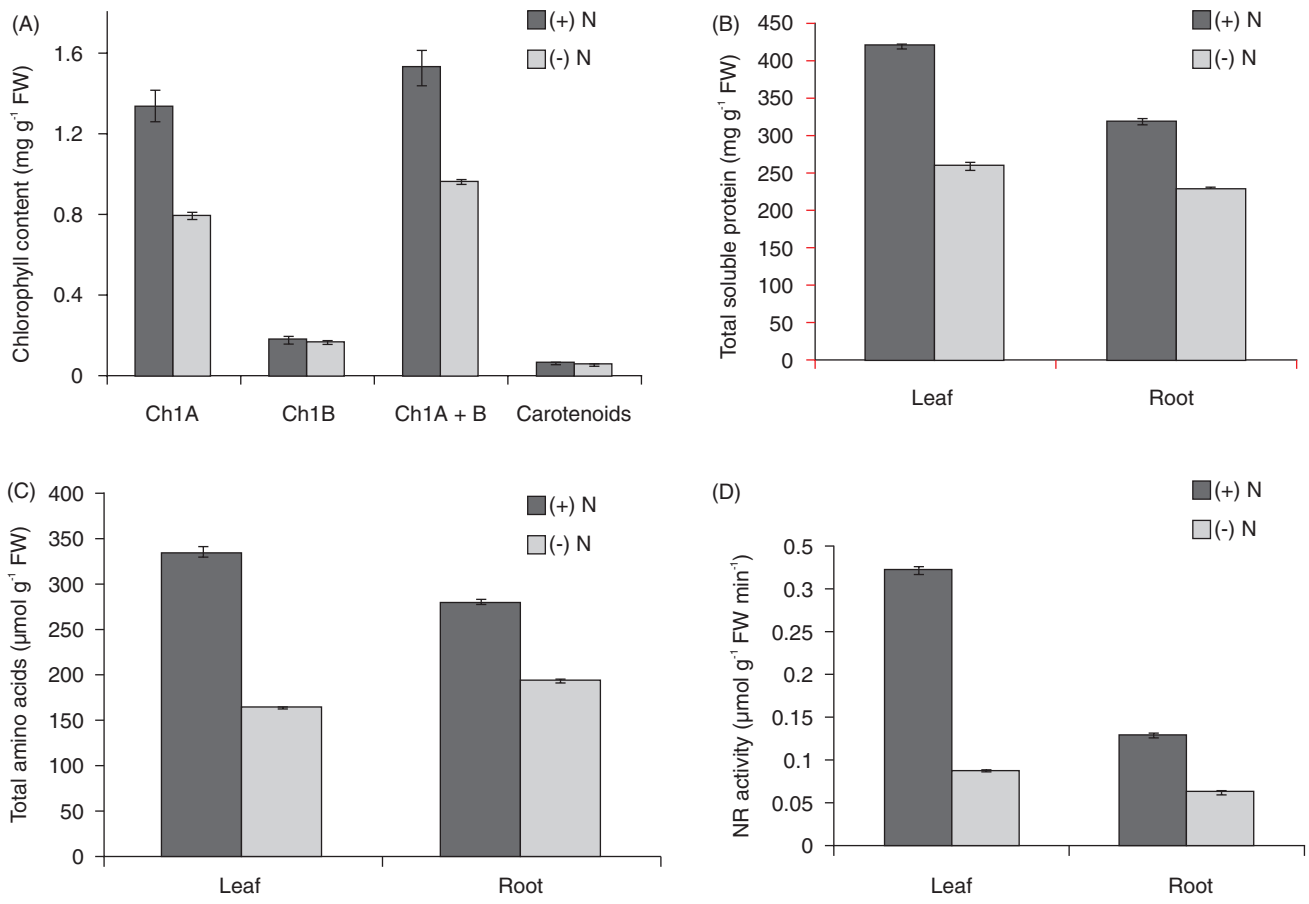

Fig 1 Effects of nitrogen deficiency on photosynthetic pigments (A), total soluble proteins (B), total amino acids (C) and nitrate reductase activity (D). The +N and –N represent maize seedling grown under nitrogen sufficient (2 mM nitrate) and deprived (0 mM nitrate) nutrient solutions, respectively for 21 days. Bar graphs represent the mean of three replicates and error bars represents standard deviation (SD) at $P<0.05$.

enzyme activity was observed in leaf (~3.63 fold decrease) and root (~2 fold decrease) in –N condition compared to +N condition. Higher activity in leaf indicated that relatively more NO_3^- reduction occurred in shoot than root. The higher NR activity and hence N assimilation (more than 90%) in maize shoot/leaf compared to root has been reported earlier. In other plant species also, higher NR activity in shoot than root has been reported (Andrews 1986, Luo *et al.* 2013). Reduction in NR activity under N-deprivation suggested that NR activity is regulated to some extent by its substrate concentration. The –N nutrient culture lacked KNO_3, the source of NO_3^-, which means non availability of enzyme substrate and hence it resulted into drastic decrease in its activity. Luo *et al.* (2013) has shown substantial increase in NR activity in roots of two poplar species with increase in nitrate concentration hydroponically. Recently, Sinha *et al.* (2015) has reported significant reduction in NR activity in two contrasting wheat genotypes under N-starved condition.

Expression analysis of selected genes involved in N assimilation and metabolism: Expression analysis of four N-assimilation and metabolism related genes (*ZmNR1, ZmGS1.1, ZmFdGOGAT1* and *ZmAS*) were performed. The genes under study were involved in nitrate reduction and incorporation of ammonia for amino acid biosynthesis. We found that expression levels of these genes varied significantly among leaves and roots. The relative expression of three N-assimilation related genes, *viz.*, *ZmNR1*, *ZmGS1.1* and *ZmFdGOGAT*, was higher in leaf than root (Fig. 2). It suggested that a large proportion of nitrate reduction and N-assimilation is carried out in leaves than roots in maize. Andrews (1986) reported that tropical and subtropical species, annual and perennial, including maize carry out a greater proportion of their nitrate assimilation in the shoot. Similarly, Scheurwater *et al*. (2002) has shown that maximum proportion that the roots can contribute to total plant nitrate reduction was 0.37 and 0.23 for the fast- and slow-growing grass species. Further, it was observed that N-deprivation caused significant down-regulation of three N-assimilation related genes, *viz. ZmNR1*, *ZmGS1.1* and *ZmFdGOGAT* in leaves as well as roots (Fig 2). Down-regulation in expression of *ZmNR1* can be correlated by reduction in nitrate transport by roots under limiting/deficient N supply conditions. Schluter *et al.* (2012) found significant down-regulation of transcripts of nitrate and nitrite reductase genes in B73 and A188 maize genotypes after 20 and 30 days of N stress treatment. Reduction in the expression of glutamine synthetase (*ZmGS1)* gene indicated low availability of substrate, i.e. glutamate,

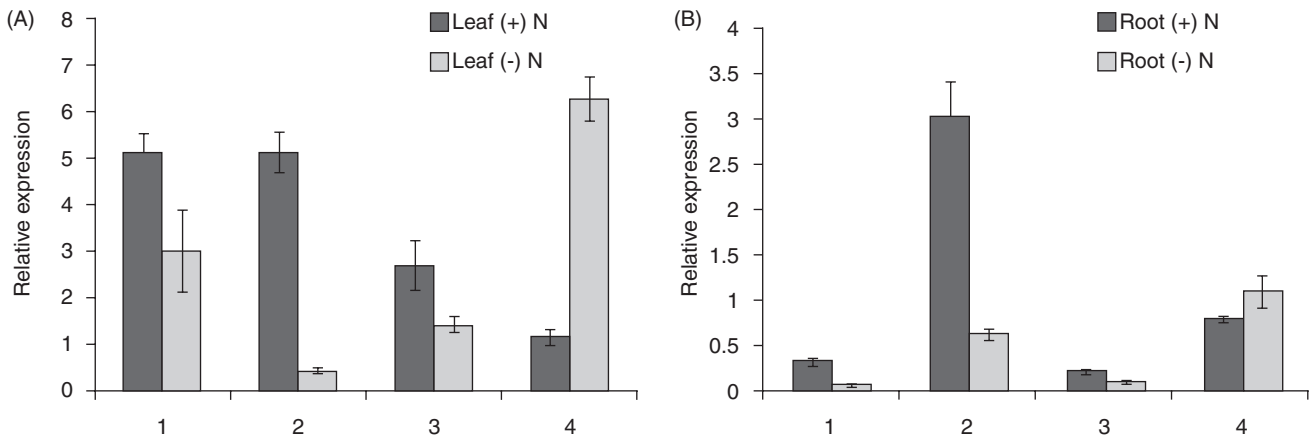

Fig 2 Expression analysis of genes related to N assimilation and metabolism in maize leaf (A) and root (B) tissues grown under N-deprivation (-N) and sufficient (+N) conditions. Number 1, 2, 3 and 4 on *X*-axis represents *ZmFd-GOGAT1, ZmGS1.1, ZmNR1*and *ZmAS1* genes, respectively.

under N-deficiency. Reduced expression of this gene may slow down the process of ammonia assimilation and thus might affect N metabolism. Apart from these three genes, expression of *ZmAS1* was found to be highly and slightly up-regulated in leaf and root, respectively (Fig 2A-B). The increased expression of *ZmAS1* gene suggested increased accumulation of asparagine under N-starvation condition. In photosynthetically active leaves, most of the N is bound in chloroplast proteins (Kant *et al.* 2011) and N-deficiency induced chloroplastic protein degradation (Soltabayeva *et al.* 2018) leading to early senescence in older leaves. The amino acids generated due to protein degradation are transported into young leaves and other growing parts. Similarly, higher level of asparagine and asparagine synthetase activity has been shown in root tip as a consequence of protein degradation under starvation. Since asparagine acts as transport form of N in phloem and source of N for biosynthesis of other amino acids in sink tissue (Gaufichon *et al.* 2013); therefore, increased *ZmAS1* gene in our study indicated its role in N recycling /remobilization during N-starvation conditions.

In conclusion, N-starvation in maize resulted in decline in contents of chlorolphyll, total amino acids, total soluble proteins and nitrate reductase activity which, in turn, accelerated leaf senescence. The expression of genes involved in nitrogen assimilation and metabolism revealed that three key genes, viz. *ZmNR1*, *ZmGS1.1* and *ZmFdGOGAT* were down regulated in leaves as well as roots under N-starvation condition and further affects N metabolism pathway. These genes might be useful in developing high N use efficient genotypes. Their role in imparting high NUE needs to be further investigated more comprehensively through over-expression and/or down-regulation using RNAi or other strategies.

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