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Nuclear localization signals, genetic characterisation and morphological study of wild type and 14 *Arabidopsis* mutant lines

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Abstract

2-oxoglutarate-dependent (2-OG) dioxygenases [2(OG)-dioxygenases] are distributed in a wide range of prokaryotes and eukaryotes, and are involved in many different important biological activities, for example biosynthesis of plant products including plant hormones and antioxidants, posttranslational modification, DNA/RNA damage repair and in certain organisms modulations of epigenetic modifications. These 2(OG) dioxygenase enzymes contain a characteristic iron (Fe)-binding active site. To understand their role in modulating mechanisms against unfavourable conditions, the present study examined nuclear localization signal (NLS), genetic status, phenotypic patterns of 14 T-DNA mutants of *Arabidopsis* containing an insert in their (2-OG) dioxygenase genes. Results showed that predicted localizations of proteins encoded by respective genes varied according to four different methods used in this analysis; seven, three, one and one of them were predicted to be located in the nucleus according to the four methods, respectively. Moreover, genotypic analysis confirmed the homozygosity of plant(s) of nine of these mutant lines [N671573 (insert in *At1g20270*), N668172 (*At1g68080*), N652869 (*At2g17720*), N679576 (*At3g06290*), N678627 (*At3g28490*), N338446 (*At4g35810*), N683883 (*At4g35820*), N666896 (*At5g18900*), N598611 (*At5g66060*)] and phenotypic analysis including vegetative, siliques and seed characteristics of those plants showed different characteristics either within each mutant (homozygous and heterozygous), or between mutants and the WT.

1. Introduction:

2(OG)-dioxygenases are one of the most functionally diverse super-families of non-haem enzymes; they are distributed in a wide range of prokaryotes and eukaryotes, and are involved in many different important biological activities including biosynthesis of plant products and antibiotics, posttranslational modification, DNA/RNA damage repair and

metabolism of lipids (Hewitson *et al.* 2005). For example, within plants one sub-class of these enzymes comprise anthocyanidin synthase, flavanone 3β-hydroxylase and flavonol synthase (Hausinger, 2015); these particular enzymes are responsible for biosynthesis of flavonoids, secondary products, which are ubiquitous in spermatophytic plants, and fulfil a multitude of physiological roles (Cheynier *et al*. 2013; Saito *et al*. 2013). These roles include adaptation to biotic and abiotic stresses in which flavonoids function in helping plants to cope with stress by modulating fertility and regulating the transport of auxin, an important plant hormone (Martens *et al*. 2010; Cheynier *et al*. 2013).

The localization of dioxygenase proteins in the nucleus may increase their possible contribution in epigenetic modifications in plants. The analysis of nuclear localization signal (NLS) is one means to predict the localization of particular proteins. A NLS consists of one or more short sequence(s) of amino acid which tags a protein to import it into the nucleus, mitochondria and plastids by cross-membrane transport. Such signals are composed of positively charged lysine and arginine residues located in peptides that are thought to bind their receptors and make loops or patches on the protein surface (Alberts *et al*. 2008). This binding mediates nuclear import of proteins, known as the karyopherins (Importins) (Jans *et al*. 2000). Through the importin α/β pathways, importin α works as an adaptor, so it links each cargo and importin β1 and subsequently discriminates NLSs within the cargos (Lange *et al*. 2007).

The analysis of nuclear localization signals (NLS) is a useful tool to predict the intracellular location of a particular protein (Uji *et al.* 2009; Lee *et al.* 2012; Geilen and Böhmer, 2015). Any protein associated with the modification of nuclear DNA should by definition be active in the nucleus. There are some epigenetic changes such DNA methylation and histone modifications in nuclear DNA that are similar to those found in animals. Recent studies of cytosine methylation in selected animals determined the role of Ten Eleven Translocation (TET) enzymes (a particular type of 2OG-dioxygenases) in producing a range of oxidised derivatives from 5-methyle cytosine (5mC). These enzymes contain a dioxygenase domain with the characteristic Fe-binding active site motif HXDXnH (where X is any amino acid). The following sequences of a subset of 14 *Arabidopsis* 2(OG)-dioxygenase proteins, were selected as being most similar to TET proteins, and the present study compared the nuclear localisation signal, genetic (homozygous and heterozygous) status and phenotypic characteristics including plant vigour, embryo and seed shapes of T-DNA lines

that contain an insert in the 14 genes encoding these proteins (Figure 1. 1). Such information formed the foundation for subsequent parts of the project that measured the amount of ascorbate in selected mutant lines and examined aspects of the response of 2(OG)-dioxygenase genes to different concentrations of 2HG and to different conditions of Fe deficiency.

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AT1G20270          DHGEGLQVLHYEAGQKYEPHYDYFVDEFNTKNGGQRMATMLMYLSDVEEGGETVFPAANM 217
AT5G66060          EHGEGLQVLHYEIGQKYEPHYDYFMDEYNTRNGGQRIATVLMYLSDVEEGGETVFPAAKG 219
AT2G17720          ENGEGLQVLHYQVGQKYEPHYDYFLDEFNTKNGGQRIATVLMYLSDVDDGGETVFPAARG 221
AT4G35810          ENGEGLQVLHYEVGQRYEPHHDYFFDEFNVRKGGQRIATVLMYLSDVDEGGETVFPAAKG 221
AT4G35820          ENGETLQVINYEVGQKFEPHFD----------GFQRIATVLMYLSDVDKGGETVFPEAKG 227
AT2G43080          ENGELIQVLRYEPQQFYKPHHDYFADTFNLKRGGQRVATMLMYLTDDVEGGETYFPLAGD 220
AT3G06300          ENGEDLQVLRYEHGQKYDAHFDYFHDKVNIARGGHRIATVLLYLSNVTKGGETVFPDAQE 179
AT5G18900          ENGEDIQVLRYEHGQKYDAHFDYFHDKVNIVRGGHRMATILMYLSNVTKGGETVFPDAEI 178
AT3G28480          ENGESMQILHYENGQKYEPHFDYFHDQANLELGGHRIATVLMYLSNVEKGGETVFPMWKG 197
AT3G28490          ENGEALQILHYENGQKYDPHFDYFYDKKALELGGHRIATVLMYLSNVTKGGETVFPNWKG 174
AT4G25600          ENGGSIKVRSYTS-EKSGKKLDYFGEEPSSVLHESLLATVVLYLSNTTQGGELLFPNSEM 182
AT2G23096          DYYESFNILRYQLGQKYDSHYDAFHSAEYGPLISQRVVTFLLFLSSVEEGGETMFPFENG 209
AT4G33910          SHGESFNILRYELGQKYDSHYDVFNPTEYGPQSSQRIASFLLYLSDVEEGGETMFPFENG 222
At1g68080          EFTG---LISWCKGASIGWHSD--DNRSYLKQRDFAAVCYLNSYEKDFIGG--LFRFQSG 135
                          .     :  :       : *             .  :    .   **   *

AT1G20270          --NFSSVPWYNELSECGKKGLSVKPRMGDALLFWSMRPDATLDPTSLHGGCPVIRGNKWS 275
AT5G66060          --NYSAVPWWNELSECGKGGLSVKPKMGDALLFWSMTPDATLDPSSLHGGCAVIKGNKWS 277
AT2G17720          --NISAVPWWNELSKCGKEGLSVLPKKRDALLFWNMRPDASLDPSSLHGGCPVVKGNKWS 279
AT4G35810          --NVSDVPWWDELSQCGKEGLSVLPKKRDALLFWSMKPDASLDPSSLHGGCPVIKGNKWS 279
AT4G35820          --IKS------------KKGVSVRPKKGDALLFWSMRPDGSRDPSSKHGKRHCLSLNLF- 272
AT2G43080          --GDCTCG------GKIMKGISVKPTKGDAVLFWSMGLDGQSDPRSIHGGCEVLSGEKWS 272
AT3G06300          FSRRSLSENKDDLSDCAKKGIAVKPKKGNALLFFNLQQDAIPDPFSLHGGCPVIEGEKWS 239
AT5G18900          PSRRVLSENKEDLSDCAKRGIAVKPRKGDALLFFNLHPDAIPDPLSLHGGCPVIEGEKWS 238
AT3G28480          ---KATQLKDDSWTECAKQGYAVKPRKGDALLFFNLHPNATTDSNSLHGSCPVVEGEKWS 254
AT3G28490          ---KTPQLKDDSWSKCAKQGYAVKPRKGDALLFFNLHLNGTTDPNSLHGSCPVIEGEKWS 231
AT4G25600          ----------KPKNSCLEGGNILRPVKGNAILFFTRLLNASLDGKSTHLRCPVVKGELLV 232
AT2G23096          ------RNMNGRYDYEKCVGLKVKPRQGDAIFFYNLFPNGTIDQTSLHGSCPVIKGEKWV 263
AT4G33910          ------SNMGIGYDYKQCIGLKVKPRKGDGLLFYSVFPNGTIDQTSLHGSCPVTKGEKWV 276
At1g68080          -----------------EPVTVAPSAGDVIMYT-------ADDRNIHSVDEVTDGERLT 170
                                        : *   : :::         *  . *        :

AT1G20270          STKWMHVGEYKI------------------------------------------------ 287
AT5G66060          STKWLRVHEYKV------------------------------------------------ 289
AT2G17720          STKWFHVHEFKV------------------------------------------------ 291
AT4G35810          STKWFHVHEYN------------------------------------------------- 290
    AT4G35820          ------------------------------------------------------------
AT2G43080          ATKWMRQKATS------------------------------------------------- 283
AT3G06300          ATKWIHVDSFDKILTHDGNCTDVNESCERWAVLGECGKNPEYMVGTPEIPGNCRRSCKAC 299
AT5G18900          ATKWIHVDSFDRIVTPSGNCTDMNESCERWAVLGECTKNPEYMVGTTELPGYCRRSCKAC 298
AT3G28480          ATRWIHVKSFERAFNKQSGCMDENVSCEKWAKAGECQKNPTYMVGSDKDHGYCRKSCKAC 314
AT3G28490          ATRWIHVRSFG---KKKLVCVDDHESCQEWADAGECEKNPMYMVGSETSLGFCRKSCKAC 288
AT4G25600          ATKLIYAKKQAR-IEESGECSDEDENCGRWAKLGECKKNPVYMIGSPDYYGTCRKSCNAC 291
AT2G23096          ATKWI-RDQTYD------------------------------------------------ 274
AT4G33910          ATKWI-RDQDQEE----------------------------------------------- 288
At1g68080          LALWFSRDSSHDEDSKL------------------------------------------- 187
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Figure 1. 1. Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/) alignment of selected dioxygenase proteins from *Arabidopsis thaliana* (courtesy of JM Dunwell)

2. Material and methods:

2.1. Nuclear Localization Signal

In order to predict the intracellular localisation of selected dioxygenase proteins in the 14 Arabidopsis mutant lines, their protein sequences were analysed using Support Vector Machine-based localization predictor (AtSuP) programme which has seven possible categories, Nucleus, Mitochondrion, Chloroplast, Cytoplasm, Plasma membrane, Golgi apparatus and Plasma membrane. The software analysis included four options namely Amino acid composition-based, Dipeptide composition-based, N-Center-C terminal based and Best hybrid-based classifier (AA+PSSM+N-Center-C+PSI-BLAST).

2.2. Seed germination under controlled environment

To determine the phenotype characteristics of WT and mutants, stratified seeds were grown and germinated in 6.5 x 6 cm mini-flower pots (2 seeds in each pot) containing potting growing medium (supplied from www.william.sinclair.co.uk) and topped with vermiculite, six pots for WT and each mutant were placed in a tray and maintained under controlled environmental conditions at 22 °C and 60% Relative Humidity (RH) with a day/night cycle of 16/8 h (at 100 µmol m^{-2} s^{-1} light intensity) in the University of Reading growth chambers (Fitotron plant growth chambers, Weiss Gallenkamp, UK). In order to avoid cross pollination each plant was covered with a transparent flower sleeve (Zwapak, Holland) prior to flowering.

2.3. Confirmation of genetic status of mutant lines

Genomic DNA of mutant lines (5 plants/mutant line) was extracted according to the DNeasy Plant Mini kit protocol (http://www.qiagen.com). 2 µl genomic DNA for each plant/line was added to 23 µl PCR mixture containing 12.5 µl 2X BioMix PCR master mix (Bioline, UK,), 0.75 µl of 0.3 µM each forward primer (or left border primer which LBb1.3 5'-ATTTTGCCGATTTCGGAAC-3', NR80 5'-GCTGATACAAAAACAAAACAACGA-3' and LB1 5'-GCC TTT TCA GAA -ATG GAT AAA TAG CCT TGC TTC C-3' were used with SALK, GK and SAIL insertions respectively) and reverse primer, and 9 µl TE water. PCR in a GeneAmp PCR system 9700 (Applied Biosystems) was conducted for each reaction mixture, the

amplification condition was one cycle of 95°C for 30 s, then 35 cycles of 95°C for 30 s, 56°C for 45 s, and 72°C for 90s at the transition speed S-9 and finally one cycle of 74°C for 5 min. The total amplification time was 102 min. The PCR products (9-10µl of each) were separated by electrophoresis in 1% agarose gels supplemented with ethidium bromide 3 µl/70 ml. Thereafter, in order to visualize the gel, it was placed on a GelDoc-It^{TS2} Imager (UVP) followed by capturing a clear picture through GeneSnap (version 6.00.19) system (SynGene, UK) and the genetic status was recognized according to the following Figure 2. 1.

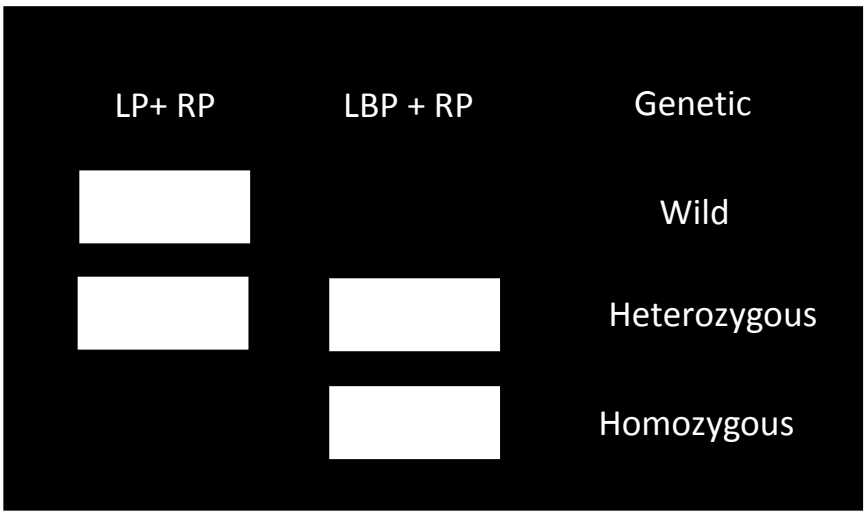


Figure 2. 1. Protocol for SALK T-DNA design primer. LP = Left or forward primer, RP = Right or Reverse primer and LBP = Left border primer.

2.4. Phenotypic measurements

Germination of mutant seeds was determined as a percentage rate (Chaudary *et al.* 2001). In order to obtain segregation or uniform patterns of mutant lines, pictures of plants at different stages of vegetative development from germination to seeding were selected and recorded. All plants from each family (NASC code) were compared together. Observation and recording of siliques and seeds under a dissecting microscope provided other parameters that were used to identify the normalities and abnormalities of silique growth (growth of embryo/seeds inside the silique), seed sizes and seed shapes of each mutant line. Phenotypic aspects were visualized using a Leica MZ95 stereomicroscope (Leica Microsystems, CH-9435, Heerbrugg) and captured using a Leica DFC290 (an integrated digital camera with Leica MZ95 and Leica application suite).

3. Results

3.1. Prediction of Nuclear Localization Signal (NLS)

NLSs were found to show different patterns of amino acid residues (Table 3. 1). The analysis predicted the presence of NLS to the nucleus for proteins encoded by seven genes namely *At1g68080, At2g17720, At3g06300, At3g28480, At4g25600, At4g35810* and *At4g35820*. Similarly, the dipeptide composition (*sequence-order*) based method predicted that proteins encoded by three genes *At1g68080, At3g28480* and *At4g25600* were likely to be transported into in the nucleus. However, only the dioxygenase protein encoded by the *At1g68080* gene was predicted to be located in the nucleus when analysed by N-Center-C terminal (*3-parts*) based. In the similar way, a protein encoded by a different gene *At4g35810* was predicted to occur in the nucleus with AA+NCC+PSI-BLAST+PSSM (*best hybrid*) based analysis. All other proteins were shown to be localised in the other organelles such as chloroplast and mitochondrion and or in an unknown location.

The *At1g68080* protein is predicted to function in the nucleus according to the N-Center-C terminal (*3-parts*) but its location is unknown according to the AA+NCC+PSI-BLAST+PSSM (*best hybrid*) based method.

A similar result was obtained with the protein encoded by the *At4g35810* gene; it was predicted to be located in the nucleus based on the hybrid test and its location was unknown based on each of N-Center-C terminal (*3-parts*) and Dipeptide composition (*sequence-order*) methods.

Table 3. 1. NLS predictions of *Arabidopsis thaliana* depending on AtSubP bioinformatics software.

Gene name	AtSubP			
	Amino Acid composition based SVM	Dipeptide composition (*sequence-order*) based	N-Center-C terminal (*3-parts*) based	AA+NCC+PSI-BLAST+PSSM (*best hybrid*) based
At1g68080	Nucleus	Nucleus	Nucleus	Unknown
At2g17720	Nucleus	Unknown	Unknown	Unknown
At3g06300	Nucleus	Unknown	Extracellular	Extracellular
At3g28480	Nucleus	Nucleus	Extracellular	Extracellular
At4g25600	Nucleus	Nucleus	Chloroplast	Chloroplast
At4g35810	Nucleus	Unknown	Unknown	Nucleus
At4g35820	Nucleus	Unknown	Mitochondrion	Unknown
At2g23096	Extracellular	Unknown	Extracellular	Extracellular
At1g20270	Chloroplast	Unknown	Mitochondrion	Mitochondrion
At4g33910	Chloroplast	Unknown	Mitochondrion	Unknown
At5g18900	Chloroplast	Unknown	Extracellular	Extracellular
At5g66060	Chloroplast	Unknown	Mitochondrion	Unknown
At2g43080	Unknown	Unknown	Extracellular	Extracellular
At3g28490	Unknown	Unknown	Extracellular	Extracellular
Total	**7**	**3**	**1**	**1**

AtSubP= http://bioinfo3.noble.org/AtSubP/?dowhat=AtSubP

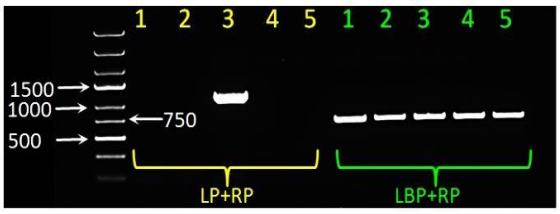
3.2. Molecular characterization of *Arabidopsis* mutant lines

In Table 3. below, at least one homozygous plant was determined in each of nine mutant lines. On the (NASC) website, each of the N671573, N683883, N679576, N668172 and N678627 mutant lines are described as being homozygous. However, results showed segregation in these lines. Moreover, despite a clear band being detected with LP+RP in the fourth replicate of N668172 mutant, no amplification was observed with LBP+RP primer sets; it appeared that there was no T-DNA insert in this plant and it should be considered as a WT. PCR confirmation represented in Figure 3. shows that in N652869, N338446, N666896 and N598611, which are described by NASC as being heterozygous, at least one homozygous plant was present. The identification of homozygous plant(s) within each mutant line allowed the generation of enough homozygous plants to permit further investigations in each line.

Table 3. 1. Identifications the genetic status of eight genes using basic PCR. H = Homozygous, T = Heterozygous and WT = Wild type, R= Replicate of plants of each mutant line.

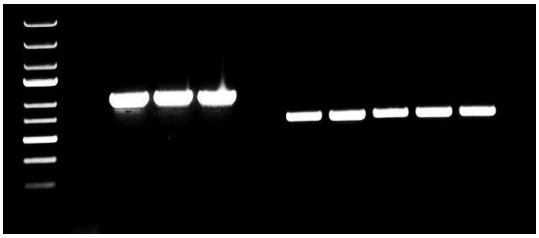
Gene	NASC code	R1	R2	R3	R4	R5	Number of homozygous plants
At1g20270	N671573	H	H	T	H	H	4
At1g68080	N668172	H	T	-	WT	H	2
At2g17720	N652869	H	T	T	T	H	2
At3g06290	N679576	T	T	T	T	H	1
At3g28490	N678627	H	H	T	H	T	3
At4g35810	N338446	H	T	H	T	T	2
At4g35820	N683883	H	H	H	H	T	4
At5g18900	N666896	H	H	H	H	H	5

At5g66060	N598611	T	T	H	T	H	2

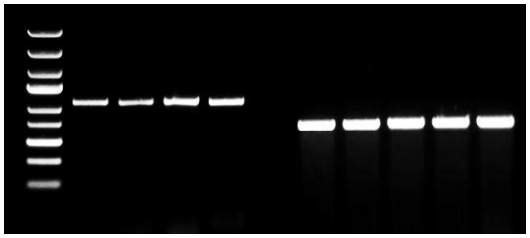


N671573 (*At1g20270*)

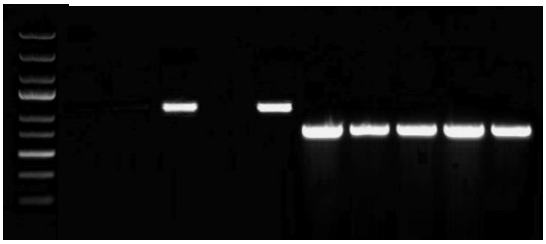
N668172 (*At1g68080*)



N652869 (*At2g17720*)



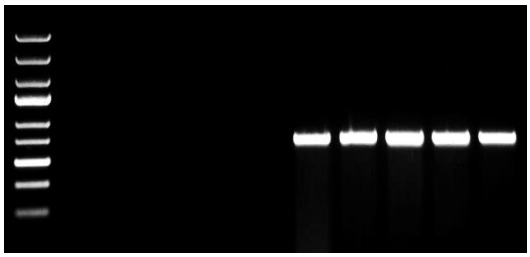
N679576 *(At3g06290)*



N678627 (*At3g28490*)

N338446 (*At4g35810*)

N683883 (*At4g35820*)



N666896 (*At5g18900*)

N598611 (*At5g66060*)

Figure 3. 1. Confirmation of genetic status (Homozygous, Heterozygous or WT) of mutant lines using SALK T-DNA design primer protocol.

3.3. Morphological study of mutants

An analysis was conducted on nine selected mutants to better understand the effect of T-DNA insertion on phenotypic characteristics including germination rates, characteristic of growth, silique and seed shapes. Results recorded from this study are described in Table 3. .

This study described the various phenotypic characteristics of nine mutant *Arabidopsis* plants under a controlled environment. As described above, observation of the various families of mutants showed that within some families there was plant to plant variation in terms of different phenotypes from germination to flowering and seed set. Seeds of the N671573 mutant showed a lower germination rate than the WT (70 % compared with 100 %), and slower germination (1d later in comparison to the WT and other mutants). Different plants of this mutant also varied in other aspects of vegetative growth; this was in line with the reduced level (84.5 %) of normal siliques. The variation in this line occurred despite the fact that it was described as being homozygous (according to NASC). Another line N668172 has an insert in chromosome 1 in the gene *At1g68080*. Generally, all plants of this mutant showed high germination rate (90 %), high normal growth (100 %), and similar morphology including plant height, normal silique and seed shapes, which suggests that this knockout did not affect the phenotype (for more detail about measurement of selected mutant lines, see Table A1 in the appendices).

Although N652869, which contains an insert in *At2g17720*, is described as being heterozygous, the results showed normal silique rates (98 %) and seeds (100 %) as well as a high rate of normal growth (100 %). Furthermore, the insert in line N679576 is present in *At3g06290*. Results from this line showed a low germination rate (50 %) and abnormalities at other growth stages such as 30.5% of abnormal siliques growth, which is the highest value that was recorded in this study. The mutant line N338446 that contains a T-DNA insertion in *At4g35810* is described as being heterozygous (according to NASC). Despite a lower germination rate (80%) in this mutant, all plantlets grew normally. Although variation in rates of vegetative growth between plants was noted, it mostly involved larger rosette diameter (7.5 cm) and much more vigour in comparison to the WT and other mutants. In all five selected siliques, the lack of an embryo or its abnormal growth (23%) was recorded; these variations were also linked to differences in seed shapes (4.5%).

Table 3. 2. Characterization of mutant lines of *Arabidopsis thaliana* showing presumed genetic status and position of T-DNA insert (According to NASC) and features of vegetative growth, silique development and seed sizes (Based on experimental results).

	NASC background information				Present study results						
Gene ID	NASC code	Homozygous Line	Heterozygous line	Position of insert	Germination Rates (%)	Normal growth of Plantlets (%)	Phenotype patterns	Siliques (%)		Seed shapes (%)	
								N	A	N	A
Wild type	-	-	-	-	100	100	Similar	100	0	100	0
At1g20270	N671573	*		7,021,373-7,022,913	70	86	Similar	84.5	15.5	97.5	2.5
At1g68080	N668172	*		25,521,372-25,522,903	90	100	Similar	90	10	97	3
At2g17720	N652869		*	7,704,595-7,706,256	60	100	Similar	98	2	100	0
At3g06290	N679576	*		1,899,129-1,907,303	50	100	Similar	69.5	30.5	95	5
At3g28490	N678627		*	10,680,280 – 10,680,452	86	83	Similar	87	13	95	5
At4g35810	N338446		*	16,968,925-16,970,509	80	100	Varied	77	23	95.5	4.5
At4g35820	N683883	*		16,971,225-16,972,433	50	50	Similar	72	28	100	0
At5g18900	N666896	*		6,304,411 - 6,306,168	100	100	Similar	95	5	100	0
At5g66060	N598611		*	26,436,707-26,438,156	60	83	Varied	94	6	100	0

Where: - N= Normal growth and A=Abnormal growth

Another mutant line (N598611) containing an insertion in the gene *At5g66060* displayed interesting morphological characteristics. Results recorded from plants of this mutant showed differences in terms of a low germination rate (60 %) and higher growth rate (83 %). These results were in line with a high normal silique production and mature seed appearance (94 % and 100 % respectively). Generally, as illustrated in Figure 3. and Table 3. 3, greater rosette diameter (7 cm) was found in heterozygous plants compared with the WT (4 cm) and slower growth (5 cm) in homozygous plants of this mutant (N598611). However, the WT appeared to flower more rapidly (13 d vs. 15 d and 19d) than heterozygous and homozygous plants, respectively. This difference was associated with a high number of flowers (13) with the WT, compared to four and one flower(s) with each heterozygous and homozygous plant respectively.

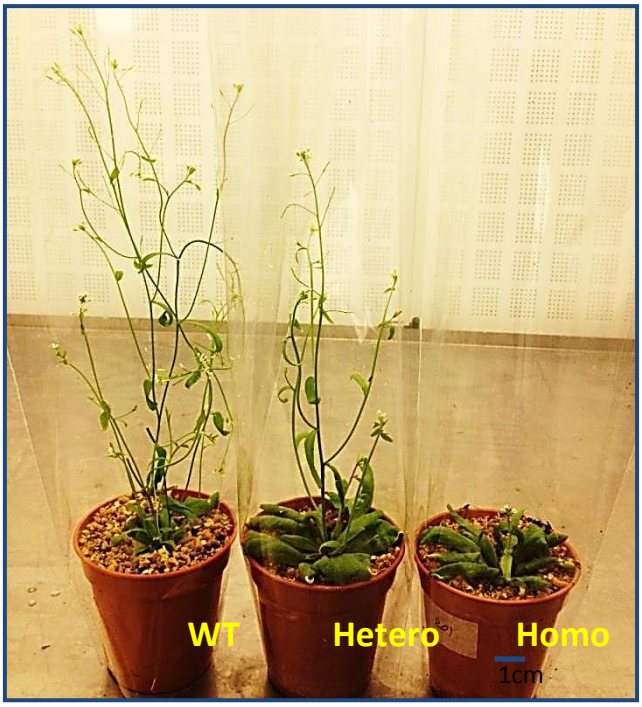


Figure 3. 2. Phenotypic comparison of WT plant with heterozygous and homozygous plants of N598611 mutant line 3 weeks after germination.

Table 3. 3. Comparison of phenotypic measurements of WT plant with heterozygous and homozygous plants (3 replicates for each genotype) of the N598611 mutant line.

	WT	Heterozygous	Homozygous
Rosette diameter (cm)	4	7	5
No. of leaves	10	12	12
Plant height (cm)	18	14	2
Flowering time (d)	13	15	19
No. of flowers	13	4	1

4. Discussion

4.1. Nuclear localisation signal (NLS)

Results from this analysis showed differences between methods used to predict the localization of respective proteins; these differences may result from the different principles of the different analytical methods, in that they test protein motifs depending on the amino acid compositions, dipeptide compositions and N, C-terminus details or on a combination of these.

These results might be useful in understanding the functional variety of proteins involved in cytosine methylation and demethylation and in regulating responses against stress conditions, including the exact role of each of the target proteins and their regulatory signals. A relevant review by Grandperret *et al*. (2014) stated that a specific nuclear signal transduction regulates expression of specific gene sets, which subsequently leads to an appropriate response to stress conditions. Interestingly, components of these pathways are subjected to post-translational modifications as well as epigenetic changes. Nuclear protein acetylation and/or deacetylation are important post-translational modifications that play major roles in the regulation of gene expression (Chen and Tian, 2007).

In light of this, in order to understand the precise localisation of those proteins in response to unfavourable conditions, it would be important experimentally to study the subcellular localisation of respective proteins, for example with techniques using green fluorescent protein (GFP) (Etchegaray *et al*. 2015). These techniques will be useful to understand mechanisms that modulate gene expression under different environmental conditions.

4.2. Confirmation of genetic status of mutant lines

The confirmation of genetic status is an important requirement in order to recognize homozygous and heterozygous plant within a mutant. According to NASC background information, N671573, N668172, N679576 and N683883 were all considered to be homozygous lines. However, only four, two, one and four homozygous plants from five plants were detected from those mutants respectively. However, all plants from N666896 (described to be homozygous) were observed to show homozygous patterns. In regards to these differences, confirming at least one homozygous plant/mutant was necessary in order to provide enough seeds for further experiments.

4.3. Morphological study of mutants

Due to the vital contribution of dioxygenase genes in several biological activities as stated previously, a single gene knock-out in every mutant can be valuable. Phenotypic variations in the mutants might result from changes in the ratios of endogenous hormones such as auxin/cytokinin (Kobayashi *et al.* 2012). In addition, proteins encoded by respective genes may influence aspects of particular metabolism including plant growth regulators such as gibberellin that promotes seed germination and internode elongation (Wang *et al.* 2013).

Moreover, phenotypic variations between plants within a mutant or between mutants may be a consequence of variations in the pathway of DNA/RNA damage/repair and posttranslational modifications (Hewitson *et al.* 2005) or from changes in important enzymatic activities causing significant changes in epigenetic markers (Aik *et al.* 2012). Future experiments may help to answer some questions regarding these morphological variations within and between mutants.

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