Analysis of splicing patterns in *Arabidopsis egy1-4* carrying argon-induced mutations in the intron 3-exon 4 region

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Eukaryotic gene expression involves the splicing of precursor messenger RNAs before the translation. The U2 spliceosome, a nuclear ribonucleoprotein complex, is important in the process of splicing, and recognizes certain conserved "canonical" sequences in introns, including a GU at the 5' end, an adenine known as the "branchpoint" located 20–50 bases upstream of the 3' end, and an AG at the 3' end. Based on the mechanism of intron excision discovered, the intron 5' splice and intron 3' splice sites are referred to as the splice donor site and the splice acceptor site, respectively. Splicing begins at the 5' splice site and involves the formation of an intron lariat at the branchpoint as the cleaved 5' end of the intron becomes attached to it. The excision of the intron lariat from the 3' splice site then allows for the ligation of the exons.

Mutations at such canonical intron sites typically cause inefficient and/or cryptic splicings.[1)] In *Arabidopsis thaliana*, mutations of the intron 3' splice site, either "AG to AA" or "AG to GG," are known to activate only cryptic 3' splice sites but not 5' splice sites.[1,2)] A 3' splice site mutation resulting in cryptic 5' splice site activation has never been reported.

In *A. thaliana*, the *Ethylene-dependent Gravitro-pism-deficient and Yellow-green 1* (*EGY1*) gene (AGI code: At5g35220) encodes a chloroplast-targeted metalloprotease and is composed of 10 exons and 9 introns. During its functional analysis, an argon-ion-induced allele, *egy1-4* (Ar-28-pg1[3)]), has been noted as it contains an AG to AC transition at the 3'-end of intron 3, in addition to 4-bp substitutions and a 5-bp deletion in the downstream exon 4.[4)] To determine whether these mutations in the intron 3-exon 4 junction region of *egy1-4* affect the splice site selection in *EGY1*, the total RNAs were extracted from 2-week-old seedlings of wild-type and *egy1-4*, and analyzed using RT-PCR with primers binding to the *EGY1* 5'-untranslated region and exon 5. The amplified PCR products were ligated to a vector and transformed into an *E. coli* DH5α strain. The resulting colonies were subsequently cultured for the propagation of plasmids, which were then Sanger-sequenced. The sequence of *EGY1* cDNAs around intron 3 was then compared between the wild-type and *egy1-4*.

Only one splicing pattern of intron 3 was observed in the wild-type by sequencing 30 clones (Fig. 1; Table 1). However, among 58 cDNA clones of *egy1-4* examined, 42 exhibited no splicing of intron 3 (unspliced pattern). The rest exhibited distinct splicing patterns from the wild-type ones: 5 and 8 clones exhibited activation of upstream and downstream cryptic 3' splice sites, respectively. Moreover, a new splicing pattern, exhibiting activation of cryptic 5' splice site alongside activation of 3' splice site, was found in 3 clones (Fig. 1; Table 1).

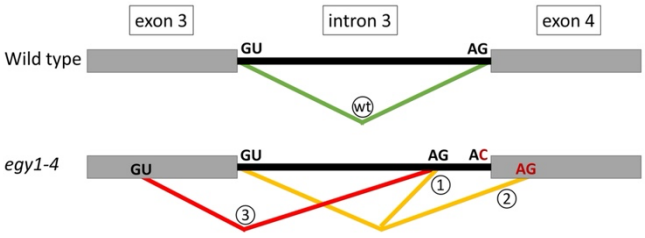

Fig. 1. Schematic comparison of *EGY1* transcript splicing patterns between the wild-type and *egy1-4*. Sole splicing pattern (wt) was observed in the wild-type. Cryptic 3' splice sites were activated without (patterns 1 and 2) or with (pattern 3) the activation of cryptic 5' splice site in *egy1-4*. The nucleotide letters in red denote that they are uniquely produced in *egy1-4* by argon-ion-irradiation.[3)]

Table 1. RT-PCR and cloning analysis of intron 3 splicing patterns in wild-type and *egy1-4*.

Plant line	Intron 3 splicing pattern	Number of cDNA clones sequenced	Relative frequency
Wild type	wt	30	100 %
	Total:	30	100 %
egy1-4	(unspliced)	42	72.4 %
	1	5	8.6 %
	2	8	13.8 %
	3	3	5.2 %
	Total:	58	100 %

As sole splicing patterns were observed in all the other introns for both wild-type and *egy1-4* (unpublished results), the pale green phenotype of *egy1-4* plants[4)] was attributed to the expression of truncated forms of EGY1. This is due to premature stop codons in the mutant exon 3 or exon 4 in any spliced/unspliced variants. Our finding demonstrates that mutations induced by heavy-ion irradiation significantly impact on the terminal phenotype of plant bodies via interference of splicing.

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