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Elaborating the Functional Roles of a Leucine-Rich Repeat Protein from *Arabidopsis thaliana*

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Abstract

Plants, just like any other living organism, naturally get attacked by various pathogenic microorganisms such as bacteria, fungi and viruses. However, unlike animals that utilize their specialized circulatory macrophage system to protect themselves, plants instead use a multi-layered complex system termed the plant innate immunity, which recognizes pathogens and transducing downstream defense responses. They have developed a unique type of transmembrane receptors or R proteins, which extracellularly, are capable of recognizing pathogen-associated molecular patterns (PAMP) such as flagellin and chitin, while intracellularly, they activate their harbored nucleotide cyclases (NCs) such as adenylyl cyclases (ACs), to generate second messenger molecules such as 3',5'-cyclic adenosine monophosphate (cAMP), which then propagates and magnifies the defense response. To date, only a single R protein from *Arabidopsis thaliana* (AtLRR) has been shown to possess AC activity as well as having the ability to defend plants against infection by biotrophic and hemi-biotrophic pathogens. Therefore, in order to further broaden information around the functional roles of this protein (AtLRR), we explored it further, using an array of web-based tools or bioinformatics. These included structural analysis, anatomical expression analysis, developmental expression analysis, co-expression analysis, functional enrichment analysis, stimulus-specific expression analysis and promoter analysis. Findings from structural analysis showed that AtLRR is a multi-domain, trans-membrane molecule that is multi-functional, and thus consistent with all known R-proteins. Findings from anatomical and developmental expression analyses showed

that AtLRR is mostly expressed in pollen grains and flowers, senescing leaves as well as during the development of seeds, shoots, roots, seedlings, leaves, flowers, and siliques, linking it to the three key plant physiological processes of reproduction, defense and development respectively. Lastly, findings from co-expression, functional enrichment, stimulus-specific expression and promoter analyses, showed that AtLRR is mostly co-expressed with several other proteins linked to disease resistance, plant reproduction and plant development. Activities and functions of such protein are also commonly regulated by cAMP via a common W-box promoter. So, all in all, our study managed to establish that besides being strongly involved in disease resistance against biotrophic and hemi-biotrophic pathogens, AtLRR also plays key roles in plant development (seed, shoot, root, seedling, leaf, and silique development) and reproduction (flowering, and pollen tube growth and re-orientation), whereby it effects its functions via a W-box or WRKY transcription factor, TTGACY, mediated by cAMP.

Keywords

Disease Resistance, Adenylyl Cyclase, R-Proteins, AtLRR, Plant Development, Plant Reproduction

1. Introduction

In nature, plants are attacked by various pathogenic microorganisms that include bacteria, fungi and nematodes [1]. However, unlike animals that utilize their specialized circulatory macrophage system to protect themselves, plants instead use a multi-layered complex system termed plant innate immunity, that recognizes pathogens and transducing downstream defense responses [1]. Ideally, this plant innate immune system is divided into two; the first-line or basal immunity and the second-line immunity [1] [2].

First-line or basal immunity comes into action when pathogen/microbial-associated molecular patterns (PAMPs/MAMPs) such as the bacterial flagellin, fungal lipopolysaccharide or oomycetic cellulose binding elicitor proteins are recognized extracellularly by plant transmembrane receptors, termed pattern recognition receptors (PRRs) [2] [3]. Once triggered, the PRRs catalyze the production of cyclic nucleotide monophosphates (cNMP) *i.e.*, 3',5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) at their cytosolic end, which then facilitate entry of Ca^{2+} ions through the cyclic nucleotide gated ion channels (CNGCs) [4]. The increase in cytosolic Ca^{2+} ion concentration is an important primary event in pathogen signalling, that triggers downstream innate immune responses [5]. As the free cytosolic Ca^{2+} increases, the amount of Ca^{2+} bound to calmodulin (CaM) or calmodulin-like (CML) protein also increases, which then triggers the synthesis of downstream signalling components such as nitric oxide (NO) and hydrogen peroxide (H_2O_2), essential

for the initiation and development of the hypersensitive response (HR) [5]. HR is essentially a collection of plant defense responses against pathogen infection that leads to rapid programmed cell death (PCD) of cells surrounding the pathogen-infected area, to prevent the spread of a disease [5]. The first-line or basal immunity is also known as the PAMPs-triggered immunity (PTI) [2] [3].

Second line immunity comes into action when the first-line immunity has been evaded, and it involves some highly specific cognate disease resistance (R) proteins that either directly or indirectly recognize pathogen effector proteins [6]. This type of immunity is also known as the effector-triggered immunity (ETI). Most R genes encode proteins that contain a nucleotide binding site (NBS) and leucine rich repeats (LRRs) or simply NBS-LRR proteins [2]. Pathogen effector molecules e.g., the *AvrPto* molecules of *Pseudomonas syringae*, typically alter the structure of NBS-LRR proteins through a direct binding or modification of other host plant proteins and allowing an exchange of ADP for ATP. The binding of ATP to the NBS domain then results in the activation of a signal transduction system through creation of binding sites for downstream signalling molecules and formation of central base binding (CBB) protein multimers. The dissociation of the pathogen effector proteins and modified effector targets from the NBS domain then results in the hydrolysis of ATP and a return of the NBS-LRR protein to its original inactive state [2] [7].

To this day, only a single NBS-LRR protein, AtLRR encoded by the At3g14460 gene in *Arabidopsis thaliana*, has been experimentally established to be an adenylyl cyclase (AC) [8] [9] with a role in defense response against the biotrophic fungus, *Golovinomyces orontii* and the hemi-biotrophic bacteria, *Pseudomonas syringae* [8]. With respect to AC activity, AtLRR was shown to display a multi-domain *in vitro* activity that is Mn^{2+}-dependent and stimulated by Ca^{2+} while at the same time, the protein could rescue AC-deficiency in a mutant (*cya*A) *Escherichia coli* or SP850 strain [8] [9]. With regard to disease resistance, knock-out mutants of AtLRR were found to have compromised immune responses to *G. orontii* and *P. syringae* but not against *Botrytis cinerea*, which is a necrotrophic fungus [8]. Therefore, in this reported study, we focused on using web-based tools or bioinformatics to elaborate further on the functional roles of this protein molecule both as an AC and NBS-LRR. Ideally, bioinformatics is simply defined as the science of data management systems in the genomics and proteomics of life forms, whereby biology, computer science and information technology merge into a single discipline.

Our work therefore, was motivated by the fact that since in nature proteins do work as a team or network to achieve common biological functions, as a result, bioinformatics can then be used as a tool with the power to both unravel and predict important information such as structure, solubility, interactions and functions of unknown and/or uncharacterized. In this study therefore, a combination of structural analysis, anatomical expression analysis, developmental ex-

pression analysis, co-expression analysis, functional enrichment analysis, stimulus-specific expression analysis and promoter analysis of the AtLRR protein was undertaken in selected mutant and wild type lines to circumscribe and further elaborate its function.

2. Materials and Methods

2.1. Determination of the Structural Features of AtLRR

The PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/) was used to predict the transmembrane topology of AtLRR [10]. The Phyre2 server (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) was used to predict the three-dimensional (3-D) structure of AtLRR based on the c2a5Yb protein as template at 100% confidence and across 30% coverage [11]. AtLRR sequence was retrieved from The Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/) and verified for presence of various functional domains using the PROSITE database located within the Expert Protein Analysis System (ExPASy) proteomics server (https://www.expasy.org/) [12] [13] [14].

2.2. Analysis of the Anatomical Expression Profile of AtLRR

In order to reveal the expression patterns of At3g14460 in various tissues of the Arabidopsis plant, the microarray database and expression-data analysis tool, GENEVESTIGATOR Version V3 (https://genevestigator.com/gv/start/start.jsp) [15] [16], was used. The tool was used because it provides the transcriptome information from the Affymetrix Arabidopsis ATH1 Genome Array platform using the 260011_At probe and At3g14460 as the query term. As a result, the arbitrary values of the expression intensity of At3g14460 in 111 Arabidopsis tissues were retrieved followed by calculation and revealing of average signals for each type of the plant tissues.

2.3. Analysis of the Developmental Expression Profile of AtLRR

The AtGenExpress Visualisation Tool (AVT) (http://allie.dbcls.jp/pair/AVT;AtGenExpress+Visualization+Tool.html) [17] was used to determine the developmental expression profile of At3g14460 in *Arabidopsis thaliana*. The analysis was made to be from the point of seedling development till the shedding off of mature seeds from siliques.

2.4. Analysis of the Co-Expressional Profile of AtLRR

In order to establish the co-expressional profile of At3g14460 with the other related Arabidopsis genes, the Arabidopsis co-expression tool (ACT) (http://www.arabidopsis.leeds.ac.uk/ACT/) [18] [19] was used. The tool analysis was performed across all experiments available on the *A. thaliana* microarray data set obtained from the Nottingham Arabidopsis Stock Centre (NASC), using At3g14460 as the driver or reference gene and leaving the gene list limit blank to

obtain a full correlation list. This tool utilizes hybridization signal intensities from microarray experiments to calculate a Pearson correlation co-efficient (r-value), which is a scale-invariant measure of expression similarity that expresses the strength and direction of the linear relationship between the driver or reference gene (At3g14460 in this case) and all other Arabidopsis genes represented on the selected chip. The tool calculates and returns both negative and positive correlations (ranging from −1 to +1), associated probability (p), and expectation (e) values, which are a measure of the statistical significance [20]. From the obtained correlation list, 50 topmost co-expressed genes (or the expression co-related gene group (ECGG50)) were considered.

2.5. Analysis of the Functional Enrichment Expression Profile of AtLRR and Its Related Proteins

After establishing the co-expression group of At3g14460 (*i.e.*, ECGG50), the "Fatigoplus" (version 4.3) compare tool in the Babelomics suite (http://babelomics.bioinfo.cipf.es) [21] was used to identify any significant enrichments in functional terms associated with the At3g14460 gene and its highly co-expressed set of 50 genes (ECGG50) in the Arabidopsis plant. Using this stimulus tool, the expression profiles of At3g14460 and its ECGG50 were screened over the ATH1:22K array Affymetrix public microarray data in the GENEVESTIGATOR V3 version (https://www.genevestigator.com/) [15] [16]. The normalized microarray data were downloaded from the GEO (NCBI) (https://www.ncbi.nlm.nih.gov/geo/), the NASC Arrays (https://arabidopsis.info/affy/link_to_iplant.html) and the TAIR GenExpress (https://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp) and subsequently analyzed for experiments that were found to induce a differential expression of the genes. All available Arabidopsis databases were selected using default options, which included the gene ontology (GO) predictions; biological process (BP), molecular function (MF) and cellular component (CC), and annotation levels 3 - 9, KEGG pathways and Swissprot keywords. For each experiment found to induce differential expression, the fold-change (log2) values were then calculated. Subsequently, expression values were generated using the Multiple Array Viewer program from the Multi-Experiment Viewer (MeV) software package (Version 4.2.01) (The Institute for Genomic Research (TIGR), wherein enrichment significances were determined using PANTHER that adjusts p-values to correct for multiple hypothesis testing [22].

2.6. Analysis of the Stimulus-Specific Expression Profile of AtLRR and Its Related Partners

The expression profiles of At3g14460 and its ECGG50 were initially screened over all of the available ATH1:22K array Affymetrix public microarray data in the Genevestigator V3 version (https://www.genevestigator.com) using the stimulus/perturbations tool [15]. In order to obtain greater resolution of gene ex-

pression profiles, the normalized microarray data were subsequently downloaded and analyzed for experiments (of over 3000 microarrays) that were found to induce differential expression of the genes. The data were downloaded from the following repository sites: GEO (NCBI) (http://www.ncbi.nlm.nih.gov/geo/) [23], NASCArrays (https://arabidopsis.info/affy/link_to_iplant.html) [24] and TAIR-ATGenExpress (https://www.arabidopsis.org/servlets/Search?action=new_search&type=expression). The downloaded array data were then analyzed, and fold-change (log2) values calculated for each experiment. An expression heat map was then generated using the Multiple Array Viewer program from the Multi-Experiment Viewer (MeV) software package (version 4.2.01) created by The Institute for Genomic Research (TIGR) [25].

2.7. Analysis of the Promoter Expression Profile of AtLRR and Its Related Partners

The promoter regions of At3g14460 and its ECGG50 were analyzed for any enrichment in potential transcription factor binding sites (TFBSs) using the web-based Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena) [26] and POBO (http://ekhidna.biocenter.helsinki.ft/poxo/pobo) [27] applications. The visualization tool in Athena performs an analysis of Arabidopsis promoter sequences and reports enrichment of known plant TFBSs. The analysis of the At3g14460 and its ECGG50 was performed using settings of 1000 bp upstream of the transcription start sites (TSSs) and not cutting off at adjacent genes. The Athena results were subsequently confirmed in POBO by uploading promoter sequences 1 kb upstream of the coding regions of the At3g14460 and its ECGG50. The analysis was run against an Arabidopsis background (clean), searching for the WRKY core motif (TTGACY) using default settings. A two-tailed p-value was then calculated in the linked online GraphPad website using the generated t-value and degrees of freedom to determine the statistical differences between the input sequences and background.

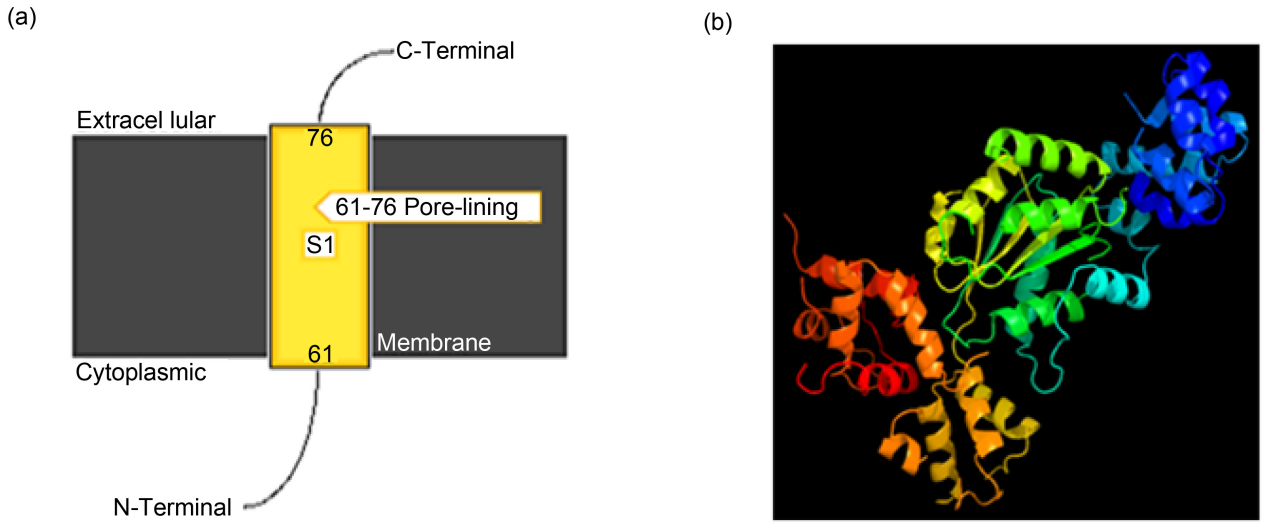
2.8. Statistical Analysis

Data was subjected to a two-tailed Student's t-test for comparisons, where a p-value of less or equal to 0.0001 was used to denote significance. Where the *t*-test revealed significant differences between treatments, means were then separated by *post hoc* Student-Newman-Keuls (SNK) multiple range test ($p \leq 0.0001$).

3. Results

3.1. Structural Features of AtLRR

AtLRR is a very large protein (~1424 amino acids) with a calculated molecular weight (mw) of 158905.2 Daltons and an isoelectric point of 5.54 [28]. Structurally, AtLRR is a trans-membrane, multi-domain and multi-functional protein (**Figure 1**).



(c)

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1                                                                  65
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RRLEDIGNDYLGDLVAQSFFQRLDITMTSFVMHDLMNDLAKAVSGDFCFRLEDDNIPEIPSTTRH
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KFYGMPRWDEWICPELEDGIFPCLQKLIIQRCPSLRKKFPEGLPSSTEVTISDCPLRAVSGGENS
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DRQTEDFDQYETQLGSLPQQFEEPAVISARYSGYISDIPSTLSPYMSRTSLVPDPKNEGSILPGS
SSYQYHQYGIKSSVPSPRSSEAIKPSQYDDDETDMEYLKVTDISHLMELPQNLQSLHIDSCDGLT
SLPENLTESYPNLHELLIIACHSLESFPGSHPPTTLKTLYIRDCKKLNFTESLQPTRSYSQLEYL
FIGSSCSNLVNFPLSLFPKLRSLSIRDCESFKTFSIHAGLGDDRIALESLEIRDCPNLETFPQGG
LPTPKLSSMLLSNCKKLQALPEKLFGLTSLLSLFIIKCPEIETIPGGGFPSNLRTLCISLCDKLT
PRIEWGLRDLENLRNLEIDGGNEDIESFPEEGLLPKSVFSLRISRFENLKTLNRKGFHDTKAIET
MEISGCDKLQISIDEDLPPLSCLRISSCSLLTETFAEVETEFFKVLNIPYVEIDGEIFS
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Figure 1. Structural features of AtLRR. (a) Transmembrane topology, showing its C-terminal end located at the extracellular region and the N-terminal end in the cytoplasmic region. The two ends are linked by a 15 amino acid trans-membrane region/pore-lining found between amino acid residues 61 and 76 [10]. (b) 3-dimensional ribbon model, showing the C-terminal end in blue, the trans-membrane region in green and the N-terminal end in orange [11]. (c) Amino acid sequence, showing the multi-domain multi-functional nature of the protein, where AC motifs capable of generating cAMP are marked in green, a domain commonly found in LLR receptor kinases with GC activity marked in yellow, the NB-ARC domain responsible for disease resistance marked in blue and scattered R residues that make AtLRR an R protein marked in purple.

3.2. Anatomical Expression Pattern of AtLRR

Our analysis of the anatomical expression profile of AtLRR, showed that besides being moderately expressed in most other tissues, the protein is explicitly expressed in pollen grains and senescing leaves (**Figure 2**). Incidentally, the noted high expression of AtLRR in pollen grains closely relates to findings of other previously undertaken studies, which showed that ACs are essential for the growth and re-orientation of pollen tubes in *Lilium longiflorum* [29], *Agapanthus umbellatus* [30], *Zea mays* [31] and *A. thaliana* [32], wherein cAMP is key as a signalling molecule [29] [30] [31]. Apparently, pollen tube growth and reorientation are a prerequisite for fertilization and seed formation [31]—two key processes of reproduction. This thus shows the involvement of AtLRR in this process (reproduction) in *A. thaliana*.

3.3. Developmental Expression Pattern of AtLRR

Expressional analysis of the At3g14460 gene showed that the AtLRR protein is

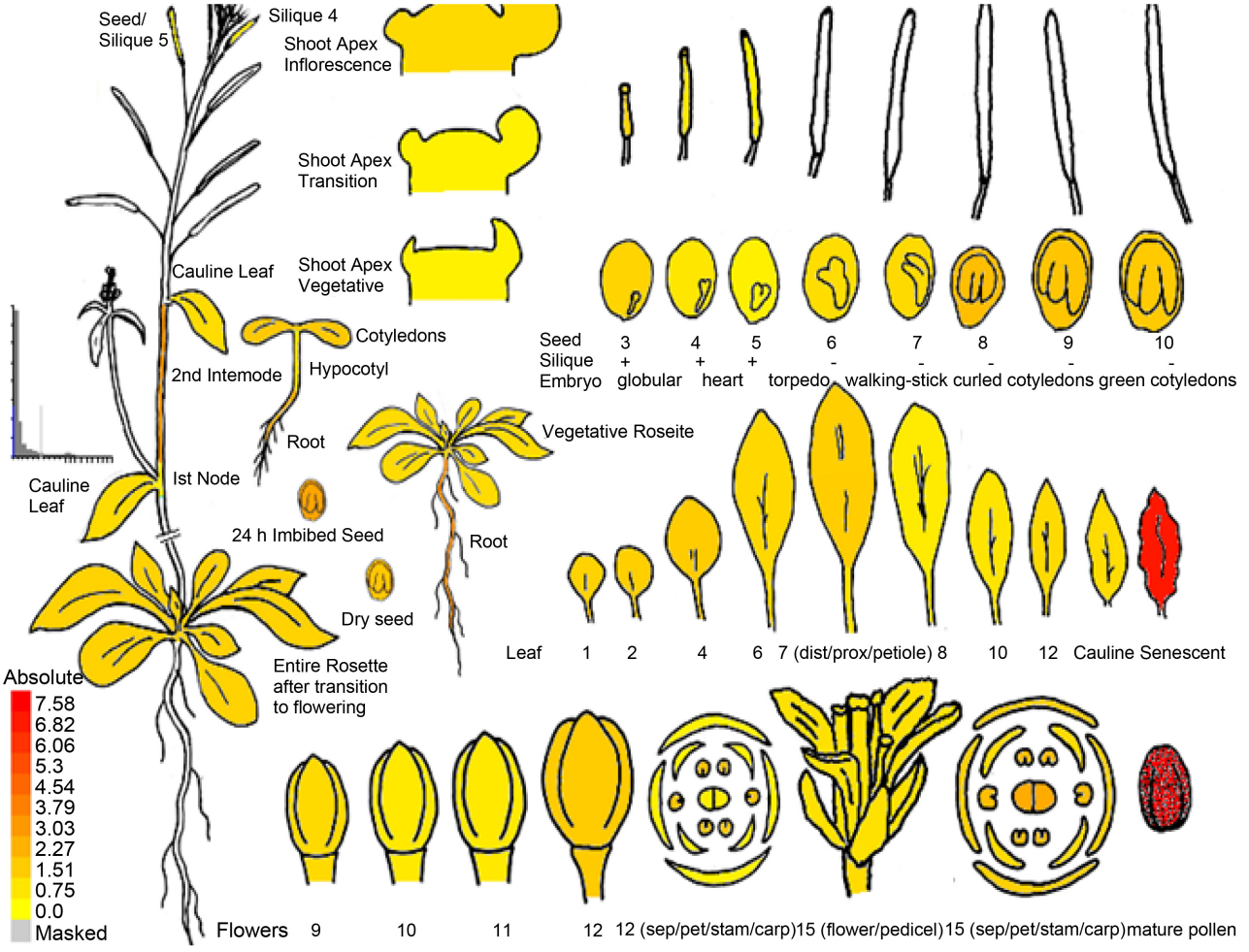

Figure 2. Expression levels of the AtLRR protein in various tissues of the Arabidopsis plant, showing moderate expression in most tissues except for the pollen grain and senescing leaf, where the intensity is so high. Additionally, expression is also at different stages of development for the various tissues (t-test: $p \leq 0.0001$).

generally expressed across all stages of growth in the Arabidopsis plant but most significantly during the flowering and seed dispersion stages (Figure 3). This thus indicates that AtLRR is not only involved in plant reproduction but plant development.

3.4. Co-Expression Pattern of AtLRR

When the At3g14460 gene was analyzed for co-expression in the Arabidopsis genome, we noted that 50 of its most correlated genes, have high r values of between 0.82 and 0.90 (Table 1). These expression-correlated genes (ECGG50) are also significantly enriched for the "biological process (BP)" gene ontology (GO) categories "response to biotic stimulus', "defense response", and "innate immune response" and "molecular function (MF)" GO categories "reproduction" and "development". Response to biotic stimulus processes includes response to bacteria, response to nematodes and response to fungi while defense response includes defense response to fungi, and innate immune response includes the PAMPS triggered immunity and SA-mediated signalling pathways. In addition,

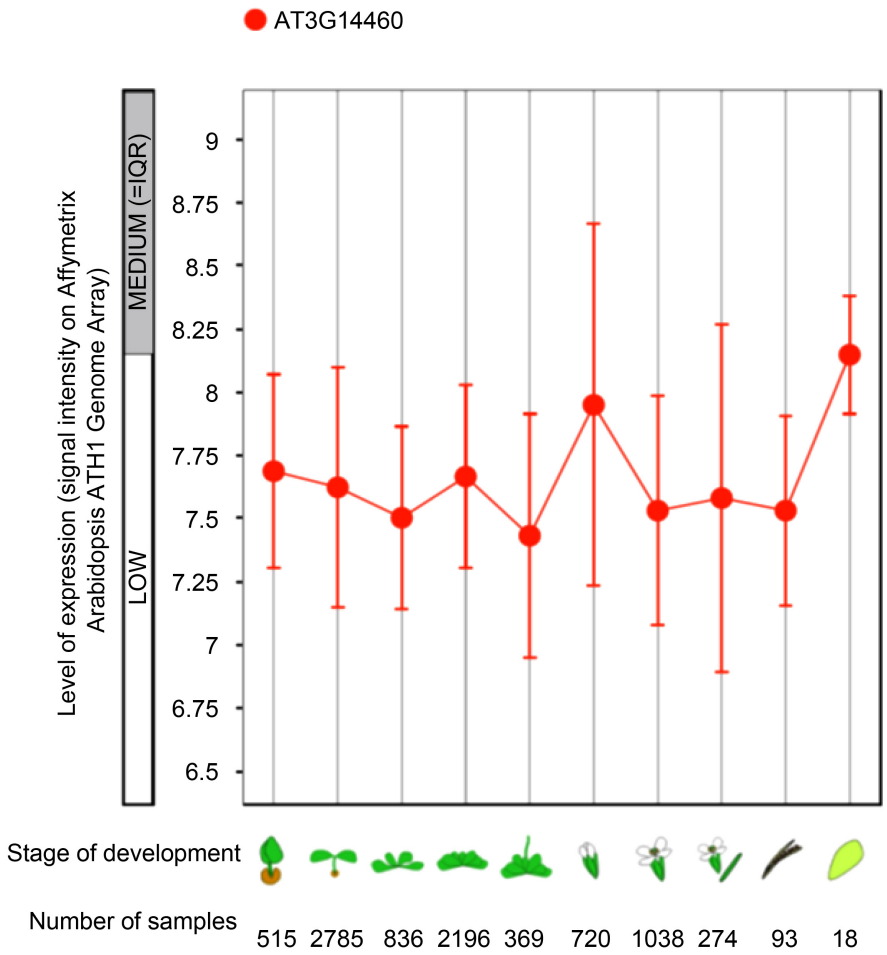

Figure 3. Expression levels of the AtLRR protein during the development of Arabidopsis plant, showing moderate expression across all stages except for the flowering and seed dispersion stages, where the levels are high (t-test: $p \leq 0.0001$).

Table 1. List of the top 50 genes co-expressed with At3g14460.

Rank	Locus	GO Terms	r-value	Annotation and Description
-	At3g14460		1.00	Disease resistance protein (TIR-NBS-LRR)/Adenylyl cyclase (AC)
1	At4g19520	DR, CRH	0.90	Disease resistance protein (TIR-NBS-LRR)
2	At3g14470		0.89	Putative disease resistance RPP 13-like
3	At4g14610		0.89	Probable disease resistance protein
4	At5g46470		0.89	Disease resistance protein RPS6
5	At5g42830		0.88	HXXXD type acyl transferase family protein
6	At1g59590		0.88	ZCF 35
7	At3g61390	PU	0.88	U-box domain-containing protein 36
8	At5g35580	PP	0.87	Serine/threonine protein kinase PBLB
9	At2g45920	PU	0.87	U-box domain-containing protein 37
10	At5g11210	RLS, IT, GCRSP	0.86	Glutamate receptor 2.5
11	At2g29065	T	0.86	Scarecrow-like protein 34
12	At5g45000	DR, ST	0.86	Disease resistance protein (TIR-NBS-LRR)
13	At1g61550	PP, IIR	0.86	G-type lectin S-receptor -like
14	At1g26420	REDOX	0.86	Berberine bridge enzyme like 7
15	At1g33880	RB	0.86	Immune associated nucleotide binding protein 2
16	At3g17700	RN	0.86	Probable cyclic nucleotide gate ion channels
17	At2g19130	RP, PP	0.85	Serine/threonine kinase
18	At2g18680		0.85	Transmembrane protein
19	At1g03660		0.85	Ankyrin repeat containing protein
20	At3g09020		0.85	Alpha 1,4-glycosyl transferase family protein
21	At5g49680	PTG, RHG	0.85	Kinky pollen protein
22	At2g19710		0.85	Regulator of VPS4 activity in the MVB pathway protein
23	At1g53620	CRH	0.84	Unknown protein
24	At5g05190	DR, RF, SA RE	0.84	Protein enhanced disease resistance 4
25	At2g35736		0.84	Unknown protein
26	At1g77890	SCA	0.84	Unknown protein
27	At3g12040	R, NCMP, RS	0.84	DNA-3-methyladenine glycosylase
28	At5g01490	CIT, RA, RD	0.84	Vacuolar cation exchanger 4
29	At2g31990		0.84	Probable xyloglucan galactosyltransferase GT15
30	At3g07600		0.84	Heavy metal-associated isoprenylated plant protein 16
31	At1g70170	RSS, RC, F, RPP, LS	0.84	Matrix metalloproteinase
32	At3g05930	RRD, RS	0.84	Germini like protein subfamily members
33	At5g25940		0.84	Early nodulin like protein
34	At5g01550	PP, DR, ABSA, SG	0.83	Lectin domain containing receptor kinase

Continued

35	At1g24140		0.83	Metalloendo proteinase 3-MMP
36	At3g51180	DB, MB	0.83	Zinc finger COOH domain containing protein 45
37	At5g01550	PP, DR, ABSA, SG	0.83	L-type lectin domain contain receptor kinase VII
38	At4g25070		0.83	Caldemon like protein
39	At4g12120	PT, PS, VDE	0.83	Protein transport SEC 16
40	At1g61560	DRF, RBS, CD	0.83	MLO-like protein 6
41	At3g26910		0.83	Hydroxyproline -rich-glycoprotein family protein
42	At1g51920		0.83	Transmembrane protein
43	At3g09010		0.83	Alpha 1,4-glycosyl transferase family protein
44	At5g11290		0.83	Unknown protein
45	At5g44990		0.83	Glutathione S transferase family protein
46	At5g66070	DR, RC	0.83	NEP 1-interacting protein like 1
47	At2g23770	RC, PTI, IIR	0.82	LYSM domain receptor like kinase 4
48	At1g72950	ST, DR	0.82	Disease resistance protein TIR-NBS class
49	At5g61010	PT, EX, PREEA	0.82	Exocyst subunit exo70 family protein E2
50	At5g67350		0.82	Unknown protein

DR = defence responses; CRH = cellular response to hypoxia; PU = protein ubiquitination; PP = protein phosphorylation; RLS = response to light stimulus; IT = ion transport; GCRSP = G-protein coupled receptor signalling pathway; T = transcription; ST = signal transduction; IIR = innate immune response; REDOX = oxidation-reduction reactions; RB = response to bacteria; RN = response to nematodes; RP = recognition of pollen; PTG = pollen tube growth; RHG = root hair cell tip growth; RF = response to fungus; SA = SA-mediated signalling pathway; RE = regulation of exocytosis; SCA = SNARE complex assemble; R = DNA repair; NCMP = nitrogen compound metabolic process; RS = response to stress; CIT = calcium ion transport; RA = response to auxin; RD = root development; RSS = response to salt stress; F = flowering; RPP = regulation of photoperiodism; LS = leaf senescence; RRD = regulation of root development; ABSA = abscisic acid activated pathway; SG = seed germination; PT = protein transport; PS = protein secretion; VDE = vesicle docking involved in exocytosis; DRF = defence response to fungi; RBS = response to biotic stimulus; CD = cell death; RC = response to chitin; PTI = PAMPS triggered immunity; EX = exocytosis; PREEA = protein regulation of extracellular exosome assembly; DB = DNA binding; MB = metal ion binding (t-test: $p \leq 0.0001$).

reproduction includes pollen tube growth and flowering while development includes root hair cell tip growth and root development. Notably, all these processes are consistent with our findings in **Figure 2** and **Figure 3**, and also in line with the role of AtLRR as an AC and disease resistance protein.

3.5. Differential Expression Pattern of AtLRR and Its Related Proteins

When we extended the analysis to identify conditions that induce At3g14460 and its ECGG50 partners (**Table 1**), we noted strong induction by various factors, which include the hemi-biotrophic pathogens, *Pseudomonas syringae* [33] and *Phytophthora parasitica* [34] [35], and their associated effector molecule, flagellin 22 [36] or its synthetic analogues, DFPM ([5-(3,4-dichlorophenyl)-2-furanyl]-1-piperidinyl-methanethione) and CPM (chlorphenamine) [37] (**Figure 4**). This further supported the role of AtLRR in

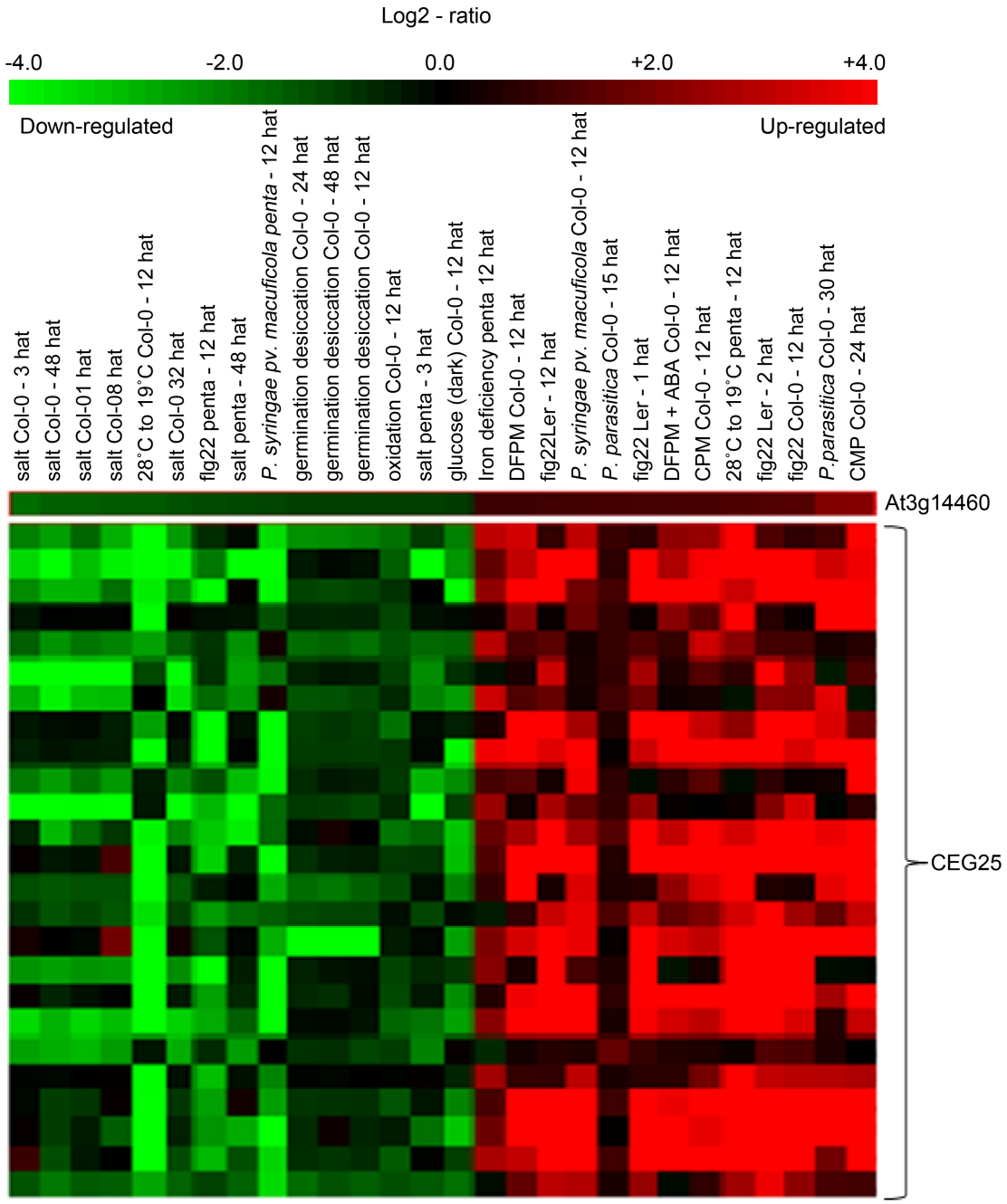

Figure 4. Heatmap constructed to illustrate the fold change (log2) in expression of At3g14460 and 25 selected expression-correlated genes (ECGG50) in response to selected microarray experiments. The experiments presented include; NaCI treatment of root stele protoplast-FACS (3, 48, 1, 8, & 32 hat: n = 3); temperature shift of leaf samples of the 35S:RPS4-HS plants from 28˚C to 19˚C (12 hat: n = 3); Flg22 treated leaf discs of Ler and Col wt and penta mt samples (12, 1 & 2 hat: n = 3); response of the transgenic mutants 35S:RPS4-HS eds1-2 vs the 35S:RPS4-HS to the virulent *P. syringae* pv. tomato strain DC3000 (12 hat: n = 3); seed desiccation of Col wt and penta mt samples (12, 24 & 48 hat: n = 3); oxidative treatment of Col wt samples (12 hat: n = 3); osmotic shock of Col wt samples (12 hat: n = 3); Fe deficiency of penta mt samples (12 hat: n = 3); DFPM treatment of Col wt samples (12 hat: n = 3); *P. parasitica* treatment of the tomato strain DC310 (30 hat: n = 2); CMP treatment of Col wt samples (15 & 30 hat: n = 3). Abbreviations: hat = hours after treatment, Flg22 = flagellin 22, Ler = Landsberg, Col = Colombia, wt = wild-type, mt = mutant-type, *P. syringae* = *Pseudomonas syringae*, *P. parasitica* = *Phytophthora parasitica*, DFPM = [5-(3,4-dichlorophenyl)-2-furanyl]-1-piperidinyl-methanethione, and CMP = 4-chloro-6-methyl-2-phenylpyrimidine (t-test: $p \leq 0.0001$).

cAMP-mediated disease resistance processes.

3.6. Promoter Enrichment Pattern of AtLRR and Its Related Partners

Generally, when genes are co-expressed and co-regulated, they are likely to share a common *cis* element in their promoter regions [38]. In our concerted Athena analysis of At3g14460 and its ECGG50 partners (**Table 1**), we identified a hexamer W-box *cis*-element, TTGACY, as the enriched TF site ($p \leq 10^{-5}$) between At3g14460 and its ECGG50 partners (**Figure 5**). In plants, W-box is a DNA *cis* regulatory element sequence that is recognized by a family of WRKY transcription factors (TFs) [39] [40]. Thus, this then implies that the WRKY TF plays a significant role in the regulation of these co-expressed genes (At3g14460 and its ECGG50). WRKY TFs are key regulators of many processes in plants that include responses to biotic and abiotic stress factors, senescence, seed dormancy and seed germination, and some developmental processes [39] [41]. In *A. thaliana*, the WRKY TF superfamily consists of 74 members that are known to play significant roles in the transcriptional reprogramming associated with plant responses to pathogens and SA-signalling [40].

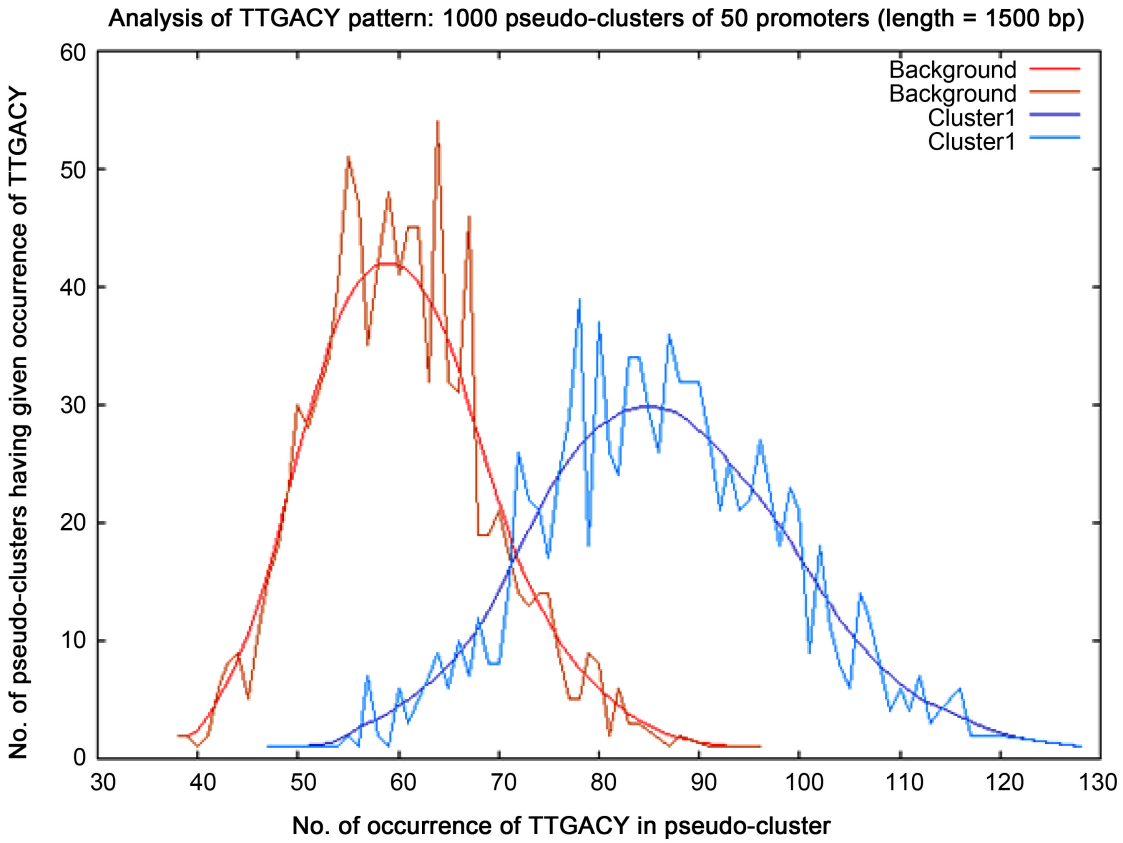

Figure 5. Frequency occurrence of the TTGACY WRKY core motif in promoters of At3g14460 and its ECGG50 partners. The motif is significantly enriched in the cluster (blue), being present in 96% of their promoters at an average of 4.8 copies per promoter compared to 88% of the whole Arabidopsis genome promoters (brown) that has an average of 3.78 copies per promoter (t-test: $p \leq 0.0001$).

4. Discussion

In plants, basal or first line immunity against pathogens provides a pre-infection resistance layer, that involves recognition of conserved structural components of pathogen such as flagellin or chitin, also referred to as pathogen-associated molecular patterns (PAMPs), ultimately leading to PAMP-triggered immunity (PTI) [42] [43]. A second layer of the plant defense system involves intracellular receptors that are products of the resistance (R) genes. These receptors recognize products of pathogen avirulence (Avr) genes, leading to rapid activation of defense responses such as the hypersensitive response (HR) at the infection sites. This layer of defense is often referred as effector-triggered immunity (ETI) [42] [43]. R genes encode proteins containing a nucleotide binding site (NBS) and leucine rich repeats (LRRs) or simply NBS-LRR proteins [2].

In *Arabidopsis thaliana*, there are approximately 150 NBS-LRR encoding genes, including At3g14460, that codes for an AtLRR protein [44]. This AtLRR protein was recently established to be an adenylyl cyclase (AC) [8] [9] with a role in resistance to infection by biotrophs and hemi-biotrophs [8]. ACs are enzymes capable of catalyzing the conversion of adenosine 5'-triphosphate (ATP) to the second messenger molecule, 3',5'-cyclic adenosine monophosphate (cAMP) [45] [46] [47]. cAMP in turn, controls various downstream plant processes such as the cell cycle [48], growth of pollen tubes [29] [30] [31], and responses to biotic and abiotic stress [49] [50] [51]. On the other hand, biotrophs and hemi-biotrophs are obligate pathogens that establish a close and long-term nutritional relationship with their host cells and continuously absorb nutrients without causing damage to cells [42] [43]. These pathogens further trigger the onset of the salicylic acid (SA) dependent signalling system, which then stimulates and controls the establishment of systemic acquired resistance (SAR) in plants [52].

In this study, our analysis of the structural features of AtLRR, showed that the protein is a trans-membrane multi-domain molecule (**Figure 1**), capable of performing multiple functions e.g., AC activity [8] [9] and disease resistance [8]. These observed structural features of AtLRR, make it an ideal candidate for the PTI and ETI, whereby it effects the recognition of the pathogen effector molecules with its extracellular domain and then instigate downstream disease resistance processes via its intracellular domain [2].

From the anatomical and developmental expression profiles of AtLRR, it was noted that the protein was mostly expressed in pollen grains and flowers, senescing leaves as well as during the development of seeds, shoots, roots, seedlings, leaves, flowers, and siliques (**Figure 2** and **Figure 3**). This was worth noting because it directly implicated AtLRR into plant reproduction, plant defense and plant development respectively—three key processes that are very important to plants. Apparently, the noted implication of AtLRR in plant reproduction, particularly as an AC, is literally not unusual because of three reasons. Firstly, cAMP has been shown to be directly involved in the growth and re-orientation of pollen tubes in *Lilium longiflorum* [29], *Agapanthus umbellatus* [30], *Zea*

mays [31] and *A. thaliana* [32]. Secondly, cAMP has been shown to regulate the development of male reproductive organs in *Marchantia polymorpha* [53]. Thirdly and lastly, cAMP has been shown to regulate flowering in *Lemma gibba* [54]. Once more, the noted implication of AtLRR in leaf senescence is also not unusual as it directly links AtLRR, both as an AC and disease resistance protein, to signal interactions that normally take place between pathogens and host plants during defense responses [55]. In this interaction, biotrophs often delay senescence to keep host cells alive, and resistance in this case is then achieved by senescence-like processes in the host while on the other hand, necrotrophs promote senescence in the host, and preventing early senescence would then be the resistance strategy for plants [55]. Hemi-biotrophs are involved in both patterns [55]. Lastly, the noted implication of AtLRR, again as an AC, in plant development is also not unusual because cAMP was previously shown to control the cell cycle in tobacco [48].

Further analysis of the expression profile of AtLRR showed that its gene (At3g14460) is mostly co-expressed and co-regulated with several other genes linked to disease resistance and cAMP signalling (**Table 1**). Of interest are At4g19520, At3g14470, At4g14610, At5g46470, At5g45000, At5g05190 and At1g72950, which all code for disease resistance proteins and At5g49680 that encodes a kinky pollen protein responsible for pollen tube growth (reproduction) and root hair cell tip growth (development). Once again, this is in tandem with the recently confirmed activities of AtLRR as an AC and disease resistance protein [8] [9].

When we extended our analysis to identify conditions that induce the expression of At3g14460 and its correlated genes (ECGG50) (**Table 1**), we noted very strong induction by the hemi-biotrophic pathogens, *Pseudomonas syringae* [33] and *Phytophthora parasitica* [34] [35], and their associated effector molecule, flagellin 22 [36] or its synthetic analogues, DFPM ([5-(3,4-dichlorophenyl)-2-furanyl]-1-piperidinyl-methanethione) and CPM (chlorphenamine) [37] (**Figure 4**). There was also strong induction in response to abscisic acid (ABA) (**Figure 4**)—a phytohormone that is chiefly involved in leaf senescence [55]. Induction by *P. syringae* and *P. parasitica* is consistent with the recently reported upregulation of the expression of AtLRR in response to the powdery mildew fungus *Golovinomyces orontii*, which is a biotroph and the Gram-negative bacterium *P. syringae* that is a hemi-biotroph [8]. In addition, ACs and cAMP have previously been directly implicated in disease resistance in various plants that include *Nicotiana benthamiana* [56], *Hippeastrum hybridum* [57], *A. thaliana* [50] and *Brachypodium distachyon* [58]. Thus, these findings are therefore, very consistent with the established role of AtLRR in plant cAMP-dependent signal transduction pathways against the biotrophic and hemi-biotrophic pathogens [52].

In addition, when we further subjected At3g14460 and its correlated genes (ECGG50) (**Table 1**) to promoter enrichment analysis, we found out that this

gene together with its co-expressed partners, have a common transcription factor binding site (TFBS) in their promoters (**Figure 5**) that then allows them to be co-regulated and ultimately co-function [59]. The identified common TFBS is the W-box hexamer, TTGACY core element, known to bind WRKY transcription factors (TFs) [39] [40]. In Arabidopsis, the WRKY TF superfamily consists of 74 members [40] that are known to play significant roles in the transcriptional reprogramming associated with plant responses to pathogens and SA-signalling [40].

5. Conclusion

This study, therefore, has managed to explore around the functional roles of AtLRR both as an AC and disease resistance protein and elaborated such an exploration. The study has unequivocally managed to establish that, as an AC and disease resistance protein, AtLRR has key roles in plant disease resistance (against the biotrophic and hemi-biotrophic pathogens), plant reproduction (flowering, pollen tube growth and re-orientation, and development of male reproductive organs) and plant development (the cell cycle, and tissue and organ development). The protein effects its functions via a W-box or WRKY transcription factor, TTGACY, mediated by cAMP.

6. Recommendations

Considering the significance and importance of the processes in which AtLRR is involved, it is crucial that this novel protein candidate is studied further to understand its exact mechanisms of action and perhaps for the possible generation of some disease resistant crops/strong cultivars in the agricultural and horticultural sectors.

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Author Contribution Statement

OR and TN conceived and designed the study; AS-M did the experimental analyses; TBD, KSS, EDB and NMM facilitated the experimental analyses; MMT and DTK prepared the heat map. All authors contributed to the writing of the manuscript and approved the final version.

Conflicts of Interest

The authors declare no conflict of interest.

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