MINI REVIEW ARTICLE

Plant Rabs and the role in fruit ripening

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Abstract

Fruit ripening is a complex developmental process that involves the synthesis and modification of the cell wall leading up to the formation of an edible fruit. During the period of fruit ripening, new cell wall polymers and enzymes are synthesized and trafficked to the apoplast. Vesicle trafficking has been shown to play a key role in facilitating the synthesis and modification of cell walls in fruits. Through reverse genetics and gene expression studies, the importance of Rab guanosine triphosphatases (GTPases) as integral regulators of vesicle trafficking to the cell wall has been revealed. It has been a decade since a rich literature on the involvement of Rab GTPase in ripening was published. Therefore, this review, sets out to summarize the progress in studies on the pivotal roles of Rab GTPases in fruit development and shed light on new approaches that could be adopted in the fields of postharvest biology and fruit-ripening research.

Keywords: cell wall; fruit ripening; Rab GTPase; Ras superfamily; vesicle trafficking

Abbreviations: DAA, days after anthesis; EC, enzyme commission number; ER, endoplasmic reticulum; GAP, GTPase activating protein; GDF, GDI displacement factor; GDI, GDP dissociation inhibitor; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GTP, guanosine triphosphate; GTPase, guanosine triphosphatases; PE, pectinesterase; PM, plasma membrane; PG, polygalacturonase; RGGT, Rab geranylgeranyl transferase; TGN, trans-Golgi network

Introduction

Fruit ripening entails the physicochemical and physiological changes that give rise to an edible fruit. During this process, changes in the cell wall structure and composition occurs (Brummell, 2006). For complete summaries of plant cell wall dynamics, the readers can refer to other excellent reviews (Brummell, 2006; Ebine & Ueda, 2015). Tomato fruit has long been used as a model system for studying this physiological process (Alexander & Grierson, 2002). It was suggested (Seymour, Lasslett & Tucker, 1987) that cell wall degradation is primarily responsible for the softening of the fruit, which is a fruit-ripening related process. The question on how the plant cell wall is synthesized and modified is still puzzling because the process occurs partly in the apoplast. As a result, several research groups have tried to alter specific cell wall-degrading enzymes related to fruit ripening through molecular genetic strategies (Hall et al., 1993; Powell, Kalamaki, Kurien, Gurrieri & Bennett, 2003; Sheehy, Kramer & Hiatt, 1988; Smith et al., 1990). In tomato fruit, gene silencing has been used to inhibit the synthesis of polygalacturonase (PG; EC 3.2.1.15). The PG levels were substantially reduced to as low as 1% thereby inhibiting pectin depolymerisation (Sheehy et al., 1988; Smith et al., 1990). However, this inhibition has been found to have only a relatively small effect upon fruit firmness (Sheehy et al., 1988; Smith et al., 1990). Even though PG activity was not the sole determinant of fruit softening, it has been shown to have led to an extended shelf life (Giovannoni, DellaPenna, Bennett, & Fischer, 1989). This unique characteristic made the PG antisense plants sufficiently different and a commercial success. Similar studies elucidating the roles of pectinmethylesterase (PME; EC 3.1.1.11) and cellulose (EC 3.2.1.4) (Brummell, Hall, & Bennett, 1999; Hall et al., 1993) have revealed the complexity of the fruit ripening process. These studies indicate that there may be interdependency among multiple enzymes that lead to fruit softening, as might be expected. For example, a study of tomato plants in which both PG and expansin were inhibited showed a synergistic effect (Powell et al., 2003).

The plant endomembrane system consisting of the Golgi apparatus, endoplasmic reticulum, endosome, trans-Golgi network (TGN) and plasma membrane (PM) work together to synthesize, modify and ship proteins and other cellular materials. An appropriate delivery to the correct destination is strictly maintained within the cell despite the influx of a vast array of gene protein products into the endomembrane system (Müntz, 1998; Zerial & McBride, 2001*)*. Whether the transport system is

maintained in a specific and coordinated manner has raised several questions; how are the cargoes selected? What elements guarantee their accurate fusion to the target membrane? How is the directionality of cargo transport maintained in spite of the interconnected pathways? Using mostly reverse genetic approaches, the Rab GTPases have been found to be primary determinants of the steps of directing traffic within endomembrane system. With only a few reports available, this mini-review sets out to summarize research findings on the roles of Rab GTPases in fruit ripening. It is expected that such currently available information could unlock the possibilities of improving biotechnological approaches to address postharvest fruit losses.

Rab GTPases as molecular switches in membrane trafficking

The first members of this protein family were originally discovered in yeasts, where they are commonly referred to as YPTs (yeast protein transport) (Gallwitz, Donath, & Sander, 1983; Salminen & Novick, 1987; Segev & Botstein, 1987). Following this discovery, the use of oligonucleotide probes to screen a rat brain cDNA library identified the first homologs in mammals (Touchot, Chardin, & Tavitian, 1987). Hence the acronym 'Rab' (Ras-related proteins in brain) was adopted (Touchot et al., 1987). The Rab GTPases oscillate between the 'active' GTP-bound and the 'inactive' GDP-bound forms in the membrane and cytosol respectively (Stenmark & Olkkonen, 2001; Zerial & McBride, 2001) (Figure 1). This conformational change accounts for their roles as 'molecular switches' (Vetter & Wittinghofer, 2001) and the ability to perform several tasks in a coordinated and regulated manner (Stenmark & Olkkonen, 2001; Zerial & McBride, 2001). Rab GTPases are initially synthesized as soluble proteins in the cytosol (Ali & Seabra, 2005). Rab escort proteins (REP) recognize and associates with the newly synthesized GDP-bound Rab proteins to form a stable Rab-REP complex (Andres et al., 1993). This interaction facilitates the prenylation of the Rab protein catalysed by the Rab geranylgeranyl transferase (RGGT) enzyme (Alexandrov et al., 1999; Seabra, 1998). Prenylation of Rab proteins is a post-translational modification which involves the addition of geranylgeranyl groups to the cysteine residues at the C terminus (Glomset & Farnsworth, 1994; Seabra, 1998). This process is essential for Rab specific membrane targeting and attachment (Casey & Seabra, 1996). Following prenylation, REP is released from the Rab-REP complex (Rak et al., 2004).

Another protein known as the Rab GDP dissociation inhibitor (GDI) protein binds to the modified Rab to maintain its stability and solubility in the cytosol by masking the prenyl group at the C- terminus of the Rab (Alexandrov, Horiuchi, Steele-Mortimer, Seabra, & Zerial, 1994). A membrane protein known as the GDI displacement factor (GDF) detaches the GDI from the Rab-GDI complex and allows the insertion of the Rab prenyl group into its target membrane (Dirac-Svejstrup, Sumizawa, & Pfeffer, 1997; Pfeffer & Aivazian, 2004). This is followed by the conversion of the Rab protein to its 'active' GTP-bound state by the guanine nucleotide exchange factor (GEF) (Figure 1).

[Figure 1 here]

The Rab family in plants

The Rab GTPase family which constitutes the largest group of the Ras (Rat sarcoma) (Cox & Der, 2010) superfamily has been found to exist in all eukaryotes studied (Stenmark, 2009). This subfamily has been extensively studied in yeasts, where they are called YPT proteins, and in humans, with at least 11 and 60 members respectively (Pereira-Leal and Seabra, 2001). Members of the Rab GTPase family have been identified in several plants (Table 1). The plant Rab GTPase family has been grouped into eight clades, namely RabA - RabH and these have been found to have a high degree of similarity with mammalian Rab classes 11, 2, 18, 1, 8, 5, 7 and 6 respectively (Pereira-Leal & Seabra, 2001; Rutherford & Moore, 2002; Vernoud, Horton, Yang, & Nielsen, 2003). The *RabA* clade is the largest of the plant Rabs with 26 members identified so far in *Arabidopsis thaliana* (Rutherford & Moore, 2002), 26 in *Solanum lycopersicum* (Flores et al., 2018; Lycett, 2008), 17 in *Oryza sativa* (Zhang, Hill, & Sylvester, 2007), 6 in *Prunus persica* (Falchi et al., 2010), 14 in *Vitis vinifera* (Abbal et al., 2008), 87 in *Gossypium raimondii* and 12 in *Lotus japonicus* (Borg, Brandstrup, Jensen, & Poulsen., 1997; Flores et al., 2018) (Table 1). The *RabA* clade is divided into six subgroups (RabA1 to RabA6) compared with only two Rab11 GTPases in mammals. The remarkably high number of Rab GTPase and their distribution across distinct membrane-bound compartments indicates their importance in plants for specific functions (Rutherford and Moore, 2002). Multiple sequence alignment analyses revealed 55% sequence homology between various subfamilies of closely related *Rab*

gene members (Agarwal, Reddy, Sopory, & Agarwal, 2009) suggesting extensive gene duplication events have occurred across and within species (Elias, Brighouse, Gabernet-Castello, Field, & Dacks, 2012; Zhang et al., 2007). The *Rab* genes in plants are highly conserved within a clade and more similar to homologs in distant species as compared to closely related Rab within the same species, which suggests functional conservation of the *Rab* genes within eukaryotes (Rojas, Fuentes, Rausell, & Valencia, 2012).

By structural analysis, the conserved and non-conserved regions have been shown to contribute to the localization and specific function of the Rab proteins (Pfeffer, 2005). The Rabs share several common structural features which include the guanine nucleotide-binding domains (Figure 2). Multiple sequence alignment analysis revealed Rab family specific regions (termed F1-F5) and Rab subfamily regions (SF1-4) respectively (Pereira-Leal & Seabra, 2001; Moore, Schell, & Palme, 1995). The Rab family regions (F1-F5) distinguish a Rab protein from other members of the Ras superfamily while the Rab subfamily regions SF1-4 facilitates the grouping of Rabs into subfamilies (Pereira-Leal & Seabra, 2001; Moore et al., 1995) (Figure 2). Rab family and subfamily regions have also been shown to play essential roles in specific effector and membrane interaction (Ali & Seabra, 2005). Despite the conserved nature of this gene family, great divergence exists at the C-terminal hypervariable domain which plays a crucial role in specific localization in the membrane (Pfeffer, 2005). The prenylation of the C-terminal sequence is also necessary for their cellular localization and interaction with effectors (Calero et al., 2003). For instance, Loraine, Yalovsky, Fabry, & Gruissem (1996) showed that LeRab1A, LeRab1B, and LeRab1C tomato mutants in which the C-terminal cysteine residues had been swapped with other amino acids were unable to complement YPT1p in yeast. However, their wild-type were able to show complementation with YPT1p. Mutations in RGGT have been shown to cause a series of developmental abnormalities, including smaller leaves, loss of apical dominance, delayed senescence and shoot gravitropic defects in *A. thaliana* (Hála, Soukupová, Synek, & Žárský, 2010) demonstrating the importance of correct prenylation of the di-cysteine motif. Recently, it has been shown that Rab5a and Rab27a mutants without a di-cysteine motif (replaced with mono-cysteine motif) led to their mistargeting to the endoplasmic reticulum/Golgi region rather than their designated cellular compartment (Shinde & Maddika, 2017). Together, these findings highlight the significance of post-translational modification for the correct targeting of Rab proteins.

Next-generation sequencing (NGS) has enabled the availability of full genome sequences. This permits robust bioinformatics analysis of orthologous Rab proteins across diverse plant groups. These technologies are powerful because they enable in-depth comparisons to be made between species. Knowledge gained from the functional information in model organisms can then be transferred to less well-studied plant groups.

[Table 1 here]
[Figure 2 here]

Rab GTPase proteins as directors of vesicle trafficking

Previous studies have revealed that there are many related Rabs groups across many species to regulate protein trafficking in different parts of the endomembrane system *(*Ebine & Ueda, 2015; Jürgens, 2004; Lycett, 2008; Saito & Ueda, 2009) (Figure 3). Distinct membrane trafficking events which span from the vesicle formation, recruitment of motor proteins, vesicle motility along the cytoskeletal tract, to vesicle tethering and fusion from the donor membrane to the acceptor membranes are carried out by Rab GTPases and their accessory proteins (Gillingham, Sinka, Torres, Lilley, & Munro, 2014). The crucial roles of the Rab GTPases have been revealed in the exocytic (Hutagalung & Novick, 2011) and endocytic pathways (Wandinger-Ness & Zerial, 2014). Although the plant Rab groups appear to maintain similar trafficking functions between membrane compartments as their mammalian counterparts (Table 2), diversification in plant post-Golgi pathway has been reported (Fujimoto & Ueda, 2012; Hanton, Matheson, Chatre, Rossi, & Brandizzi, 2007). This could be due to the distinctive features of the plant cell, such as the presence of the plant cell wall (Kim & Brandizzi, 2014) and chloroplast (Saito & Ueda, 2009). The plant cell wall components and proteins that are involved in cell wall-building events are synthesized in different parts of the cell. Cell wall-modifying enzymes are produced on the endoplasmic reticulum and cell wall polysaccharides are made in the Golgi apparatus or cell membrane (Romanovicz, 1982; Somerville, 2006).

These synthesized cargo materials are transported by vesicles to the TGN and eventually to the PM of the plant cell (Kim & Brandizzi, 2014) (Figure 3). The RabA

group have been shown to be localized in the TGN (Chow, Neto, Foucart, & Moore, 2008) and subsequently transported to the PM. The diversity of the RabA group suggested that distinct functions unique to plants may have evolved amongst them (Pereira-Leal & Seabra, 2001; Rutherford & Moore, 2002), some of which are demonstrated by recent studies. For example, Chow et al., (2008) showed the involvement of RabA2 and RabA3 in cell plate formation as mutations of these resulted in altered cell wall formation. In another study, functional analysis of the RabA group in *A. thaliana* stem tissue indicated that null mutations of the *RabA1, RabA2 and RabA4* sub-clades altered the cell wall composition (Lunn, Gaddipati, Tucker, & Lycett, 2013a). Choi et al., (2013) observed that in the leaf epidermal cells of *Nicotiana benthamiana*, RabA1b and RabA4c are involved in anterograde and retrograde trafficking between the TGN and PM respectively. These results suggested that the functions of RabA members have diverged. The RabB and RabD sub clades are related to mammalian Rab1 and 2 respectively (Pereira-Leal & Seabra, 2001). Plant studies have shown that these members are associated with ER to Golgi transport (Batoko, Zheng, Hawes, & Moore, 2000; Cheung et al., 2002). Mutations in a maize *Rab2*, (*ZmRab2A1*) were shown to induce wart-like structures on leaf surfaces, suggesting a role in cell wall secretion during expansion (Zhang et al., 2007). RabE is involved with Golgi to PM transport (Speth, Imboden, Hauck, & He, 2009). RabF and RabG are associated with endosomal trafficking (Ebine et al., 2014). RabC has been linked to the Golgi (Li & Guo, 2017; Dejgaard et al., 2008) and cell membrane (Li & Guo, 2017). Studies in plants have shown that RabC is involved with fruit abscission (Corbacho, Romojaro, Pech, Latché, & Gomez-Jimenez, 2013; Gil-Amado & Gomez-Jimenez, 2013) and stress response (Jiang et al., 2017).

[Table 2 here]
[Figure 3 here]

The involvement of Rab GTPases in fruit ripening

Fruit is one of several plant systems where the key role of vesicle trafficking in cell-wall related events has been well characterized (Lycett, 2008). Gene expression pattern of the Rab GTPase associated with fruit ripening offers insights into understanding the

possible functions of Rabs during fruit development. Fruit softening is a ripening-related process which involves physiological changes to produce an edible fruit of desired quality. These changes are caused by the synthesis and secretion of cell wall polymers and enzymes (Brummell, 2006). It is clear that the process of cell wall disassembly requires multiple enzymes working in concert (Brummell, 2006). On the basis of this, it has been suggested that regulating the trafficking route is a promising strategy that might drastically reduce softening and increase shelf-life, while reducing wastage. This would be through a range of enzyme activities being reduced in a coordinated fashion through reducing or eliminating normal trafficking (Lycett, 2008). The preferential expression of the Rab GTPases during fruit ripening has been reported (Abbal et al., 2008; Falchi et al., 2010; Liu et al., 2014; Loraine et al., 1996; Lu, Zainal, Tucker, & Lycett, 2001; Lunn, Phan, Tucker, & Lycett, 2013b; Park, Sugimoto, Larson, Beaudry, & van Nocker, 2006; Zainal, Tucker, & Lycett, 1996; Zegzouti et al., 1999). Thus establishing their possible role in fruit ripening.

It has been suggested that Rab GTPases are involved in the secretion and targeting of the enzymes that alter the cell wall components during fruit ripening (Loraine et al., 1996; Zainal et al., 1996). Zainal et al., (1996) carried out a pioneer investigation for the RabA subclade in mango fruit mesocarp and reported an expression in ripe fruit but not in green unripe fruit. A tomato orthologue *RabA1a* was silenced using antisense technology. The transgenic tomato fruit was found to remain firm for a longer period, with decreased levels of PG and PE enzymes (Lu et al., 2001). Recently, quantitative PCR (qPCR) data showed that the tomato *RabA1a* is highly expressed during early fruit development, suggesting a possible role in cell wall deposition (Lunn et al., 2013b). This author went further to investigate the composition of the cell wall at different tomato fruit developmental phases and compared between wild-type and antisense lines. The results showed that the antisense fruit in which *RabA1a* had been silenced accumulated less pectin at the breaker stage compared to the wild type. Taken together, this supports the hypothesis earlier raised (Tucker & Seymour, 1990; Brummell, 2006) that cell wall composition and/or modifying enzymes contribute to fruit softening. The RABA subclade provides a good illustration of how altered expression of a RAB GTPase could be used to effect cell wall events necessary for expansion and loosening during ripening (Lu et al., 2001; Lunn et al., 2013b). Selected *Rab* genes from *V. vinifera* were shown to be strongly expressed in grape

berries (Abbal et al., 2008). The expression for selected *VvRabs* were observed from early green to fully ripe stages of the fruit. However, they exhibited various expression patterns. For *VvRabA5e, VvRabB1c, VvRabC1* and *VvRabE1c*, expression remained almost constant while for the other tested genes, the expression pattern tended to be either down regulated after the onset of ripening (*VvRabA1c, VvRabA1e, VvRabG3a*) or fluctuated (*VvRabA2a, VvRabB1d, and VvRabD2c*) during berry development. These results contrast with the northern blot data from early studies which were only able to show a single combined expression level of the target *Rab A* and *D* genes in tomato (Loraine et al., 1996; Lu et al., 2001), of *RabA* in mango (Zainal et al., 1996) and *RabG* (Mbeguie-A-Mbeguie, Gomez, & Fils-Lycaon, 1997) in apricot as ripening progressed. Difference between these results is probably due to those early results being influenced by other *Rab* gene members (Rutherford & Moore, 2002). Previous studies have undertaken the characterization of the peach *Rab* gene families during fruit development and ripening (Falchi et al., 2010). The authors have shown that the *Prunus persica Rab* (*PpRab*) transcripts exhibited transient *up*-regulation from 72-86 days after anthesis (DAA) and from 86-107 DAA respectively. A study by Liu et al., (2014) demonstrated an increased expression of RabF from 10 DAA to 70 DAA in mango fruit. Together, these studies reveal the complexity of the gene expression pattern *and* support the hypothesis that Rab GTPase is required for the phases of fruit development and ripening. Genetic redundancy has often been used to explain the lack of a discernible phenotype after the inactivation of single genes. Pinheiro et al., (2009) reported distinct but overlapping functions of the RabD1 and RabD2 GTPases in the early secretory pathway. More recently, a study by Lunn et al., (2013a) showed that single gene knockouts of the *AtRabA1*, *AtRabA2* and *AtRabA4* sub-clades influenced the cell wall composition in stem tissue of *A. thaliana*. These results suggest that individual Rab proteins may have non-overlapping functions. The hyper variable region of the Rab protein has been reported to play a pivotal role in their functions for specific localization and membrane targeting (Pfeffer, 2013). Cell wall composition and extent of modification differs among fruit species such as apple and tomato thus contributing to the variation of fruit texture observed (Brummell, 2006; Redgwell et al., 1997). Different softening behaviours have also been observed within fruit cultivars (Jha et al., 2011; Ng et al., 2013). According to Brummell (2006), the abundance, timing, activity and the types of ripening associated with genes expressed during ripening contributes to

the diversity of cell wall changes between species. However, the correlation between the level of *Rab* gene expression and the different softening characteristics across plant species and within cultivars is yet to be established. Studies that can provide an insight into this biological question will broaden our general knowledge of plant cell wall biosynthesis and modification during ripening.

Conclusion

A wealth of information is now available for the Rab GTPase, which are a large family of the small GTP-binding proteins displaying functional diversity in plants. It is now clear that the trafficking of cell wall polymers and modifying enzymes is required during fruit softening, a ripening-related event. Inhibition of gene expression approaches implicated several Rab GTPases in gene product trafficking to the cell wall, in particular, the RabA clade. Although our understanding of plant vesicular trafficking is rapidly expanding, the participation of Rab proteins in fruit ripening cannot be overemphasized and as such more high-throughput studies on identification of Rab proteins during fruit ripening would be useful. Further extensive research employing genomic tools on these proteins, their regulators, effectors and linkage with fruit ripening will enhance our understanding of their importance in postharvest fruit studies. This, in turn, will open up new possibilities to address challenges faced in post-harvest losses.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the CFF-UNMC Doctoral Training Partnership under the FoodPlus programme [Food P1-016].

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Table titles

Table 1. Distribution of Rab GTPase proteins identified so far in selected plant species.
Table 2. Localization and functions of plant Rab GTPases.

Statement: Tables were created by me based on the publicly available information.

List of figure captions and notes

Figure 1. Schematic model of the Rab GTPase cycle
Note: (Figure 1).
Rab escort proteins (REP) interacts with newly synthesized GDP- bound Rab proteins and presents the complex to the Rab geranylgeranyl transferase (RGGT) enzyme for post translational modification of Rab protein (step1). Prenyl groups are indicated by the orange lines on Rab protein. Membrane cycling of the prenylated GDP-bound Rab is facilitated with the binding of GDI and release of Rab escort proteins (REP) (step 2). GDI displacement factor (GDF) catalyzes the release of GDP dissociation inhibitor (GDI) from the GDP-bound Rab-GDI complex and aids in membrane insertion (step 3). Following this, the guanine nucleotide exchange factor (GEF) facilitates the activation of the Rab GTPase through an exchange of GDP to GTP (step 4). Activated GTP-bound Rab recruits effector proteins necessary for diverse trafficking functions. Once the Rab completes its function, GTPase activating protein (GAP) stimulates GTP hydrolysis (step 5) thus generating a GDP-bound Rab. GDP dissociation inhibitor (GDI) interacts with the GDP-bound Rab and extracts it from the membrane into the cytosol awaiting the next cycle. The box with dashed lines represent. Adapted from Seixas, Barros, Seabra, & Barral, (2013); Stenmark, (2009).

6724

Statement: Figure 1 was created by me based on the available information and these have been acknowledged accordingly.

Figure 2. Multiple sequence alignment of selected Rab subfamily proteins from *A. thaliana.*

Note: (Figure 2).

The conserved guanine nucleotide-binding domains named 'G box' sequences are identified and boxed with rectangles (G1, G2, G3, G4, and G5) respectively according to Jiang and Ramachandran, (2006). Rab family (F) and subfamily (SF) regions (defined according to Pereira-Leal and Seabra, 2001) are indicated and included in boxes with dashed lines. HVR identifies the hypervariable region. The alignment was generated using clustal omega (Sievers et al., 2011). In the consensus line, asterisks (*) indicate amino acids with 100% homology in all sequences. Colons (:) and dots (.) represent conserved substitutions and weakly conserved sites respectively. TAIR accession numbers: *At*RabH1B (At2G44610), *At*RabA1C (At5G45750), *At*RabB1C (At4G17170), *At*RabE1A (At3G53610), *At*RabF1 (At3G54840), *At*RabC1 (At1G43890), *At*RabG3F (At3G18820), *At*RabD2B (At5G47200). Adapted from Falchi et al., (2010).

Statement: Figure 2 was created by me using the Clustal Omega program (Sievers et al., 2011) on selected publicly available Rab sequences of *Arabidopsis thaliana.*

Figure 3. Simplified illustration of the trafficking pathways involved with cell wall softening.

Note: (Figure 3)

The Rab family members involved at each step are indicated in parentheses. Arrows indicate pathways to and fro the cell wall respectively. ER, endoplasmic reticulum; PM, plasma membrane; TGN, trans-Golgi network. Adapted from Lycett, (2008).

Statement: Figure 3 was created by me based on the available information and source of data has been acknowledged accordingly.