*Himachal Journal of Agricultural Research 48(1): 1-7 (2022)*

# *Ascochyta rabiei* infections modify expression of chickpea invertase genes differentially in contrasting genotypes

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Manuscript Received: 14.10.2021; Accepted: 12.11.2021

## Abstract

**Sucrose is the main form of assimilated carbon and energy source in plants. Sucrose in sink tissues is hydrolyzed by invertases to glucose and fructose that act as major carbon molecules for plant metabolism. Initial studies in some crops suggested role of invertases in plant disease resistance however, no information on these genes is available for chickpea biotic stresses. To identify the role of invertases in resistance/susceptibility to ascochyta blight (causal organism: *Ascochyta rabiei*) in chickpea, expression of six invertase genes (two cell wall invertases, one vacuolar invertase and three alkaline/neutral invertases) was evaluated in *A. rabiei* infected susceptible (GPF2) and resistant (HC1) genotypes of chickpea. Of these six gene, only one overexpressed in susceptible GPF2 whereas in resistant HC1 five genes overexpressed. The study suggested that down regulation of invertase genes was associated with susceptibility of chickpea to *A. rabiei* whereas over expression was associated with resistance.**

**Key words:** Ascochyta blight, *Cicer arietinum*, cell wall invertase, vacuolar invertase, alkaline/neutral invertase, gene expression, *Ascochyta rabiei*

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop grown in the world. It ranks first in the Indian sub-continent and forms an integral part of human diet where consumers are primarily vegetarian and pulses are the main source for dietary protein requirements. One of the major problems associated with the higher production of chickpea in Himachal Pradesh (India) and other cool humid regions of the world is ascochyta blight, which is caused by a fungus called as *Ascochyta rabiei* (Pass.). Chickpea yield losses due to *A. rabiei* infection range from 10% to 100% (Gaur and Singh 1996). The blight spots on chickpea seeds also lowers the quality of produce (Gaur and Singh 1996). Some chickpea genotypes possess resistance to blight and hence, yield better than the susceptible ones under blight epiphytotic conditions. Upon infection, plants activate defences to protect them from disease by reorienting transcription and ultimately changing the metabolite profile. While considerable information is available for defence molecules such as phytoalexins, no information is available on changes in sugars in infected plants of chickpea with respect to resistance or susceptibility.

Sucrose is the primary form of assimilated carbon that is produced during photosynthesis in leaves (source tissues). It is then transported through the phloem to sink tissues (stem, reproductive organs, roots. etc). The products (glucose and fructose) of this disaccharide are known to have crucial roles in cell signalling as these are involved in several metabolic processes and also act as signal molecules (Gomez-Ariza *et al*. 2007; Moghaddam *et al*. 2012). Sucrose activates the immune responses of plants against pathogens during infection by reallocating the sugar contents. A sucrose hydrolyzing enzyme known as invertase (EC 3.2.1.26) is involved in hydrolysis of sucrose to glucose and fructose and activities of this enzyme change during plant-pathogen interactions in some systems (Roitsch *et al*. 2003; Roitsch and Gonzaez 2004; Siemens *et al.* 2011). It was also suggested that the invertases in addition to

establishing a defense response also control sugar-signalling in plant-pathogen interactions (Tauzin and Giardina 2014). The activity of invertases increase significantly in the infected sites of plants (Long *et al*. 1975; Tang *et al*. 1996). In addition, an increased apoplastic invertase activity was found to be associated with a higher degree of resistance to powdery mildew in barley (Swarbrick *et al*. 2006). Our knowledge on the role of invertases in the infection processes of plants is, however, still vague (Parrent *et al*. 2009) and it needs further studies to explore the role of invertases in host pathogen interactions. Limited information on changes in invertase activities is available with respect to cold stress in chickpea (Kiran *et al*. 2021; Sharma *et al*. 2021). Since, carbohydrates and invertases are integral components of plant growth and development and role of these molecules in abiotic stress tolerance is adequately described.

There is a need to understand the role of invertases in plant-pathogen interactions. The present study explored the expression of invertase genes in ascochyta blight resistant and susceptible chickpea after inoculation with blight fungus *A. rabiei* and compared it with expression in control plants. The study demonstrated that increased expression of invertases was associated with blight resistance in chickpea.

## Materials and Methods

### Plant materials and growth conditions

The plant material included an ascochyta blight resistant genotype (HC1) and an ascochyta susceptible genotype (GPF2) of chickpea. The plants were grown in plastic pots (25 cm diameter) with two plants per pot under controlled conditions of 23±1°C/18±1°C day/night for 21 days and inoculated with *A. rabiei*. The pathogen was isolated on potato dextrose agar medium and mass-multiplied on kabuli chickpea seeds medium. For preparing the medium, kabuli chickpea seeds were soaked over-night in water followed by transfer of 100 g seeds per 250 ml flask and sterilization by autoclaving twice at 121°C (15psi) for 25 minutes. Bits of pathogen were transferred onto the medium aseptically and allowed to grow at 25°C for 20 days. The flasks were hand shaken every alternate day to ensure spread of pathogen evenly across the medium. After 20 days, 100 ml autoclaved distilled water was added to each tube, tubes were shaken vigorously and the liquid (inoculum) was passed through four layers of muslin cloth. The spore concentration of inoculum was adjusted to $2\times 10^5$ with sterile distilled water and plants were sprayed with the spore solution and maintained at high relative humidity ($\approx$ 100%) for 24 h to facilitate infection. Disease severity was assessed after 14 days of inoculation. The leaf samples (100 mg per replication, three replications per treatment) from both genotypes were harvested at 0 h (control) and 72 h post inoculation (*A. rabiei* infected) and the samples were immediately snap frozen in liquid nitrogen for RNA extraction.

### Gene retrieval and primer synthesis

The cDNA sequences of all six invertase genes were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/) (see Table 1 for names of the genes and gene IDs). To design gene specific primers, primer3 software was used (http://bioinfo.ut.ee/primer3/) using coding region sequences of these six target genes. *ATP-binding cassette transporter* (*ABCT*) and *Clathrin adaptor complexes subunit family protein* (*CAC*) genes were used as reference genes. The reference genes were also used for normalization of gene expression. The primers were custom synthesized by Integrated DNA Technologies (www.idtdna.com). Details of primer sequences, amplicon size, Tm, etc. are given in Table 1.

### RNA extraction and cDNA synthesis

Total RNA was extract edusing Trizol method according to the manufacturer's instructions (Ambion Life Sciences, Thermo Fisher, USA). The RNA pellet was dissolved in nuclease free water and treated with RNase-free DNase-I. The quantity and quality of RNA was measured using nanodrop (Thermo scientific) at 260 nm and 1% denaturing agarose gel electrophoresis, respectively. The quantity of RNA was normalized in all samples. Total RNA, oligo (dT) 18 primer (Thermo scientific), and MMLV Reverse transcriptase enzyme (Promega, USA) were used to synthesize cDNA by incubating these in the reaction tubes at 42°C for 60 minutes as per the instructions of the manufacturer.

**Table 1. Primer sequences of invertase genes of chickpea**

| Sr.No. | Gene name | Gene ID | Primer sequence 5'-3' | Melting temperature (Tm) | Amplicon size (bp) |
|---|---|---|---|---|---|
| 1. | *Beta-fructofuranosidase, insoluble isoenzyme CWINV3-like* | 101500539 | Forward: ATATTGTGGGGATGGGTGA Reverse: GGCCCACTGCATCAATCTCTT | 63.7°C 64.2°C | 128 |
| 2. | *Beta-fructofuranosidase, cell wall isozyme-like* | 101504789 | Forward: GGTGCTCATGTGTTGTGACA Reverse:CCCAAAACTCTCCACCACAG | 63.5°C 64.5°C | 148 |
| 3. | *Acid beta-fructofuranosidase 1, vacuolar-like* | 105852298 | Forward: AGGAGTTTCAGCCAAGTCCA Reverse: TTGGGGTTGAAAGTGGAAAG | 59.84°C 59.84°C | 106 |
| 4. | *Probable alkaline/neutral invertase F* | 101490083 | Forward: CAGGGTTGCTCCTGTTGATT Reverse: ATCAAACGCATACCCTCCTG | 60.11°C 59.96°C | 120 |
| 5. | *Neutral/alkaline invertase 3 chloroplastic* | 101511142 | Forward: CAGGTTCTGTCCAGGGTTGT Reverse: TTGACTGGCTTTGAAACACG | 60°C 59.88°C | 145 |
| 6. | *Alkaline/neutral invertase CINV2* | 101512400 | Forward: CTCGTTGGGATGAGTTGGTT Reverse: CTGGCCAAGATCCTCCATTA | 59.97°C 60.03°C | 138 |

**Quantitative real time polymerase chain reaction**

The transcript abundance of the genes was analyzed using quantitative real time polymerase chain reaction (qPCR). The qPCR was carried out in Step One™ Real-Time PCR System (Applied Biosystems, USA) with SYBR Green PCR Master Mix (TB Green™ Premix Ex Taq™, Takara Clontech, USA) (see Table 2 for components of reaction mixture). The thermal cycle profile for qPCR was 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute and included 40 cycles during which fluorescence was measured. The experiment had three replications. Two reference genes (*ATP-binding cassette transporter* (*ABCT*) and *Clathrin adaptor complexes subunit family protein* (*CAC*), were used to normalize the transcript amounts. The data were collected and analysed as described by Livak and Schmittgen (2001) and Taylor *et al*. (2019).

**Table 2. Components of master mix of qPCR**

| Sr.No. | Component | Volume (µL) |
|---|---|---|
| 1. | Sybr Green | 5.00 |
| 2. | Rox | 0.20 |
| 3. | Forward Primer | 0.20 |
| 4. | Reverse Primer | 0.20 |
| 5. | cDNA | 1.00 |
| 6. | Nuclease free water | 3.40 |
| 7. | Total reaction volume | 10.00 |

## Results and Discussion

### Quality and quantity of RNA

RNA isolated from control and *A. rabiei* infected leaves was of high quality which was explicit from absorbance ratio at 260/280 nm (1.85 to 2.10, close to ideal value of 2.0 for RNA) (Table 3). RNA was also electrophoresedusing gels to check its quality. All the samples yielded discrete rRNA bands showing that the quality of the RNA was high.

### Expression of invertase genes in *A. rabiei* inoculated and uninoculated chickpea plants

Six genes were used in the current study, of which two were cell wall invertases (*beta-fructofuranosidase insoluble isoenzyme CWINV3-like and Beta-fructofuranosidase cell wall isozyme-like*), one was vacuolar invertase (*Acid beta-fructofuranosidase 1 vacuolar-like*) and three were alkaline/neutral invertases (*Probable alkaline/neutral invertase F, Neutral/alkaline invertase 3 chloroplastic* and *Alkaline/neutral invertase CINV2*).

#### *Expression of cell wall invertase genes*

Between the two genotypes (resistant and susceptible), the expression of two cell wall invertase genes varied under control conditions (Fig 1A, 1B) suggesting that the two genotypes differed for carbohydrate metabolism. Upon blight infection, the expression of *beta-fructofuranosidase insoluble isoenzyme CWINV3-like* decreased in susceptible GPF2 and increased in resistant HC1 (Fig 1A) whereas the expression of *Beta-fructofuranosidase cell wall isozyme-like* enhanced in both the genotypes (Fig 1B). The fold change in gene expression vis-à-vis GPF2 under control further confirmed upregulation of both the genes in HC1 and upregulation of only one gene i.e., *Beta-fructofuranosidase cell wall isozyme-like* in GPF2 (Fig 2). This upregulation of cell wall

**Table 3. Absorbance ratio and quantity of RNA isolated from leaves of chickpea genotypes**

| S.N. | RNA sample | Absorbance (A260/280) ratio | Quantity (ng/µL) |
|---|---|---|---|
| 1. | GPF2 Control | 1.85 | 1040.00 |
| 2. | GPF2 *A. rabiei* infected | 2.10 | 145.00 |
| 3. | HC1 Control | 1.90 | 1360 |
| 4. | HC1 *A. rabiei* infected | 2.00 | 245 |

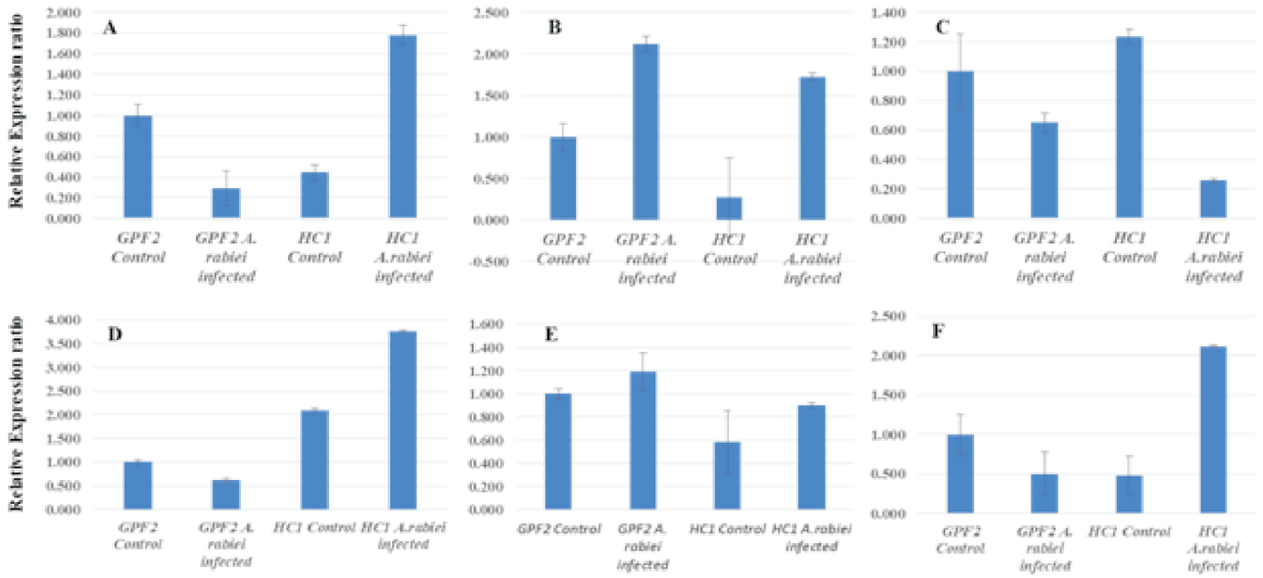


**Figure 1.** Relative expression of invertase genes in *Ascochyta rabiei* infected leaves of chickpea after 72 hours of inoculation. Two genotypes, GPF2 (susceptible) and HC1 (resistant) were used. Relative expression was calculated with respect to GPF2 (control). A. *Beta-fructofuranosidase, insoluble isoenzyme CWINV3-like* gene; B. *Beta-fructofuranosidase, cell wall isozyme-like* gene; C. *Acid beta-fructofuranosidase 1, vacuolar-like* gene; D. *Probable alkaline/neutral invertase F* gene; E. *Neutral/alkaline invertase 3 chloroplastic*gene; F. *Alkaline/neutral invertase CINV2* gene

invertase genes is important for sugar partitioning and supply of sugars in the resistant HC1 after pathogen interaction. Repression of cell wall invertase in the leaves of tobacco and tomato was associated with damage by disease and delay in the defence reactions to pathogens like *Phytophthora nicotianae* and *Xanthomonas campestris* (Essmann *et al*. 2008; Kocal *et al*. 2008). Similarly, several cell wall invertases of *Populus* such as *PtCWINV1*, 2, 3, 4 were downregulated when plants were infected by *B. dothidea* (Chen *et al*. 2015). The role of invertases in pathogen defence was also highlighted using RNA mediated gene silencing (Essmann *et al*. 2008). Silencing of cell wall invertase gene in tobacco disturbed the ability of the plants to respond to pathogen aptly and also perturbed the defense induced reaction of the plants (Essmann *et al*. 2008). The present study and that of Essmann *et al*. (2008) clearly highlighted the role of cell wall invertases in plant defences including that of chickpea upon infection with *A. rabiei*.

***Expression of vacuolar invertase gene***

The vacuolar invertase (*Acid beta-fructofuranosidase 1, vacuolar-like*) downregulated in the susceptible (GPF2) as well as resistant (HC1) genotype after *A. rabiei* infection (Fig 1C). The fold changes in expression of vacuolar invertase gene in infected GPF2 was 0.6 and in infected HC1, it was 0.2 (Fig 2). Vacuolar invertase genes have already been shown to express at higher levels in leaves of grapes, cassava (Nonis *et al*. 2008; Yao *et al*. 2015) and chickpea (present study). Vacuolar invertases play role in cell expansion, storage of sugars and regulation of cold induced sweetening (Roitsch and Gonzalez 2004). Similar to our observations, a vacuolar invertase gene of *Populus* (*PtVINV3*) was also shown to down regulate upon infection with *B. dothidea* (Chen *et al*. 2015).

***Expression of alkaline/neutral invertase genes***

Of the three alkaline/neutral invertase genes (*Probable alkaline/neutral invertase F, Neutral/alkaline invertase 3 chloroplastic* and

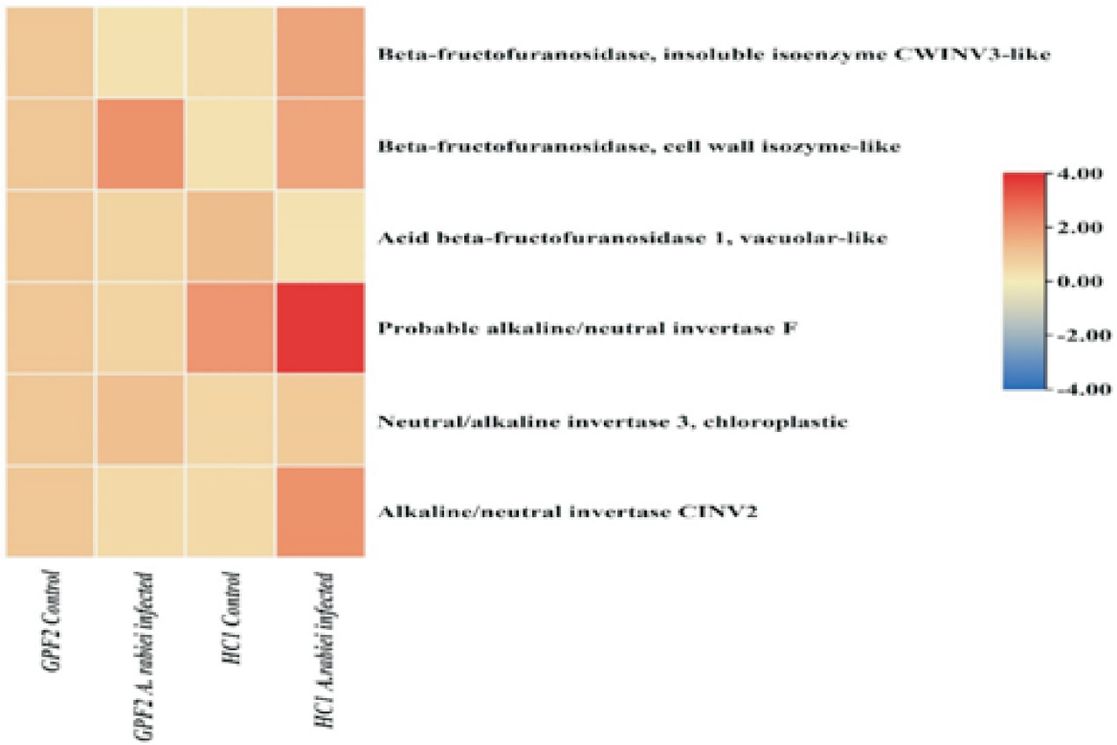


**Figure 2.** Heat map depicting fold change relative to GPF2 control in the expression of invertase genes in chickpea leaves infected with *Ascochyta rabiei*. Plants of chickpea genotypes, GPF2 (susceptible) and HC1 (resistant) were inoculated with *A. rabiei* and harvested 72 h post inoculation and were analyzed for gene expression

*Alkaline/neutral invertase CINV2*), the expression of all the three genes increased in *A. rabiei* infected HC1 as compared to control with a fold change of 1.76, 1.5 and 4.0 for *probable alkaline/neutral invertase F, neutral/alkaline invertase 3 chloroplastic* and *alkaline/neutral invertase CINV2*, respectively (Fig 1D, 1E, 1F, Fig 2). In contrast to this, the alkaline/neutral invertase genes in *A. rabiei* infected GPF2 either downregulated or expression did not change (Fig 1D, 1E,1F, Fig 2). The *probable alkaline/neutral invertase F* (0.6-fold) and *alkaline/neutral invertase CINV2* (0.5-fold) were downregulated when infected with the pathogen while the expression of *neutral/alkaline invertase 3 chloroplastic* did not change (1.1-fold) (Figure 1E, Fig 2). The upregulation in relative expression of all the alkaline/neutralinvertase genes in resistant HC1 suggested role of these genes in plant defence against *A. rabiei* infection.

*A. rabiei*, the blight pathogen, lands on chickpea surfaces after inoculation and establishes organic relationships by 72 h of inoculation. In other host pathogen systems, the down regulation of invertase genes following infection has been reported (Chen *et al*. 2015). The present study established that following *A. rabiei* infections, chickpea plants modify invertase gene expression. It was also established that down regulation of cell wall invertases, vacuolar invertase and alkaline/neutral invertases was associated with blight susceptibility. Contrary to it, over expression of majority of the invertase genes was associated with resistance as observed for blight resistant genotype HC1. It is concluded that the host invertases respond differentially to *A. rabiei* infections in resistant and susceptible genotypes of chickpea and over expression of these genes is required for host resistance.

**Conflict of interest:** There is no conflict of interest among the authors in this research paper.

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